Activation of the Tumor Suppressor Merlin by Mosin/Moesin Phosphatase

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Abstract

One of the most important characteristics of normal cells as compared to cancer cells is the inhibition of growth upon cell-cell contact. The tumor suppressor merlin plays a critical role in the establishment and maintenance of this contact inhibition. Merlin becomes active at high cell density and mediates contact inhibition by interfering with the transduction of Ras dependent proliferation signals. This growth inhibiting ability of merlin is associated with its dephosphorylation at serine 518. When merlin is phosphorylated by PAK-2 or PKA at this site, it cannot inhibit cell growth.

In order to identify the activating phosphatase of merlin, RT4 cells were engineered to express C-terminal merlin (C-merlin, 300-595). This C-terminal part of merlin contains the critical serine 518 site and is regulated similarly to full-length merlin in these cells. At first, PP1 rather than PP2A was identified as the catalytic subunit of the phosphatase, since the dephosphorylation of merlin was inhibited only by high concentrations of okadaic acid and purified PP1 could dephosphorylate C-merlin *in vitro* more efficiently than PP2A. Myosin/moesin phosphatase consisting of PP1δ as the catalytic subunit and MYPT-1 as the target subunit was then identified as the phosphatase of merlin. i) Merlin interacted directly with MYPT-1 both *in vivo* and *in vitro*. This direct interaction was mediated by a region from residue 312 to 341 in merlin and the leucine zipper domain in MYPT-1. ii) A naturally occurring mutation of the *nf-2* gene (L339F) in this interacting region could impair the interaction of merlin with MYPT-1. iii) Under conditions when merlin was dephosphorylated, this phosphatase was activated via its dephosphorylation at threonine 696. iv) Furthermore, merlin could not be dephosphorylated and lost its growth inhibitory ability in the presence of CPI-17, a specific inhibitor of myosin/moesin phosphatase. Finally, CPI-17 was able to induce transformation by inhibiting the activation of merlin and is therefore proposed to be a novel oncoprotein.

In conclusion, this study reveals that myosin/moesin phosphatase is the activating phosphatase of merlin and plays a central role in mediating contact inhibition.

Aktivierung des Tumorsuppressor-Proteins Merlin durch Myosin/Moesin Phosphatase

Zusammenfassung

Eine der wichtigsten Eigenschaften von normalen Zellen im Unterschied zu Krebszellen besteht in der Hemmung des Wachstums unter Zell-Zell-Kontakt Bedingungen. Das Tumorsuppressor-Protein Merlin spielt in der Etablierung und Aufrechterhaltung dieser Kontaktinhibition eine entscheidende Rolle. Merlin wird bei Erreichen einer hohen Zelldichte aktiviert und vermittelt die Kontaktinhibition, indem Ras-abhängige Proliferationssignale gehemmt werden. Diese wachstums-inhibitorische Fähigkeit Merlins ist mit seiner Dephosphorylierung am Serinrest 518 verbunden. Erfolgt durch die Kinasen PAK-2 oder PKA eine Phosphorylierung an dieser Stelle kann Merlin das Zellwachstum nicht mehr inhibieren.

Um die Merlin-aktivierende Phosphatase zu identifizieren, wurden RT4 Zellen verwendet, die den C-Terminus von Merlin (C-Merlin, 300-595) künstlich überexprimieren können. Der C-Terminus enthält den kritischen Serinrest 518 und wird in diesen Zellen ähnlich dem vollständigen Merlin reguliert. Die Dephosphorylierung von Merlin konnte erst durch hohe Konzentrationen Okadainsäure inhibiert werden, welches eher auf PP1 als auf PP2A als katalytische Untereinheit schließen ließ. Weiterhin konnte C-Merlin *in vitro* durch aufgereinigte PP1 effizienter dephosphoryliert werden als durch PP2A. Die Myosin/Moesin Phosphatase, welche aus PP1δ als katalytischer und aus MYPT-1 als regulatorischer Untereinheit besteht, wurde dann als Phosphatase von Merlin identifiziert. i) Merlin interagierte *in vitro* und *in vivo* direkt mit MYPT-1. Diese direkte Interaktion wird in Merlin durch die Aminosäuren 312 bis 341 und in MYPT-1 durch die Leucinreißverschluss-Domäne vermittelt. ii) Eine natürlich vorkommende inaktivierende Mutation des *nf-2* Gens (L339F) in dieser Interaktionsregion verhindert die Bindung von MYPT-1 an Merlin. iii) Unter Bedingungen, unter denen Merlin dephosphoryliert ist, ist die Myosin/Moesin Phosphatase durch Dephosphorylierung am Threonin 696 ebenfalls aktiviert. iv) Schließlich konnte gezeigt werden, daß Merlin in Gegenwart von CPI-17, einem spezifischen Myosin/Moesin Phosphatase Inhibitor, nicht mehr dephosphoryliert werden und als Tumorsuppressor wirken kann. Konsequenterweise induziert CPI-17 durch Inhibition der Merlinaktivierung Transformation von Zellen und wird daher als neues Onkoprotein vorgeschlagen.

Diese Studie zeigt, daß die Myosin/Moesin Phosphatase die Merlin-aktivierende Phosphatase ist und eine zentrale Rolle in der Vermittlung der Kontaktinhibition spielt.

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Table of Contents

Abbreviations

A	adenosine
aa	Amino acid
APC	Adenomatous polyposis coli
APS	ammonium persulfate
bp	Base pair
BSA	bovine serum albumin
C	cytidine
°C	degrees celsius
CD	cluster of differentiation
CPI-17	Protein kinase C potentiated inhibitor of 17 kDa
DMEM	Dulbecco´s modified eagles medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E.coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylendiamine-N,N-tetracetate
e.g.	Example given
ERM	ezrin/radixin/moesin
et al.	and others
FCS	foetal calf serum
FERM	domain 4.1, ezrin, radixin, moesin-like domain
g	gram
G	guanosine
G418	geneticin
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor

GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	hyaluronic acid, hyaluronate
hr	hour
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IP	immunoprecipitation
KD	kilodalton
kb	Kilo-base
L	liter
m	milli
M	molar
mAb	monoclonal antibody
merlin	moesin, ezrin, radixin-like protein
mg	milligram (10-3 gram)
min	minute
ml	milliltre (10^{-3} l)
MT	mutant
MW	molecular weight
NF2	neurofibromatosis type-2
OA	Okadaic acid
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	phosphoinositide
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
RNA	ribonucleic acid
RNAi	RNA interference

SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
T	thymidine
TEMED	N,N,N´,N´-tetramethylethylenediamine
TRIS	tris-(hydoxymethyl)-aminomethane
U	unit
UV	ultra violet
WT	wild type
w/o	without

1. Introduction

1.1 Cancer and carcinogenesis

1.1.1 Cancer and cancer cells

Cancer is one of the major health problems of human beings. More than 10 million people are diagnosed with cancer every year. It is estimated that there will be 15 million new cases every year by 2020. Even worse, cancer causes 7 million deaths every year, accounting for 12% of deaths worldwide.

After a quarter century of rapid advances, cancer research has generated a large body of knowledge, revealing cancer cells to be a manifestation of six essential alterations in normal cell physiology that collectively dictate abnormal growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), unlimited potential of replication, sustained angiogenesis, and/or tissue invasion and metastasis (Hanahan and Weinberg, 2000; Ponder, 2001). These six capabilities are shared in common by most, if not all, types of cancers. One of the most distinct *in vitro* growth characteristics of cancer cells differing from normal cells is the loss of contact inhibition. The contact inhibition of growth was firstly described 50 years ago (Abercrombie and Heaysman, 1954). It was used to refer to the inhibition of continued growth and division of a normal cell due to physical contacts with other cells or extracellular matrix (ECM). This regulation of cell growth is very important to control tissue organization. However, cancer cells show no contact inhibition. They can continue to divide after reaching confluency in culture dishes, piling up into mounds even if the surface of the dish has already been covered (two dimension culture mode) and growing into huge colonies in semi-solid culture medium such as soft agar (three dimension culture mode) (Abercrombie, 1970; Abercrombie and Ambrose, 1962; Weber and Rubin, 1971; Weiss, 1970).

Each of these six novel capabilities represents the successful breaching of an anticancer defense mechanism in normal cells and tissues. Thus cancer seems to

develop via a process formally analogous to Darwinian evolution, in which a succession of epigenetic and genetic changes, each conferring one type of growth advantage, finally leads to the progressive conversion of normal cells into cancerous cells (Foulds, 1954; Jakobisiak et al., 2003; Macaluso et al., 2003; Nowell, 1976; Renan, 1993). The genetic changes are related to alterations in DNA sequence while the epigenetic changes are changes of chemical modifications to DNA or chromosomal proteins but do not involve alterations in DNA sequence *per se* and can lead to the deregulation of gene expressions.

1.1.2 Epigenetic mechanism in carcinogenesis

The discovery of epigenetic mechanisms in carcinogenesis arose from the observations that some sporadic cancers are primarily linked to certain environmental factors without any obvious mutagenicity. DNA modification, mainly methylation, is the most well defined epigenetic mechanism in tumorigenesis (Holliday, 1979; Jones, 2002). DNA methylation, the covalent addition of a methyl group to cytosine within the context of a CpG dinucleotide, has profound effects on the mammalian genome, such as transcriptional repression via the inhibition of transcription factors binding (Goelz et al., 1985; Robertson and Jones, 2000; Robertson and Wolffe, 2000; Tran et al., 1988). Well-controlled DNA methylation is essential for proper embryonic development. In cancer cells, however, DNA methylation patterns are frequently disrupted with global hypomethylation accompanying region-specific hypermethylation (Goelz et al., 1985; Robertson and Jones, 2000; Robertson and Wolffe, 2000; Tran et al., 1988). Therefore, DNA hypomethylation is believed to be responsible for the transcription activation of genes during tumor development. DNA hypermethylation, on the other hand, can also contribute to cancer development in that the protein coded by the regulated gene acts as an inhibitor of tumor growth (Baylin, 1992; Clark and Melki, 2002). Histone modification is another epigenetic change occurring during the process of cancer evolution (Jones, 2002; Kim and Huang, 2003; Peters et al., 2001). Histones are mainly modified by reversible acetylation. Similar to methylation of DNA, both hyperacetylation and

hypoacetylation of histones are important events during cancer development, depending on the affected target genes (Archer and Hodin, 1999). Both changes of DNA and histone modification can affect the remodeling of chromatin and the subsequent transcription of key genes involved in tumor progression (Clark and Melki, 2002; Davis and Brackmann, 2003; Esteller, 2003; Hake et al., 2004; Jaffe, 2003; Jones, 2002; Momparler, 2003; Nephew and Huang, 2003; Oligny, 2003). A third epigenetic mechanism called tissue disorganization has been proposed recently based on the fact that tumors in parenchyma can be induced indirectly by carcinogen treated stroma or connective tissue (Sonnenschein and Soto, 2000). The molecular mechanism of how disorganizations in the microenviroment could influence cellular programs of the cell proliferation and differentiation is not clear. Aberrant cytoskeleton reorganizations alone or together with abnormal DNA and histone modifications are expected to be responsible.

1.1.3 Genetic mechanism in carcinogenesis

Genetic changes can affect two gene families important for cancer development, oncogenes that act dominantly by a gain of function mutation and tumor suppressor genes that are recessive by loss of function mutations. Both classes of cancer genes have been identified by their alterations in cancer cells and their elicitations of cancer phenotypes in experimental models (Bishop, 1996; Knudson, 1971).

1.1.3.1 Oncogene

Hundreds of oncogenes have been identified so far. Proteins encoded by these genes (oncoproteins) usually govern fundamental processes of cell proliferation and differentiation through constitutively deregulating certain growth controlling signaling pathways (Bishop, 1987; Knudson, 2002; Lynch et al., 1997). The activating switch from protooncogene to oncogene is mainly caused by gene mutations such as substitutions, deletions, insertions, gene amplifications or chromosome rearrangements (Barbacid, 1986; Pearson and Van der Luijt, 1998). Of course the elevated activity of the oncoproteins can also be due to the deregulated gene

expression, which in some cases result from some epigenetic changes such as DNA hypomethylations, as described above (Tycko, 2003).

1.1.3.2 Tumor suppressor gene

Following the discovery of oncogenes, a distinct class of cancer genes called tumor suppressor genes or antioncogenes was found via somatic cell fusion experiments (Ephrussi et al., 1969; Harris et al., 1969; Stanbridge, 1989). Tumor suppressors can antagonize the effects of oncoproteins and block tumor development. The identification of tumor suppressor genes has revolutionized our understanding of cancer. Since a single functional copy of a gene is usually enough to maintain the anti-cancer effect of the tumor suppressor gene, prototypic tumor suppressor genes are thought to be recessive, in contrast to oncogene, requiring 'two-hit' inactivations of both alleles (Knudson, 1971). Alterations by loss of function are much more common than by gain of function, presumably because they could be compensated by the remaining normal allele and thus kept within families whereas the gain of function mutations might lead to severe development defects. However, individuals from these families have a higher possibility to develop cancer, since they carry one mutated allele in all of their cells and only one allele is necessary to be inactivated to cause the disease (Ahuja and Issa, 2000; Ponder, 2001). This is the reason that most tumor-suppressor genes were identified in inherited cancer syndromes. Loss of function of a tumor suppressor gene often involves a loss of chromosome material, ranging in extent from a subband to the whole chromosome. Such events are called loss of heterozygosity (LOH) and can be demonstrated by comparing the polymorphic loci in DNA of normal and tumor cells (Cavenee et al., 1983).

Most of tumor suppressor proteins control cell growth directly and therefore are termed 'gatekeepers' (Kinzler and Vogelstein, 1997). The dysfunctions of these classic tumor suppressor genes always lead to a very specific tissue distribution of the disease. For example, the inherited mutation of *retinoblastoma* or *adenomatous polyposis coli* gene will lead to tumors of the retina of colon, respectively. The underlying mechanism of this phenomenon is not yet clear. One plausible explanation

could be that each cell type has only one (or a few) restricted vital gatekeeper(s). There is another category of tumor suppressor genes called 'caretakers', which function in maintaining the integrity and fidelity of the genome (Kinzler and Vogelstein, 1997). Although caretakers cannot control the cell growth directly, they are important as well to support normal cell growth. The dysfunctions of these genes can lead to the higher spontaneous mutation rates in the genome. This is called 'mutator phenotype' (Loeb, 1998). The unstable genome can provide more mutations as the source of clonal selection to pick up more aggressive cancer cells with increased proliferation advantages and thereby speed up cancer development.

1.2 The tumor suppressor gene *nf-2*

One of the tumor suppressor genes called *neurofibromatosis type-2 (nf-2)* is the focus of my thesis. Several lines of evidence showed that the *neurofibromatosis type-2 (nf-2)* gene is a tumor suppressor gene. First, the disease termed neurofibromatosis type-2 is associated with high incidence of certain tumors such as schwannoma. A characteristic LOH could be found in patients with this disease (Twist et al., 1994). Second, both copies of the *nf-2* gene are rendered nonfunctional by mutations in tumors from both sporadic and inherited NF-2 patients (Twist et al., 1994; Ueki et al., 1999). Epidemiological data indicated that two independent somatic mutations occurred in sporadic patients while only one in patients with an inherited family history (Evans et al., 2000; Ueki et al., 1999). This fits well with Knudson's 'two-hit' model. Third, reintroduction of this gene into tumor cells could reverse the transformation phenotypes. Several signal transduction pathways important for cell growth were impaired by the expression of *nf-2* gene, suggesting that it might act as a gatekeeper tumor suppressor (Gutmann et al., 1999b; Morrison et al., 2001; Sherman and Gutmann, 2001; Tikoo et al., 1994). Fourth, loss of merlin function is sufficient to promote tumor formations in experimental animal modes. The *nf-2* heterozygous mice developed a variety of malignant tumors (McClatchey et al., 1998). Schwannoma was formed in conditional *nf-2* knockout mice, where merlin expression was specifically disrupted in Schwann cells (Giovannini et al., 2000). Taken together, *nf-2* matches all

of the criteria to define a classical tumor suppressor. In the following the general information about this tumor suppressor will be introduced.

1.2.1 NF-2, the disease

The name of *nf-2* comes from a disease called 'neurofibromatosis'. At least two types of neurofibromatosis exist, neurofibromatosis type-1 (NF-1) and neurofibromatosis type-2 (NF-2), respectively. Both NF-1 and NF-2 display autosomal dominant inheritance and are characterized by the presence of tumors mainly in the nervous system. However, they are two different disorders with distinct clinical and genetic features (Mulvihill, 1988; Mulvihill et al., 1990).

Neurofibromatosis type-2 is a much less common type of neurofibromatosis than neurofibromatosis type-1. It is estimated to affect approximately one in 40,000 individuals without regard to sex or race (Evans et al., 2000; Gutmann et al., 1997). The hallmark of NF-2 is the presence of bilateral vestibular schwannomas with a paucity of cutaneous features, which present frequently in NF-1. Although quite variable regarding the age of onset and the severity of symptoms, NF-2 is associated with significant morbidity and decreased life span (Gutmann et al., 1997).

Unfortunately, very few effective approaches are available to treat this disease. Destructive surgical resection is the main treatment for NF2 patients (Baser et al., 2003; Turgut et al., 1998). No chemotherapeutic drugs have been shown so far to be effective to control this disease. Novel drugs could, however, be developed soon with the advances in NF2 research (Hirokawa et al., 2004; Utermark et al., 2003a; Widemann, 2004).

1.2.2 *nf-2*, the Gene

1.2.2.1 The Structure of the *nf-2* Gene

The human *nf-2* tumor suppressor gene was cloned in 1993 independently by two groups (Rouleau et al., 1993; Trofatter et al., 1993). It was mapped to chromosome 22q12.2 and spans a region of 110kb. The gene encompasses 17 exons (scheme in Figure 1.1). Thirty introns follow the typical gt-ag rule; the others are atypical with less frequent rules such as gc-ag, ct-gg and ct-ag. The analysis of 5'-flanking sequence in the human *nf-2* gene revealed that multiple regions are required for the full promoter activity (Kino et al., 2001). A GC-rich sequence (position -58 to -46) is the most crucial regulatory element to which the transcription factor Sp1 can bind (Chang et al., 2002).

The *nf-2* gene is conserved phylogenetically and can also be found in *C. elegans* and *Drosophila* (Turunen et al., 1998). In 1994, the mouse *nf-2* was isolated and mapped on chromosome 11 in a region homologous to human Chromosome 22; the cloned mouse gene shows more than 90% cDNA sequence identity with the human *nf-2* gene (Haase et al., 1994).

1.2.2.2 The regulation of the *nf-2* Gene

The *nf-2* gene is widely expressed despite the restricted phenotype of the NF2 disease. Northern blotting results revealed that the *nf-2* gene is expressed at high levels during embryogenesis and early postnatal life in most tissues but becomes restricted to the brainstem, cerebellum, dorsal root ganglia, spinal cord, adrenal gland, kidney, lung, breast and testis in adult animals (Gutmann et al., 1995; Hara et al., 1994; Rouleau et al., 1993; Trofatter et al., 1993).

The *nf-2* cDNA was primarily found to have an open reading frame of 1785 nucleotides and encode a protein composed of 595 amino acids (Rouleau et al., 1993; Trofatter et al., 1993). Further studies found three mRNAs with different sizes of approximately 7kb, ~4.5kb, and ~2.6kb. These mRNAs encode at least two major alternative isoforms, consisting of exon 1 through 15 and 17 or exon 1 through 17 (Bianchi et al., 1994; Trofatter et al., 1993). Alternative splicing of other exons such as 2, 3, 8, 10 and 15 have also been reported, but their physiological significance

remains unclear (Arakawa et al., 1994; Pykett et al., 1994; Schmucker et al., 1999). The splice variants of *nf-2* gene demonstrate a tissue-specific and development-specific expression pattern, indicating the possible additional functions of this tumor suppressor gene (Schmucker et al., 1999). Besides alternative splicing, differential usages of multiple initiation and poly-adenylation sites contribute to the complexity of human *nf-2* transcripts; at least 16 different transcripts have been reported so far (Chang et al., 2002).

1.2.2.3 Mutations of the *nf-2* gene

Although there are some additional mechanisms accounting for the inactivation of the *nf-2* gene such as methylation of a promoter region or histone modification, gene mutation remains the main mechanism (Alonso et al., 2004; Gonzalez-Gomez et al., 2003; Kino et al., 2001). A large number of germline and somatic mutations of *nf-2* gene have been found in NF2 patients (Gusella et al., 1999; MacCollin et al., 1993; MacCollin et al., 2003; Zucman-Rossi et al., 1998).

Mutations of *nf-2* gene are observed throughout most of the coding sequences except exon 16 and 17 (Gusella et al., 1999). Most mutations have been found in the first half of the gene, through exon 1 to 8 (Louis et al., 1995). The mutations are mainly frameshift and nonsense mutations, but also some missense mutations have also been discovered. Interestingly, germline mutations differ from somatic mutations. The most frequent germline mutations are point mutations that either alter splice junctions or create new stop codons. Somatic mutations are mainly deletions or insertions. These alterations produce either a frameshift mutation with a subsequent premature stop codon or alternatively disrupt proper splicing. Therefore, most of these mutations, either germline or somatic mutations, are predicted to cause premature translation termination and the subsequent protein truncation (MacCollin et al., 1994; Parry et al., 1996).

Apart from the hereditary NF-2, *nf-2* mutations are involved in the development of other cancers including colorectal cancer, melanoma and breast cancer (Bianchi et al.,

1994; Rustgi et al., 1995). Strikingly, inactivating mutations of the *nf-2* gene are frequently detected in malignant mesothelioma, a rare disease caused as the result of growing malignant cancerous cells lining the patient's body cavities such as the chest, abdominal region or the area surrounding the heart. (Baser et al., 2002; Bianchi et al., 1995; Cheng et al., 1999; Fleury-Feith et al., 2003; Lee and Testa, 1999; Murthy and Testa, 1999; Pylkkanen et al., 2002; Schipper et al., 2003). The fact that individuals with a germline mutation in one *nf-2* allele are more susceptible to develop malignant mesothelioma implicates further the involvement of *nf-2* inactivation in the development of this aggressive disease (Baser et al., 2002; Carbone et al., 2002). However, inactivation of the *nf-2* gene seems to be involved in the progression rather than the initiation of mesothelioma development, since malignant mesothelioma is not a characteristic feature of NF-2.

1.2.3 Merlin, the Protein encoded by the *nf-2* gene

1.2.3.1 The Structure of merlin

The protein encoded by the *nf-2* gene is termed merlin (**m**oesin, **e**zrin and **r**adixin **l**ike prote**in**) because it demonstrates sequence similarity with the ERM proteins, **e**zrin, **r**adixin and **m**oesin, three highly homologous membrane-cytoskeleton linker molecules (Trofatter et al., 1993). ERM proteins belong to a larger superfamily of erythrocyte band 4.1 proteins, that includes the prototype erythrocyte band 4.1 protein and many homologues (Mangeat et al., 1999). Merlin, together with ERM proteins, constitutes the MERM (Merlin and ERM) family (Murthy et al., 1998). The NF2 protein is also called schwannomin for its role in preventing schwannoma formation (Rouleau et al., 1993).

Two major isoforms of merlin exist as a result of alternative splicing of exon 16. Isoform 1 is a 595-residue protein encoded by exons 1-15 and 17. Isoform 2 contains exon 16, which inserts 11 unique C-terminal residues followed by a stop codon that prevents translation of exon 17, generating a 590-residue protein in which the first 579 residues are identical to isoform 1 (Bianchi et al., 1994). Other splice variants have

also been identified at the RNA level, but not detected at the protein level. It is not known whether these other isoforms are expressed under normal physiological conditions and whether they contribute to the functions of merlin (Bianchi et al., 1994). Only isoform 1 has been confirmed to have tumor suppressor function therefore the term merlin is used here to mainly describe isoform 1.

A.
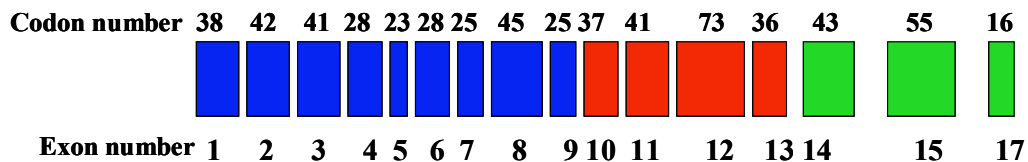
B.
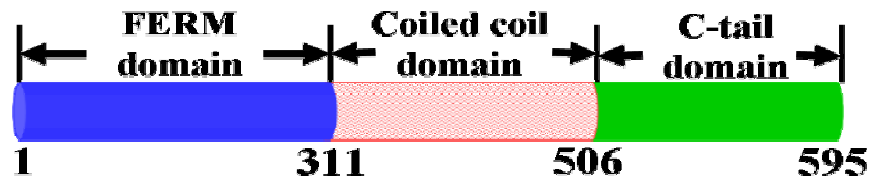
Figure 1.1 The structure of *nf-2* gene and merlin. A. The structure of *nf-2* gene. The *nf-2* gene is composed of 16 constitutively exons with one variably inserted exon (exon 16), which is inserted between exons 15 and 17. The insertion of exon 16 results in a novel carboxyl terminus of merlin with 11 unique amino acid residues and a premature termination codon, eliminating exon 17 sequence. **B.** The structure of merlin. Merlin contains a globular N-terminal domain or FERM domain, a coiled-coil domain and a short C-tail domain.

Merlin shares the predicted overall domain structure of ERM proteins, which consists of a globular FERM domain (erythrocyte band **F**our point one protein, **E**zrin, **R**adixin, **M**oesin) or N-terminal domain (1-311 amino acids in merlin), a coiled-coil domain (312-506 amino acids) and a C-tail domain (507-595 amino acids) (scheme in Figure 1.1) (Rouleau et al., 1993). The FERM domain is responsible for interaction with trans-membrane proteins and membrane binding in a phosphatidylinositol 4,5-bisphosphate (PIP2) - dependent manner (Hamada et al., 2000). Merlin demonstrates homology in its FERM domain to the ERM proteins with over 63% identity, while the homology in the coiled-coil domain and the C-tail domain is only about 20-30% (Gusella et al., 1999; Tsukita and Yonemura, 1997; Turunen et al., 1998). Strikingly, merlin lacks the C-terminal actin-binding site conserved on ERM proteins (den Bakker et al., 2000; Xu and Gutmann, 1998). The strong structural similarity of merlin with the ERM proteins results in many common features (Bretscher et al., 2002; Gautreau et al., 2002; Tikoo et al., 1994). However, merlin is the only member protein in MERM family that functions as a tumor suppressor protein. In Western blot analysis merlin is detected as a ~66 KD protein, close to the molecular weight predicted from its actual sequence while the ERM proteins migrate with their molecular weight bigger than predicted from their sequences. The localization of merlin is also different from ERM proteins (Gonzalez-Agosti et al., 1996). The different gel migration and intercellular localization of merlin and ERM proteins have been ascribed to the distinct structure of their carboxy-terminus. The potential to inhibit cell growth is associated with the isoform 1 containing this carboxy-terminus, whereas neither isoform 2, nor constructs truncated at amino acid 547 or amino-terminus alone, possess such a function, confirming that this unique carboxy-terminus seems indispensable for merlin to be a tumor suppressor (Morrison et al., 2001; Sherman et al., 1997; Tikoo et al., 1994).

1.2.3.2 The distribution of merlin

Although the detection of *nf-2* mRNA indicated its wide distribution, merlin is found mainly in epithelia, mesothelium, smooth muscle, endothelial cells, neurons of the central nervous system, Schwann cells, arachnoid cells and faintly in glial and

ependymal cells (den Bakker et al., 1999; Huynh et al., 1997; Stemmer-Rachamimov et al., 1997a). There is no clear explanation about the discrepancy between protein and mRNA analysis results. One plausible reason could be the low expression level of merlin in these tissues.

Concerning the intercellular distribution, ERM proteins are concentrated in actin-rich specialized plasma membrane structures, such as microvilli, filopodia and membrane ruffles (Berryman et al., 1993; Bretscher et al., 1997; Serrador et al., 1997). Merlin co-localizes with F-actin in motile regions, such as the leading edge (Gonzalez-Agosti et al., 1996). Merlin was also shown to be located at cell/substrate adhesion sites where merlin is associated with stress fibers (Schmucker et al., 1997) and at adherens junctions (AJs) where it physically interacts with AJ components in confluent cultured cells (Lallemand et al., 2003). This indicates that part of merlin's tumor suppressor function could be a direct effect on the formation or stabilization of AJ or could transduce signals from AJ and cell-substrate adhesion sites.

The bulk of cellular merlin were found to localize within the detergent-resistant membrane fraction (DRM), that mainly consists of lipid rafts; virtually no ezrin associates with lipid raft (Stickney et al., 2004). Lipid rafts is the description of the microdomains of the plasma membrane which are resistant to solubilization in nonionic detergents because of their uniquely tight packing of glycosphingolipids and cholesterol moieties (Brown and London, 1998; Henderson et al., 2004; Jacobson and Dietrich, 1999; Munro, 2003; Simons and Ikonen, 1997; van Meer, 2002). The raft residence of merlin could therefore account for its previously reported detergent resistance (den Bakker et al., 2000; Simons and Ikonen, 1997). Lipid rafts are believed to be important for the regulation of signal transduction and membrane trafficking by providing a physical "platform" (Nabi and Le, 2003; Simons and Ikonen, 1997). The raft residence of merlin could therefore indicate a potential role of merlin in signal transduction and the regulation of membrane trafficking (Stickney et al., 2004). Additionally, although merlin is constitutively localized to lipid rafts, the buoyant density of merlin-containing lipid rafts changes with cell density (den Bakker

et al., 2000). This change most likely reflects a dissociation of merlin-containing lipid rafts from the cytoskeleton and could be related to the function of merlin as a negative regulator of cell growth.

Merlin appears not to be localized in the nucleus. In that it seems to be an exception as compared to other tumor suppressors (Fabbro and Henderson, 2003; Levine, 1990; Monteiro and Birge, 2000). However, certain isoforms, such as isoforms with the exclusion of exon 2 or exon 15, were proposed to be able to enter the nucleus (Bischoff et al., 2002; Fornerod and Ohno, 2002; Fried and Kutay, 2003; Kressel and Schmucker, 2002; Serrador et al., 1997; Ullman et al., 1997; Yoshida and Horinouchi, 1999). A putative effect of merlin as a transcriptional regulator therefore remains to be elucidated.

1.2.3.3 The interaction partners of merlin

Despite the lack of the C-terminal actin-binding site presented in ERM proteins, merlin was shown to be associated with the cytoskeleton. First, merlin can bind to polymerized actin directly via its N-terminal domain including residues 178-367 *in vitro* (Brault et al., 2001; den Bakker et al., 2000; James et al., 2001; Xu and Gutmann, 1998). Second, merlin can interact with the cytoskeleton *in vivo* indirectly through oligomerization with ERM proteins (Gronholm et al., 1999; Huang et al., 1998; Meng et al., 2000; Nguyen et al., 2001). Third, an actin-interacting protein called beta II-spectrin (also known as fodrin) can directly bind to merlin through the interaction between the carboxy-terminal domain of merlin and its ankyrin-binding region (Scoles et al., 1998). In addition, merlin can associate with polymerized microtubules *in vitro* by virtue of a novel microtubule binding region in the N-terminal region (Xu and Gutmann, 1998).

Very few binding partners have been known to interact with the unique C-terminus of merlin. Syntenin is one of such interaction partners of merlin (Jannatipour et al., 2001). Syntenin is an adapter protein that couples transmembrane proteins to cytoskeleton and plays a role in intracellular vesicle transport (Grootjans et al., 1997). This interaction seems to be important to regulate the function of merlin because

fibroblast cells deficient of syntenin display alterations in the subcellular distribution of merlin (Jannatipour et al., 2001). Another merlin binding partner is HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), a potential coordinator of endosomal receptor sorting and signaling (Clague and Urbe, 2001; Gutmann et al., 2001; Scoles et al., 2000; Sun et al., 2002a). The interaction with HRS seems to be indispensable for merlin to exert its tumor suppressor function since merlin can only inhibit growth of *HRS* $^{+/+}$, but not *HRS* $^{-/-}$, mouse embryonic fibroblast cells (Sun et al., 2002a).

In contrast to only few binding partners known to interact with the C-terminal part of merlin, many proteins have been reported to interact with N-terminus of merlin (Bretscher et al., 2000; Bretscher et al., 2002; Gautreau et al., 2002; Gusella et al., 1999; Herrlich et al., 2000; Sun et al., 2002b). Such merlin interacting proteins include membrane associated cell adhesion molecules such as CD44 proteins and integrins (Obremski et al., 1998; Sainio et al., 1997). CD44 is the designation of a family of polymorphic transmembrane glycoproteins that function as receptors for hyaluronic acid (HA), a mucopolysaccharide that surrounds cells and is a major component of extracellular matrix (Peach et al., 1993). CD44 proteins are believed to be involved in cell adhesion and trafficking as well as in tumorigenesis and tumor metastasis (Herrlich et al., 2000; Isacke and Yarwood, 2002; Jothy, 2003; Ponta et al., 2003). Merlin interacts with the cytoplasmic tail of CD44 proteins and this association forms a molecular switch controlling cell growth arrest or proliferation (Morrison et al., 2001; Sainio et al., 1997). The association of merlin with integrins have been shown in differentiating Schwann cells (Obremski et al., 1998). The significance of this interaction is not yet known. Elevated integrins levels as well as increased cell spreading were detected in human schwannoma cells lacking the expression of merlin, suggesting that the lack of merlin could lead to the aberrant endocytosis and degradation of integrins (Utermark et al., 2003b). Other cell adhesion molecules such as CD43, intercellular adhesion molecules (ICAM)-1, 2, 3, vascular cell adhesion molecule (VCAM)-1 and E-cadherin might also bind to merlin, since their bindings to ERM proteins via the conserved FERM domain have already been shown (Barreiro et

al., 2002; Hamada et al., 2003; Maeda et al., 1999; Serrador et al., 1998; Serrador et al., 2002). Other merlin interacting proteins include Na^+-H^+ exchanger regulatory factor (NHE-RF), Rho-GDI, schwannomin interacting protein (SCHIP-1) and Paxillin (Fernandez-Valle et al., 2002; Goutebroze et al., 2000; Maeda et al., 1999; Murthy et al., 1998). As a scaffold protein, Paxillin acts as a focal adhesion adapter protein implicated in growth factor- as well as integrins-mediated signaling pathways (Sattler et al., 2000; Schaller, 2001; Tumbarello et al., 2002; Turner, 1994; Turner, 1998; Turner, 2000). The interaction with Paxillin brings merlin to the plasma membrane, where it associates with integrins (Fernandez-Valle et al., 2002). NHE-RF is known to regulate the function of Na^+-H^+ exchanger (NHE), G-protein coupled receptors, platelet-derived growth factor receptor and ion transporters (Ladias, 2003; Shenolikar and Weinman, 2001; Stemmer-Rachamimov et al., 2001; Voltz et al., 2001; Weinman et al., 2000; Weinman et al., 1995). NHE-RF interacts with the FERM domain of merlin through its carboxy-terminal region, at actin-rich structures such as membrane ruffles, microvilli, and filopodia (Murthy et al., 1998). Thus, NHE-RF, through the interaction with merlin, acts as a multifunctional adaptor protein and may play a role in the assembly of signal transduction complexes, linking membrane receptors and ion channels to the actin cytoskeleton. The Rho guanine nucleotide-dissociation inhibitor (RhoGDI) is a general regulator of Rho-family GTPases by forming a complex with the GDP-bound form of Rho and suppressing their activation (Olofsson, 1999; Sasaki and Takai, 1998). ERM proteins bind to RhoGDI with their FERM domain and release Rho from RhoGDI for the subsequent activation. The formation of the complex between RhoGDI and ERM proteins is therefore an important step in the regulatory cycle of Rho activation (Hamada et al., 2001; Takahashi et al., 1997). Merlin can also bind to RhoGDI. The consequence of this interaction, however, seems to be different since no obvious dissociation of Rho from Rho-GDI can be detected after the binding of merlin (Maeda et al., 1999).

One of the cAMP-protein kinase A (PKA) regulatory subunits (RIß) was found to be a novel binding partner of merlin (Gronholm et al., 2003). RIβ and merlin demonstrated a similar expression pattern in the central nervous system and an overlapping

subcellular localization could be detected (Diaz-Enrich et al., 2003; Gronholm et al., 2003; Solberg et al., 1994; Tasken et al., 1993). This interesting interaction suggests a novel function for merlin in connecting the cytoskeleton to PKA signaling.

1.2.3.4 The function of merlin

The ubiquitous distribution of merlin expression during embryogenesis indicated that merlin could play a role in early development. Indeed merlin seems to be required for the extra-embryonic development immediately prior to gastrulation (Gutmann et al., 1995; McClatchey et al., 1997). Mouse embryos homozygous for mutations at the *nf-2* locus failed to develop between embryonic days 6.5 and 7.0, exhibiting a collapsed extra-embryonic region and the absence of organized extra-embryonic ectoderm (McClatchey et al., 1997). At the later stage of development merlin seems to be also necessary for some organogeneses such as the development of heart and eye (Huynh et al., 1996).

The requirement of merlin for normal embryonic development reflects the house-keeping nature of this tumor suppressor. Merlin can also inhibit tumor cell growth both *in vivo* and *in vitro* (Bretscher et al., 2002; Gautreau et al., 2002; Gutmann et al., 1999b; Morrison et al., 2001; Tikoo et al., 1994). The molecular mechanism for the function of merlin remains largely unclear. Numerous results implicated that merlin could be involved in the regulation of signal transduction. Several signaling pathways such as Ras/MAPK (Mitogen Activated Protein Kinase), Rac/PAK (P21 Activated Kinase), JNK (Jun N-terminal Kinase) and STAT (Signal Transducers and Activators of Transcription) are regulated by merlin (Gronholm et al., 2003; Kaempchen et al., 2003; Lim et al., 2003; Morrison et al., 2001; Scoles et al., 2002). There are two main mechanisms that could account for the involvement of merlin in so many divergent signaling pathways. First, merlin could regulate multiple pathways in general at the membrane receptor level through its regulation of membrane trafficking (Fraenzer et al., 2003; McCartney and Fehon, 1996; Scoles et al., 2000). Second, merlin could regulate signal transduction indirectly, via its influence on the cytoskeleton organization since the concordant cytoskeleton organization may be important for the

propagation of signals (Bauch et al., 2000; Carpenter, 2000; Carraway and Carraway, 1995). In addition, merlin could regulate signal transduction pathways separately via interactions with certain signaling molecules in different pathways.

Results from our group showed that Merlin could disrupt the assembly of signaling complex containing Ras and its major activator SOS (Son Of Sevenless) and thereby inhibit the activation of Ras. The association of Ras with its main downstream effectors Raf-MEK was also impaired by merlin, potentiating the inhibitory effect of merlin on this important pathway (Helen Morrison, unpublished data). This novel function of merlin could be due to the replacement of ERM proteins in the association with transmembrane adhesion molecules. The complex composed of ERM proteins and the adhesion molecules was found to be necessary for growth factor induced Ras activation (Helen Morrison, unpublished data)..

Merlin deficiency can also contribute to the activation of Rac/PAK and JNK pathway. The enhanced activation of Rac1 and increased nuclear level of phosphorylated JNK could be detected in merlin deficient schwannoma cells (Kaempchen et al., 2003). The activation of JNK could be the consequence of Rac activation while the activation of Rac could be a downstream event of Ras dependent signaling pathways. Interestingly merlin can interact directly with one of Rac effectors PAK-1 especially under growth non-permissive conditions (Kissil et al., 2003). This interaction could prevent the activation of PAK-1 by active Rac while merlin deficient cells exhibit an elevated PAK-1 activity (Hirokawa et al., 2004; Jaffer and Chernoff, 2002; Kissil et al., 2003). These findings directly lead to the development of the specifc inhibitors of PAK-1 as therapeutic drugs for *nf-2* related diseases (Widemann, 2004).

1.2.3.5 The regulation of merlin

For the ERM proteins, an intra-molecular binding of the N- terminus to the C-terminus is believed to negatively influence their activities (Bretscher et al., 1997; Pearson et al., 2000). Merlin seems to be regulated in a similar way (Gutmann et al., 1999a; Sherman et al., 1997). In order to function as a tumor suppressor, merlin must

form two intra-molecular associations. The first requires the binding of the N-terminus to the C-terminus, whereas the second involves the interaction within the N-terminal domain. The association of the N- and C-terminus of merlin involves residues 302-308 within the FERM domain and residues 580-595 in the C-terminus, whereas residues within sub-domain A and C participate in the intra-N-terminal interaction (Gutmann et al., 1999a; Sherman et al., 1997). The intra-N-terminal interaction is required for the proper localization of merlin beneath the plasma membrane and influences the interaction of merlin with actin cytoskeleton (Brault et al., 2001). The association of the N- and C-terminus is dynamic and relatively weak, since the C-terminus of merlin has a relatively higher affinity for the N-terminus of ezrin rather than for its own N-terminus (Nguyen et al., 2001). This could be important for the regulation of merlin and ezrin activities, perhaps by forming complexes that differentially modulate the abilities to bind to their critical effectors or regulatory molecules (Meng et al., 2000).

Phosphorylation and phospholipid binding is known to weaken the self-association of the ERM proteins and stimulate their abilities to bind to transmembrane proteins and F-actin (Bretscher et al., 2000). Merlin was believed to be regulated in a similar way. An association of merlin with phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) was suggested by the crystal structure of merlin (Shimizu et al., 2002). More importantly, merlin was found to be regulated by phosphorylation as well. Merlin is phosphorylated and inactive in growing cells while it is dephosphorylated and active under non-permissive growth conditions (Morrison et al., 2001; Shaw et al., 1998). The target phosphorylation site was mapped to serine 518 (Xiao et al., 2002). The substitution of serine 518 by phosphorylation defective alanine (S518A mutant) conferred merlin to be constitutively inhibitory cell growth while the substitution of serine 518 by phosphorylation mimicking aspartic acid (S518D mutant) abolished the growth inhibitory function of merlin (Kissil et al., 2002; Surace et al., 2004).

The phosphates are transferred usually from adenine triphosphate (ATP) by protein

kinases and are taken off by protein phosphatases. The dynamic state of cellular protein phosphorylation is therefore dependent on the antagonistic interplay between protein kinases and corresponding phosphatases (Cohen, 2002a; Hunter, 1995; Lechward et al., 2001). One kinase responsible for the phosphorylation of merlin at serine 518 is PAK-2 (<u>P</u>21-<u>A</u>ctivated-<u>K</u>inases-<u>2</u>) (Kissil et al., 2002; Shaw et al., 2001; Xiao et al., 2002). PAK-2 is one of the downstream effectors of Rho family proteins, e.g. Rac1 and Cdc42 (Bagrodia and Cerione, 1999; Jaffer and Chernoff, 2002; Manser and Lim, 1999; Symons, 2000). Recently, merlin was found to be phosphorylated at serine 518 by PKA independent of PAK-2 (Alfthan et al., 2004). Therefore merlin seems to be downstream of both Rac/PAK and cAMP/PKA pathways. The corresponding phosphatase for the dephosphorylation at serine 518 and the subsequent activation of merlin has not yet been identified. Merlin can be dephosphorylated under conditions of high confluence or serum deprivation but not by the treatment of growth-suppressive agents such as staurosporine, hydroxyurea, nocodazol (Shaw et al., 1998). This indicates that this unknown phosphatase is activated specifically under conditions of high confluence or serum deprivation.

1.3 Aims of my project

The principle aim of my PhD work is to identify the phosphatase that dephosphorylates merlin at the serine 518 site and thereby activates merlin. Merlin plays a central role in growth control by mediating contact inhibition. The loss of function of merlin seems to be involved in the development of many cancers. The identification of the phosphatase that activates merlin would therefore not only lead us to understand further how merlin is regulated and how the loss of function of merlin contributes to tumorigenesis but also help us in developing important targets for therapeutic intervention of *nf-2* related disease. Merlin is dephosphorylated under conditions of confluency or serum deprivation. Thus, under these conditions the phosphatase of merlin should be active. In contrast, this phosphatase is inactive under conditions when merlin is phosphorylated, e.g. serum stimulation. First, I would propose to establish a suitable system that could be used to monitor the activity of

merlin specific phosphatase under these conditions. Subsequently, I would screen the candidate phosphatase in this system by using phosphatase inhibitors. Afterwards, I will investigate the interaction of merlin with the putative phosphatase both *in vivo* and *in vitro*. If possible, I will try to identify the interaction region in merlin and also in the phosphatase. A further goal will be to confirm the effect of the identified phosphatase on merlin by investigating the regulation of the phosphatase *in vivo* and its relationship with merlin phosphorylation, growth control, and tumorigenesis.

2. Materials and methods

2.1 Materials

2.1.1 General Chemicals

Name	Supplier
Acetic acid	Merk, Darmstadt
Agarose	Peqlab, Erlangen
Ammonium persulfate	Sigma, Deisenhofen
Ampicillin	Roche Diagnostic, Mannheim
Bacto-Agar	Roth, Karlsruhe
Bacto-Yeast Extract	Roth, Karlsruhe
Bacto-Peptone	Roth, Karlsruhe
Bromophenol blue	Serva, Heidelberg
Bovine serum albumin (BSA)	Serva, Heidelberg
Chloroform	Merck, Darmstadt
Coomassie Brilliant Blue G 250	Serva, Heidelberg
Dimethyl Sulfoxide (DMSO)	Fluka, Neu-Ulm
Dithiothreitol (DTT)	Sigma, Taufkirchen
Ethanol	Roth, Karlsruhe
Ethidium bromide	Sigma, Taufkirchen
G418	GIBCO,Eggenstein
Glycerol	Merck, Darmstadt
Hydrochloride (HCL)	Merck, Darmstadt
Hyaluronic acid (HA)	Sigma, Deisenhofen
Isopropanol	Merck, Darmstadt
Methanol	Roth, Karlsruhe
Nonidet P-40 (NP-40)	Boehringer, Mannheim
Puromycin	Merck, Darmstadt
Rotiphorese® Gel30:	Roth, Karlsruhe

Acrylamide/bis-Acrylamide

Sodium Chloride   Roth, Karlsruhe

Sodiumdodecylsulfate (SDS)   Roth, Karlsruhe

Sodium hydroxide   Roth, Karlsruhe

Tris   Roth, Karlsruhe

Tris Hydrochloride   Roth, Karlsruhe

Triton-X100   BioRad, München

Tween-20   Roth, Karlsruhe

All other chemicals were, unless otherwise stated, supplied with highest purity grade by Merck (Darmstadt), Carl Roth GmbH & Co (Karlsruhe) and Sigma Chemie GmbH (Deisenhofen).

2.1.2 Primers

All of the primers were synthesized by MWG Biotech GmbH (München, Germany) with HPSF purity grade.

2.1.2.1 Standard primers

Name	sequence
GST-C-merlin FW	5'-TTCCACGGATCCAAAGCCGATTCTTTG-3'
GST-C-merlin RV	5'-GACGCGAATTCCTCTTCAAAGAAGGCC-3'
GST-C-merlin Δ FW	5'-TATAGGATCCGAGAAGCAGATGAGGGA-3'
GST-C-merlin Δ RV	5'-GACGCGAATTCCTCTTCAAAGAAGGCC-3'
GST-C-merlin ΔCC FW	5'- CAAGAGGGATCCCTGTCTTTCGACTTC-3'
GST-C-merlin ΔCC RV	5'-GACGCGAATTCCTCTTCAAAGAAGGCC-3'
pcDNA-C-merlin Δ FW	5'-TATCCGCCACCATGGAGAAGCAGATGA-3'
pcDNA-C-merlin Δ RV	5'-CTAGAGCTCTTCAAAGAAGGCCACTCG-3'

2.1.2.2 Mutagenesis primers

Name	sequence
merlin S518A1	5'-CTGACATGAAGCGGCTTGCCATGGAGATAGAGAAAG-3'
merlin S518A2	5'-CTTTCTCTATCTCCATGGCAAGCCGCTTCATGTCAG-3'
merlin S518D1	5'-CTGACATGAAGCGGCTTGACATGGAGATAGAGAAAG-3'
merlin S518D2	5'-CTTTCTCTATCTCCATGTCAAGCCGCTTCATGTCAG-3'
merlin -Q324L1	5'-CAGATGAAAGCCCTGGCCAGGGAGGAGA-3'
merlin -Q324L2	5'-TCTCCTCCCTGGCCAGGGCTTTCATCTG-3'
merlin -L316F1	5'- GGAAAGCCGATTCTTTCGAAGTTCAGCAGATGAAAG-3'
merlin -L316F2	5'-CTTTCATCTGCTGAACTTCGAAAGAATCGGCTTTCC-3'
merlin -L339F1	5'-GAGCGGCAGCGCTTCGCTCGAGAGAA-3'
merlin -L339F2	5'-TTCTCTCGAGCGAAGCGCTGCCGCTC-3'

2.1.3 Plasmids

Constructs encoding GST-C-merlin and its truncations were generated by inserting corresponding PCR products into pGEX 4T.1 empty vector in frame using multiple cloning sites of EcoR I and BamH I. Other constructs used here are listed below with the name, insert and source.

Name	Insert/usage	Source
pUHD 10.3 Nf2-C	human Nf2-C cDNA	Dr. Morrison, Karlsruhe
pUHD 10.3 Nf2-FL WT	human NF2-FL cDNA	Dr. Morrison, Karlsruhe
pUHD 10.3 Nf2-FL S518A	human NF2-FL cDNA	Dr. Morrison, Karlsruhe
pUHD 10.3 Nf2-FL S518D	human NF2-FL cDNA	Dr. Morrison, Karlsruhe
pGEX4-MYPT-1 WT	WT human MYPT-1 cDNA	Dr. Surks, Boston
pGEX4-MYPT-1 MT	MT human MYPT-1 cDNA	Dr. Surks, Boston
pcDNA3.1-CPI-17	Human CPI-17 cDNA	Dr. Aitken, Edinburgh

2.1.4 Enzymes

All restriction endonucleases and other modifying enzymes were purchased from Invitrogen GmbH (Karlsruhe, Germany), Promega (Mannheim, Germany) and New England Biolabs (Beverly, USA) unless otherwise stated.

2.1.5 Cell culture regents

Dulbecco's Modified Eagle's Medium (DMEM) and Phosphate Buffered Saline (PBS) were purchased from GibcoBRL Life Technologies (Karlsruhe, Germany). Foetal calf serum (FCS) was provided from PAA Laboratories GmbH (Linz, Austria).

2.1.6 Cell lines

Name	Description	Culture Medium
RT4-D6P2T (RT4)	Rat schwannoma cell line	DMEM, 10% FCS
RT4-54	RT4 expressing dox-inducible full-length merlin	DMEM, 10% FCS
RT4-C	RT4 expressing dox-inducible C-merlin	DMEM, 10% FCS
NIH-3T3	Mouse fibroblast cell line	DMEM, 10% FCS

2.1.7 Antibodies

2.1.7.1 Primary antibodies

Name	Supplier
NF2 (C-18), rabbit polyclonal	Santa Cruz,USA
NF2 (A-19), rabbit polyclonal	Santa Cruz, USA
MYPT-1, goat polyclonal	Santa Cruz, USA
Phospho-MYPT-1, goat polyclonal	Santa Cruz, USA
PP1c, mouse monoclonal	Santa Cruz, USA
PP2A,goat polyclonal	Santa Cruz, USA

PP1β, goat polyclonal	Santa Cruz, USA
Actin, goat polyclonal	Santa Cruz, USA
Histone, goat polyclonal	Santa Cruz, USA
Anti-flag, mose monoclonal	Sigma, Germany
Phosphor-merlin (serine518), rabbit polyclonal	Abcam, UK

2.1.7.2 Secondary antibodies

Name	Supplier
HRPconjugated goat anti-rabbit IgG	DAKO, Denmark
HRPconjugated rabbit anti-goat IgG	DAKO, Denmark
HRPconjugated goat anti-mouse IgG	DAKO, Denmark

2.2 Methods

2.2.1 Preparation of competent bacteria

2.2.1.1 Preparation of chemically competent *E.Coli*

A single colony of *E.Coli* (DH5α) from LB-Agar plate (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% Agar) was inoculated into 3 ml LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) without selection pressure and incubated overnight at 37ºC with shaking (220 rpm). One ml of this medium was diluted to 100 ml LB medium and grown to an OD_{600} of 0.3-0.5. Bacteria were collected by centrifugation at 4000 rpm for 10 minutes at 4°C. The pellet was washed with 25 ml of chilled sterile $MgCl_2$ (100 mM) and recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. Then the pellet was resuspended in 5 ml chilled sterile $CaCl_2$ (100 mM), incubated on ice for at least 30 min and precipitated by centrifugation at 4000 rpm for 5 min at 4°C. Finally, all of the bacteria were resuspended in 6 ml chilled $CaCl_2$/glycerol (4.8 ml 100 mM $CaCl_2$ plus 1.2 ml 86% glycerol). After 5 minutes incubation on ice, the bacteria were dispensed in 200 µl aliquots to thirty pre-chilled eppendorf tubes and snap-frozen in liquid nitrogen before being stored at - 80°C.

2.2.1.2 Preparation of electrocompetent *E.Coli*

As above, a single colony of *E.Coli* (DH5α) was inoculated into 1 ml of YENB (7.5g/l Bacto yeast extract, 8.0g/l Bacto Nutrient broth) medium and cultured overnight at 37°C with shaking. This is very important as it eliminates all the steps needed to remove the salts. Salts are known to reduce the efficiency of electrotransformation and cause arcing in the electroporation cuvette. One ml of this medium was taken to further culture in 500 ml of fresh YENB medium until it reached an OD600 of 0.5-0.9. The bacteria were harvested as described above. The pellet was resuspended in 2 ml of cold 10% glycerol after two times washing with 100ml cold sterile water and one time washing with cold 10% glycerol. Finally, the bacteria were dispensed in 40 µl aliquots to 50 pre-chilled eppendorf tubes and snap-frozen in liquid nitrogen before being stored at - 80°C.

2.2.2 Transformation of *E.Coli*

2.2.2.1 Chemical transformation

Depending on the application, 5 ng of super coiled plasmid or 1 µl of a ligation mix (usually a 1/10 of the ligation) was added to 200 µl of competent cells and left on ice for a period of 30 minutes. Following this, the cells were heat-shocked at 42°C for 90 seconds before rapidly return of the tube to ice for a few minutes. After addition of 1 ml antibiotic free SOC medium (2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO4 and 20 mM glucose), the bacteria were incubated for 45 min at 37°C with shaking. The cells were then pelleted lightly by a short centrifugation (3 min at 1000x g) and 1 ml of the supernatant was removed before resuspending the cells in the remaining 200 µl. A volume of 50-200 µl was plated out on LB-agar plates supplemented with the correct antibiotic and the plates incubated 18-24 hours at 37°C.

2.2.2.2 Electroporation transformation

One to five µl of DNA or 1/10 of a ligation product was mixed with one 40 µl aliquot of competent cells (Salt can be removed from ligation mix by phenol/chloroform extraction and then ethanol precipitated). The mixture was transferred to a cold 0.2-cm electroporation cuvette (Bio-Rad) and electroporated in a Bio-Rad Gene Pulser R according to the manufacturer's instructions (1.8 Kv). The cuvette was removed from the chamber and immediately 1 ml of YENB medium was added to the cuvette. The cells were re-suspended and transferred to a polypropylene tube (17 x 100 mm) for 1 hour incubation at 37°C with shaking. The cells were then plated onto the antibiotic containing LB-Agar plates as described previously.

2.2.3 Plasmid preparation

2.2.3.1 Small scale plasmid preparation (minipreparation)

Individual colonies were picked from LB-Agar plate and inoculated into 3 ml of antibiotic containing LB medium. After overnight incubation at 37°C with shaking, one ml of bacteria was pelleted by centrifugation at 13,000 g for 10 sec. The pellet was resuspended compeletly in 50 µl of solution I (50 mM glucose, 25 mM Tris·Cl, pH 8.0, 10 mM EDTA, pH 8.0) and incubated on ice for 5 min. One hundrend µl of solution II (200 mM NaOH, 1 % SDS, made freshly) was then added and mixed gently by inverting the eppendorf tube for 6 times. After 5 min incubation, 75 µl solution III (3M sodium acetate, pH 4.8) was added, mixed gently and incubated on ice for 5 min. The protein-DNA complex was cleared off by centrifugation at 13,000 g for 3 min, and the supernatant was transferred to another eppendorf tube, mixed well with 2 volume of 100% ethanol, and incubated at room temperature for 5 min. The plasmid was finally recovered by centrifugation at 13,000 g for 1 min, washed with 70% ethanol once, and dissolved in 20 µl of TE (10 mM Tris·Cl, pH 8.0, 1 mM EDTA pH 8.0) containing RNase A (20 µg/ml).

2.2.3.2 Large scale plasmid preparation (maxipreparation)

The Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) was used following the

manufacturer's instructions. Usually, a volume of 200-400 ml of LB medium supplemented with the relevant antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37°C overnight till the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 4000 g for 10 min and the pellet resuspended in 10 ml of solution (10 mM EDTA, 50mM Tris-HCL pH 8.0 and 400 µg/ml of RNase A). Following 5-10 min incubation at room temperature, the cells were lysed by addition of 10 ml of solution II (200mM NaOH and 1% SDS). Once the solution had taken an opaque appearance, the mixture was neutralized with 10 ml of solution III (3 M potassium acetate pH 4.8) the entire contents gently inverted to aid mixing of the solutions. After an additional 10-20 min on ice, the cell wall fragments and the bacterial chromosomal DNA were sedimented by centrifugation at 13000 x g for 20 minutes at 4°C in a fixed angle rotor. The retained supernatant was then added directly to a pre-equilibrated Quiagen-tip 500 column (Qiagen Inc.) and the plasmid DNA was recovered using the manufacturer's supplied buffers. The purified DNA was precipitated using 0.8-1.0 volumes of isopropanol, washed twice in 70% ethanol before resuspending the DNA to a final concentration of 1-3 mg/ml in bi-dest H_2O and stored at -20°C.

2.2.4 Determination of nucleic acid concentration

The concentration of DNA was determined by spectroscopic measurement of their optical density (OD) at 260nm and OD280nm. The OD value of one at 260nm is equivalent to 50µg/ml of double standed DNA. Pure DNA in aqueous solution should have an OD260/OD280 ratio of 1.6-1.8.

2.2.5 Restriction endonuclease digestion of DNA

Usually 2-3 units of a restriction enzyme for each µg DNA were used. DNA was digested at a concentration of 1 µg/10 µl in a buffer recommended by the supplier. The reaction was carried out for 2 hours to overnight at 37°C (unless otherwise recommended by the supplier) and was stopped by a phenol/chloroform extraction.

The DNA was precipitated with ethanol. The quality of the digest was controlled by gel electrophoresis.

2.2.6 Nucleic acid analysis by agarose gel electrophoresis

The required amount of agarose (SeaKem, Biozym Diagnostik, Hameln, final concentration between 0.8 and 2%) was dissolved in 50 ml electrophoresis buffer (TBE: 90mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Ethidium bromide was added at a concentration of 0.3μg/ml. The molten gel was poured into a horizontal (13.5 × 8 cm) chamber. Combs with the appropriate number and size of the teeth were used to make the loading slots. The gel (when set) was with 200 ml electrophoresis buffer and run at 35-45 mA (50-100 V) at room temperature for the required time. Samples were loaded onto the gel in loading buffer (10 mM EDTA, 10% glycerol, 0.1% SDS, 0.02% bromophenol blue). DNA was visualized by transillumination with 302 nm ultraviolet radiation.

2.2.7 Isolation/purification of DNA from agarose gels

In general, the EasyPure DNA purification Kit (Biozyme, Oldendorf, Germany) was used to isolate the appropriate DNA fragments in the agarose gel (e.g. restriction-digested vectors and PCR products). The DNA band of choice was cut out from the gel, under long wave UV light with the aid of a scalpel. DNA containing gel strip was added to 3x its weight to volume of "salt buffer" (all reagents provided in the kit). The gel piece was melted in the buffer by incubation at 55°C before the binding resin was added. After two subsequent washing steps the resin with bound DNA was air dried and the DNA eluted by addition of bi-dest H_2O.

2.2.8 Phenol/Chloroform extraction of nucleic acid

To remove unwanted protein contaminants from nucleic acids, an equal volume of Tris-buffered phenol, chloroform and isoamylalcohol (2-propanol) at a ratio of 25:24:1 was added and mixed with vortex. The two phases were separated by

centrifugation at 10000g for 10 min. The aqueous nucleic acid containing upper phase was transferred to a new reaction tube and subjected to a further round of extraction with chloroform/isoamylalcohol (24:1).

2.2.9 Ligation

In all cases, the insert and vector were loaded on agarose gel to check the DNA content before ligation, and ligated at the amount ratio of about 4:1 (insert:vector). Ligation was performed in a total volume of 20 μl with insert, vector, 1x ligation buffer and 1 μl of T4 ligase (Invitrogen, Karlsruhe, Germany) and incubated at 16°C overnight.

2.2.10 Precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration was adjusted to 200 mM with 3 M sodium acetate (PH 5.0) and then 2 volumes of cold ethanol was added. After 2 hours to overnight incubation at - 20°C or 30 min at - 80°C, the nucleic acids was precipitated by centrifugation with 13000 rpm for 15 min. The pellet was dried after one time washing with 70% ethanol. Finally, the nucleic acids was re-suspended in sterile water or TE buffer (10mM Tris-HCL, 1mM EDTA, PH 8.0).

2.2.11 Polymerase Chain Reaction (PCR)

All PCR were performed in a total volume of 50 μl, in the presence of 10 ng templates, 200 μM dNTPs, 0.2 μM of each primer, 1× supplier's reaction buffer, 10% DMSO and 1 μl GoTaq polymerase (Promega, Mannheim, Germany). The reactions were carried out in PCR thermocycler (Perkin Elmer, Norwalk, USA), using the following cycling parameters: 94°C, 2 min, 1 cycle; 94°C, 30 sec, 55°C, 30 sec and 72°C, 45 sec for a total of 30 cycles. The PCR products were analyzed by agrose gel electrophoresis.

2.2.12 Site-directed mutagenesis

Mutagenesis was performed according to the instruction manual of QuickChange™Site-Directed Mutagenesis kit (Stratagene, La jolla, USA). The reaction was set up as indicated below: 5 µl of 10× reaction buffer, 50 ng of DNA template, 2×125 ng of primers, 1 µl of dNTP mix (provided in the kit), 1 µl of pfu Turbo DNA polymerase (2.5 µg/µl) and ddH$_2$O was added to make a final volume of 50 µl. The reaction was subject to the following PCR program: segment 1: 95°C, 30 sec; segment 2: 95°C, 30 sec; 55°C, 1 min; 68°C, 10 to 14 min (depending on the vector used); 12 cycles totally. After PCR amplification, 1 µl of the *DpnI* restriction enzyme (10 U/µl, provided in the kit) was directly added to reaction and incubated at 37°C for 1 hr. Ten µl of *DpnI* treated DNA was transferred to 100 µl of *E. Coli* XL1-Blue supercompetent cells. After heat pulsed at 42°C for 45 sec, the transformation reaction was placed on ice for 2 min. One ml of LB medium (ampicillin free) was then added All the bacteria were plated on LB plate (100 µg/ml of ampicillin) after 1 hour incubation at 37°C with shaking and incubated overnight at 37°C. Two single colonies were picked for plasmid mini-preparations and subsequent sequencings by GATC biotech (Konstanz, Germany).

2.2.13 TOPO TA cloning

The plasmid vector (pCR2.1) is supplied linearized with single 5'-deoxythymidine (T) overhangs for TA cloning and with a covalently bound topoisomerase I. Because Taq polymerase has a non-template dependent terminal transferase activity, it can add a single deoxyadenodine (A) to the 3' end of the PCR products. This allows the PCR products to efficiently ligate with the vector's overhanging 5'-T residues. The reactions for TA cloning were performed according to protocols provided in the TOPO (TA) cloning Kit (Invitrogen, Groningen, Netherlands).

2.2.14 Cell culture

All cells were maintained at 37°C in the incubator (Forma Scientific Labotect GmbH, Göttingen, Germany) with 5% CO_2 and 95% air humidity. All cells were grown in petri dishes (Greiner Labortechnik, Flikenhausen, Germany) of varing sizes depending on the application. Storages of cells were done by harvesting cells and adding 1 ml of freezing medium (10% DMSO, 50%FCS). The cells were transferred to cryovial, left on ice for 1hr and transferred to –80°C for 1 day before finally to liquid nitrogen. To thaw cells, the cryovial was removed from liquid nitrogen and placed at 37°C for 5 to 10 minutes. The cells were transferred to 10 ml of pre-warmed fresh medium followed by light centrifugation (to remove DMSO) before being replated on petri dishes in fresh medium.

2.2.15 Transfection

Cells used for transfection were plated 24 hours before transfection. Usually, stable transfections were performed in 6 well petri dishes containing 3×10^5 cells, giving a confluency of 60-80%. Transfection was performed with the liposomal transfection reagent FuGENE 6 (Roche, Mannheim, Germany) as described in the protocol provided. For stable transfection, the transfected cells were reseeded 24 hours after transfection and placed under appropriate antibiotic selection to generate clones. After a period of 1-2 weeks, visible clones were picked and placed in 24 well petri dishes and propagated further under selection until sufficient cell number had been reached. For transient transfections, the transfectant cells were cultured without antibiotic selection for further experiments.

2.2.16 Cell extracts preparation for Western Blot analysis

Cells were either lysed directly in the SDS-PAGE sample buffer (120mM Tris-HCl, pH 6.8, 4%SDS, 50mM DTT, 20% v/v glycerol and 0.01% bromophenol blue) before a sonication to break down the chromosomal DNA, or lysed in buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 0.5% NP-40, $1 \times$ protease inhibitor cocktail), and the resulting cell extract was mixed with equal volume of $2 \times$ sample buffer. For both preparations, all of the samples were boiled for 5 min before loading in a SDS-PAGE

gel.

2.2.17 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For most applications a polyacrylamide separating gel of 7.5-12.5% and a 5% stacking gel were made. Reagents for a separating gel were 8.3 ml of acrylamide/bis-acrylamide (30:0.8, Carl Roth GmbH & Co, Karlsruhe), 6.25 of 1.5 M Tris-HCl (PH 8.8), 0.125 ml of 20% SDS, and 10.05 ml of H_2O (The amount of acrylamide/bis-acrylamide and H_2O were adjusted accordingly to prepare separating gels with different percentages of acrylamide). To the mixture 250 µl of 10% ammonium persulphate (APS) was added and the reaction initiated with 20 µl of TEMED (Tetramethylethylenediamine). The gel mix was poured between two glass plates with spacers between and left for a while. Upon the completion of the polymerization, a stacking gel is poured on top. This was made up of 1.7 ml of acrlamide/bisacrylamide, 2.5 ml of 0.5 M Tris-HCl (PH 6.8), 50 ml of 20% SDS, 5.65 ml of H_2O, 100 µl of 10% APS and 7.5 µl of TEMED. The gel was then run in 1× laemmli-running buffer (25mM Tris-HCl pH 8.3, 0.2 M glycine and 0.1% SDS) until the desired distance had been reached.

2.2.18 Staining of gels

In general, Coomassie Brillant Blue G-250 was used for gel staining. Gels were stained with staining solution for overnight (at least 3 hours) after being fixed 1 hour in fixation solution (40% methanl, 10% acetic acid), and then destained carefully only with water until clear background was obtained. The staining solution was prepared as following: mix 98% solution A (20 g 85% H_3PO_4, 100g $(NH_4)_2SO_4$ and add H_2O to final volume of 980 ml) and 2% solution B (5% w/v Coomassie brilliant blue G250), and shake it intensive overnight, the resulting solution was called solution C. The staining solution was obtained by mixing 80% solution C and 20% methanol and should be used at the same day of preparation.

2.2.19 Western blotting

Proteins resolved by SDS-PAGE were transferred to Immunobilon-PVDF membrane (Millipore) using BioRad Transfer chambers containing transfer buffer (24 mM Tris base, 193 mM glycine, and 10% methanol). Transfer was normally performed at 4°C overnight.

2.2.20 Probing

The membrane was blocked for 1 hr in blocking buffer (PBS with 0.3% Tween 20 and 5% dry milk or BSA) and incubated with primary antibody with variant dilutions (1:500 to 1:1500, depending on the antibody) in blocking buffer for 1 hr at room temperature or overnight at 4°C. After 4 times of washing with 0.3% Tween 20 in PBS, the membrane was incubated with HRP labelled secondary antibody (1:2000) at room temperature for 1 hr and washed again with 0.3% Tween 20 in PBS for 3 times. The protein of choice finally were visualized by enhanced chemiluinescence using ECL Western blotting reagents and ECL-Hyperfilm (Amersham Life Science, Buckinghamshire, UK) following the manufacture's instructions.

2.2.21 Stripping Western blot membrane

To utilize Western blots more than one time, the PVDF membrane were stripped as following: incubate the membrane with 50 ml Strip solution (62.5 mM Tris, pH 6.8, 2% SDS, 0.8% DTT) at 50°C for 45 min with shaking. Wash twice with PBS containing 0.3% Tween 20 and then block as usual and proceed for the normal western blot probing protocol.

2.2.22 Immunoprecipitation

2.2.22.1 Cell lysis and pre-clearing

Cells were grown to confluency (2×10^6), washed once with ice-cold PBS before lysing the cells on ice with 1 ml of lysis buffer (50 mM Tris pH7.4, 100 mM NaCl, 3 mM MgCl$_2$, 0.5% NP-40) containing $1\times$ Complete^{TM} protease inhibitors cocktail (Roche, Mannheim, Germany). The lysed cells were then scraped and collected into a

reaction tube. The cell lysate was homogenized by passing it several times through the pipette tip and incubated on ice for 30 minutes before clarification by centrifugation for 5 min 10,000× g at 4°C. The clarified supernatant was then transferred for pre-clearing into a new reaction tube. The tubes were rotated end over end at 4°C for 1 – 2 hr after the addition of 30 µl of 50% protein G-agarose beads slurry into the lysate. The recovered supernatant by centrifugation at 12,000g at 4°C for 5 min was used for immunoprecipitation.

2.2.22.2 Immunoprecipitation

Certain amount of the antibody (normally 1 to 5 µg) was added to the precleared cell lysate and the tube was rotated at 4°C. Two to four hours later, 30µl of 50% protein G-agarose beads slurry was added and the samples was rotated at 4°C for further 4 to 6 hours. Immuncomplexes were then recovered by 3 min of gentle centrifugation (4500 rpm). After 4 times of wash with cold lysis buffer, 50 µl of 2 × Laemmli sample buffer was added and the samples were subjected to SDS-PAGE analysis.

2.2.23 *In vitro* dephosphorylation assay

After immunoprecipitation, the beads were washed twice with different reaction buffers (depending on the phosphatase used). Certain amounts of CIP (calf intestine phosphatase), purified PP1 catalytic subunit or PP2A catalytic subunit was added to the beads respectively. The dephosphorylation occurred at 37°C after gentle mix. The reaction was stopped by centrifugation after one hour. Finally, the beads were sampled for SDS-PAGE analysis.

2.2.24 Preparation of GST and GST fusion proteins (large scale)

The bacteria containing GST (Glutathione-S-Transferase) or GST fusion constructs were inoculated into 20ml LB medium and incubated at 37°C with shaking (200 rpm) until it reached the logarithmic growth phase. Further incubation after 1:50 dilution with the fresh medium was carried out until it reached the OD about 0.5. The

expression of GST or GST fusion protein was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG, 0.1mM). After more than 3 hours of induction at room temperature with shaking (200 rpm), the bacteria were harvested by 5 min of centrifugation at 5000 rpm. The bacteria was washed twice with cold PBS and then frozen at - 80°C for one hour to overnight. Appropriate sonication was performed to lyse the bacteria in 10 ml protease inhibitors containing cold PBS by using the Branson Sonifier (Heinemann, Germany). One ml of 10% Triton-X 100 (BioRad, München, Germany) was added into the lysate and the lysate were then rotated for 20 min at 4°C. Subsequently, the lysate was cleared at 4°C by 10 min of centrifugation at 12,000 rpm and the supernatant was transferred to a fresh tube containing 400 µl 50% slurry of Sepharose 4B beads (Amershem Pharmacia, Uppsala, Sweden). The beads were finally harvested after at least two hours of incubation with gentle rotation at 4°C. The beads were then dispensed into 20 vials after 3 times of wash with cold PBS and stored at - 80°C.

2.2.25 GST pull down assay

In principle, it was performed as immunoprecipitation. However, the Sepharose beads with GST or GST fusion protein, but not antibody and protein G coupled agarose beads, were added into the cell extracts. The incubation time varied from 1 hour to overnight. The beads was recovered by gentle centrifugation and sampled by adding 2 × Laemmli sample buffer for SDS-PAGE analysis.

2.2.26 Cleavage of the GST tag from GST fusion protein

To obtain GST free purified protein for *in vitro* binding assay, thrombin was used to remove GST tag from the GST fusion protein since it contains a Thrombin recognition site. Two units per mg fusion protein of thrombin (Amershem Pharmacia, Uppsala, Sweden) were added to the fusion protein anchored beads. The digestion occurred after gently mix at room temperature. After 2 to 16 hours of complete digestion (depending on the protein of interest), the GST free purified protein was collected

simply by centrifugation at 4°C. The supernatant was then stored as the source of the GST free purified protein for the *in vitro* binding assay.

2.2.27 *In vitro* binding assay

In principle, it was performed as GST pull down assay. However, the GST free purified protein, but not the whole cell extract, was used as the source of input. The binding was allowed to take place for one hour at 4°C with gentle rotation. The beads was recovered by gentle centrifugation and sampled by adding 2 × Laemmli sample buffer for SDS-PAGE analysis.

2.2.28 Soft agar assay

Cells were detached from culture plates with 0.25% trypsin, resuspended in complete media and counted. Ten thousand of cells were resuspended in 3.6 ml of medium containing 10% FCS. Resuspension were divided into two and Dox (Doxycycline) was added to one. To each mixture 200 µl of warm stock agar solution (3.3% in sterile PBS) was added and mixed. The mixture was rapidly dispensed into 24 well plates at 500 µl volume and cooled for 2 min at 4°C. Colony was counted after 7 to 10 days.

3. Results

In order to identify the phosphatase of merlin, a system needs to be established that can be used to monitor the activity of this merlin specific phosphatase. In the MERM family merlin is the only member which functions as a tumor suppressor (Bretscher et al., 2002; McClatchey, 2003). But also merlin is a structurally distinct member of MERM family mainly because it differs considerably from other members in its carboxy-terminus, which is particularly well conserved throughout evolution (Haase et al., 1994; McCartney and Fehon, 1996; Rouleau et al., 1993; Stemmer-Rachamimov et al., 1997b). Previous results in our group showed that this unique carboxy-terminus was actually indispensable for merlin to execute its tumor suppressor function (Morrison et al., 2001). Furthermore, the critical phosphorylation site of merlin, serine 518, is indeed located in this unique carboxy-terminus (Kissil et al., 2002; Xiao et al., 2002). I therefore choose to focus on the carboxy-merlin as the target to establish the system.

3.1 C-merlin is regulated similar to full-length merlin

RT4-D6P2T cell line is a N-ethyl-N-nitrosourea (ENU)-induced rat schwannoma cell line (Bansal and Pfeiffer, 1987). It has been found that the full-length merlin in this cell line, both endogenously and ectopically expressed (this engineered cell line was named RT4-54), could be activated by dephosphorylation at serine 518 in response to different stimuli such as cell-cell/ECM contacts and serum deprivation (Morrison et al., 2001). This indicates that the activating phosphatase of merlin should exist and be functional in this cell line. It was therefore engineered to express an epitope flag tagged carboxy-terminus of merlin (300-595, C-merlin) only in the presence of doxycycline (Dox) by using the Tet-On System (Morrison et al., 2001; Shockett et al., 1995). This modified cell line is named RT4-C.

3.1.1 Phosphorylation of C-merlin

The expression of C-merlin upon Dox treatment of RT4-C cells reached considerably high levels (Figure 3.1A). Interestingly, C-merlin appeared as a doublet upon SDS-PAGE separation and subsequent Western blotting with both the anti-merlin antibody (Figure 3.1A) and the anti-flag antibody (data not shown). Since full-length merlin is

A
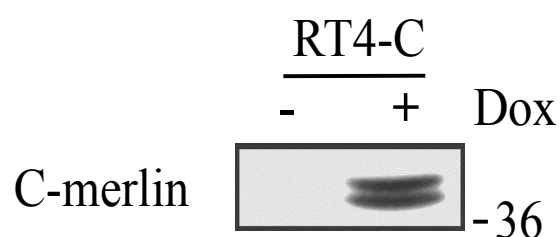
B
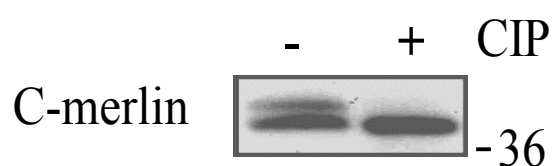
Figure 3.1. C-Merlin can be phosphorylated. (A) RT4-C cell line expresses C-merlin only after Dox treatment. The expression of C-merlin in RT4-C cells in the absence or presence of Dox (8 hours) was detected by Western blotting using the antibody against merlin (C-18). **(B)** CIP alters the electrophoretic migration abilities of C-merlin. Immunoprecipated C-merlin was treated with or without CIP for 1 hour at 37°C and then analyzed by Western blotting with the merlin specific antibody as A.

a phospho-protein and the phosphorylation site is located in the C-terminus, the two bands could reflect two different phosphorylation states. To test this, C-merlin was enriched by immuno-precipitation (IP) with the anti-flag antibody and then treated for one hour at 37°C with calf intestinal phosphatase (CIP) before being separated by SDS-PAGE. Western blot revealed that after CIP treatment the slower migrating C-merlin band was merged into the faster migrating band, suggesting that the slower migrating form of C-merlin was indeed phosphorylated (Figure 3.1B). Taken together, C-merlin, like full-length merlin, can also be regulated by phosphorylation in RT4 cells.

3.1.2 Regulation of C-merlin phosphorylation

The phosphorylation state of full-length merlin is regulated by cell density, serum conditions and HA incubation (Morrison et al., 2001; Shaw et al., 1998). Similar to full-length merlin, the phosphorylation of C-merlin was increased dramatically upon serum addition after overnight starvation of RT4-C cells (Figure 3.2A). In addition, the phosphorylation of C-merlin was also decreased considerably after either 5 minute starvation or 5 minute HA incubation of RT4-C cells (Figure 3.2B), suggesting that the activation of the unknown phosphatase was indeed involved during these processes. Furthermore more dephosphorylated C-merlin was detected in cells cultured with increased cell density (Figure 3.2C). This could be due to activation of phosphatase or/and inactivation of kinase during the switch from low cell density (LCD) to high cell density (HCD). All these results show that the phosphorylation of C-merlin is regulated similar to that of full-length merlin.

3.1.3 Identification of the phosphorylation site in C-merlin

To evaluate whether the critical phosphorylation site S518 is also the target site in C-merlin as found in the full-length merlin, the respective serine 518 residue in C-merlin was changed into phosphorylation defective alanine (S518A mutant) or phosphorylation mimicking aspartic acid (S518D mutant). The gel motilities of both mutants were determined by SDS-PAGE in comparison with the wild type C-merlin.

A

B

C
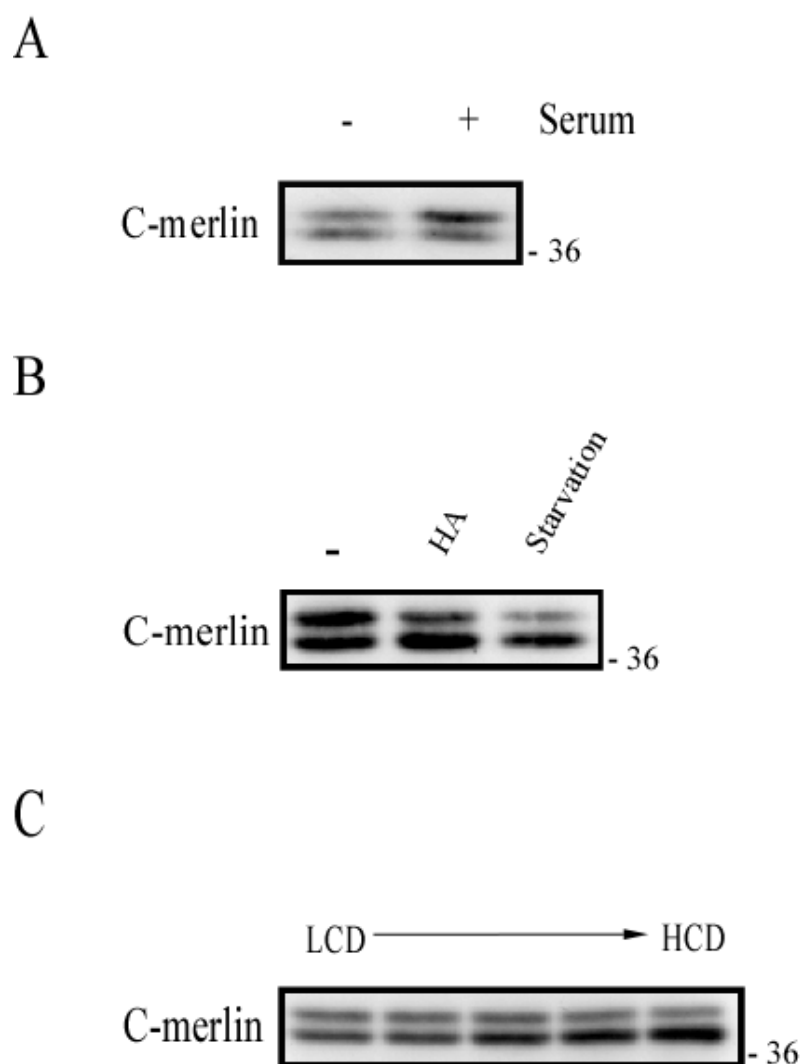
Figure 3.2. The phosphorylation of C-merlin is regulated similar to that of full-length merlin. (A) C-merlin gets phosphorylated after serum addition. Two milliliters of FCS were added into the culture medium (10 ml) after overnight starvation of RT4-C cells. The cells was then sampled and subjected to SDA-PAGE (9.5%) separation. The anti-merlin antibody (C-18) was used for Western blotting. **(B)** HA treatment and serum withdrawal induces the dephosphorylation of C-merlin. RT4-C cells after incubation of 100 μg/ml HA or deprivation of serum for 5 minutes were sampled for C-merlin detection as A. **(C)** Cell density influences the phosphorylation of C-merlin. The phosphorylation states of C-merlin in RT4-C cells growing at different cell densities were monitored by Western blotting as A and B.

Either S518A or S518D mutant showed only a single band and S518A mutant migrated similar to the dephosphorylated form of C-merlin whereas S518D mutant mimicked the phosphorylation form (Figure 3.3A), indicating that the phosphorylation of C-merlin occurs at serine 518 site. To further confirm this, the levels of serine 518 phosphorylation in C-merlin before and after serum starvation were directly compared using a specific antibody that can recognize only serine 518 phosphorylated but not dephosphorylated merlin. After serum starvation of the cells for 5 minutes, the amount of serine 518 phosphorylated C-merlin was dramatically reduced (Figure 3.3B), indicating that the phosphorylation site in C-merlin is indeed serine 518.

Taken together, C-merlin is regulated similar to full-length merlin. This suggests that RT4-C cell line can be used as the system to identify the phosphatase responsible for the serine 518 dephosphorylation and the subsequent activation of merlin.

3.2 PP1 but not PP2A is the catalytic subunit of merlin phosphatase

Based on their substrate specificity, protein phosphatases are divided into three classes, protein serine/threonine phosphatase, protein tyrosine phosphatase and protein phosphatase with dual specificity, respectively (Oliver and Shenolikar, 1998). The class of protein serine/threonine phosphatase is mainly concerned here since the target phosphorylation site in my work is a serine residue (serine 518 of merlin). Most of the serine/threonine phosphatases are holoenzymes consisting of at least a catalytic subunit and a target subunit. The target subunit can not only target the catalytic subunit to substrates and thereby define the specificity but also regulate the activity of the catalytic subunit. The catalytic subunit can be utilized by different target subunits to constitute different functional phosphatases. The names of phosphatases are therefore usually defined by the target subunits. However, there are many target subunits in mammalian cells while the number of catalytic subunits is quite limited. Therefore, I set out primarily to identify the catalytic subunit of the phosphatase. After

A

MT

WT S518A S518D

C-merlin

- 36

B

-          +   Starvation

Phos-merlin

- 36

actin

- 55
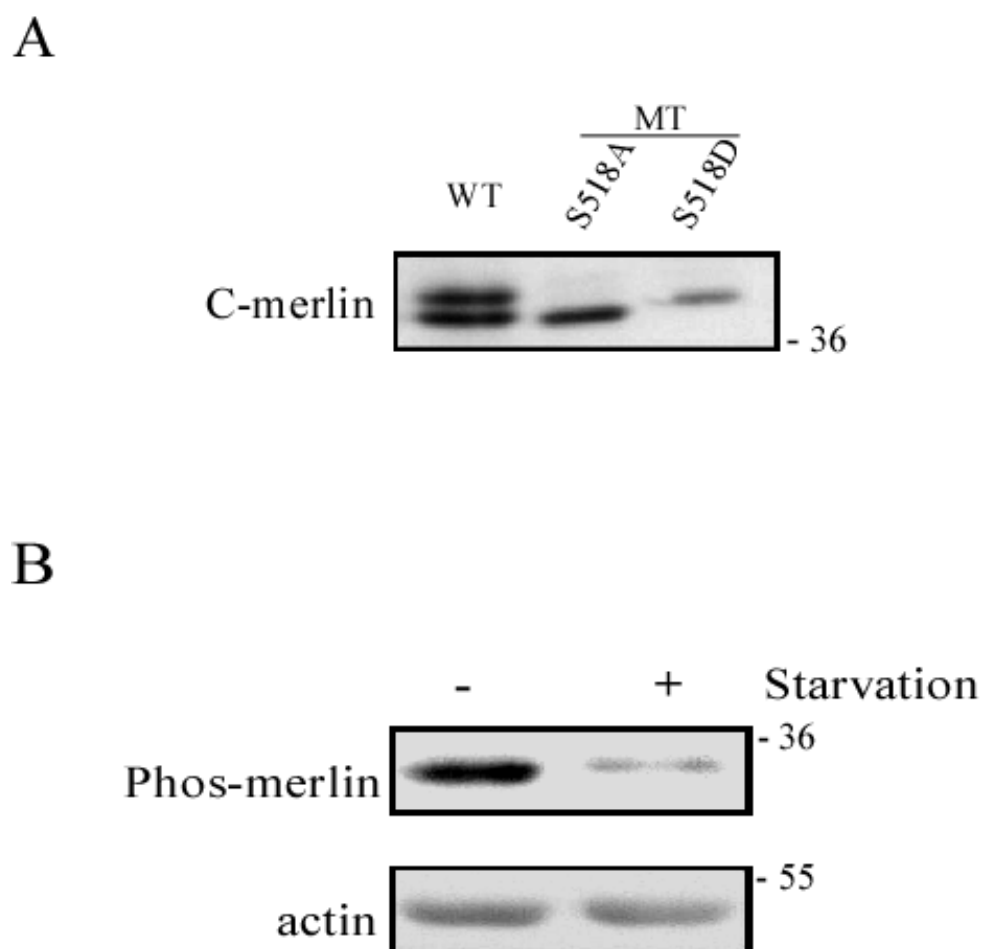
Figure 3.3. The phosphorylation site in C-merlin is indeed serine 518. (A) Both mutant of C-merlin migrate differently from the wild type C-merlin. The residue of serine 518 in C-merlin was mutated to alanine or aspartic acid, respectively. Both mutants, as well as the wild type C-merlin, were expressed in RT4 cells and detected by Western blotting with the antibody against merlin (C-18). **(B)** Serum starvation induces serine 518 dephosphorylation of C-merlin. Serine 518 phosphorylated C-merlin in serum starved or non-starved RT4-C cells were monitored by Western blotting with the antibody specifically recognizing serine

this identification, I could then narrow down and finally find out the target subunit of the merlin phosphatase. According to their biochemical properties, the catalytic subunits of protein serine/threonine phosphatase are further classified into two families designated PPP (Protein Phosphatase P), and PPM (Protein Phosphatase M). While PPM comprises Mg^{2+}-dependent phosphatase PP2C (Protein Phosphatase 2C), PP1 (Protein Phosphatase 1) together with PP2A (Protein Phosphatase 2A), PP4 (Protein Phosphatase 4), PP5 (Protein Phosphatase 5), and PP6 (Protein Phosphatase 6) constitute the PPP family (Aggen et al., 2000; Ceulemans and Bollen, 2004; Cohen, 2002b; Lechward et al., 2001; Liu et al., 2003; Lohse et al., 1995; Oliver and Shenolikar, 1998). PP1 and PP2A containing phosphatases contribute to more than 90% of the global serine/threonine phosphatase activities in mammalian cells (Oliver and Shenolikar, 1998), these two classes of catalytic subunits were therefore mainly considered in my further experiments.

3.2.1 The effect of okadaic acid on merlin phosphorylation

To distinguish between PP1 and PP2A, the serine/threonine phosphatase inhibitor okadaic acid (OA) was used. Okadaic acid is a cell-permeable toxin that can inhibit both PP1 and PP2A but at different concentrations. It can inhibit PP2A at nano-molar concentrations but PP1 at tens to hundreds times higher concentrations (Dounay and Forsyth, 2002). To investigate the effect of okadaic acid on the phosphorylation of C-merlin, RT4-C cells were subjected to Western blotting analysis after the treatment with different concentrations of okadaic acid. The phosphorylation state of C-merlin changed only after the treatment with high concentrations of okadaic acid (Figure 3.4). The ratio of phosphorylated C-merlin to dephosporylated C-merlin was completely reversed after 2μM okadaic acid treatment. However, the increased phosphorylation of C-merlin could also be due to the activation of a merlin specific kinase. To confirm the involvement of okadaic acid on the inhibition of phosphatase of interest, RT4-C cells were pretreated with high or low concentrations of okadaic acid prior to serum withdrawal. The phosphorylation status of the C-merlin was monitored by Western blotting as described before. The C-merlin in low concentrations of okadaic acid

pretreated RT4-C cells could still be dephosphorylated after serum starvation, the same as C-merlin in the okadaic acid non-treated cells (Figure 3.5A). However, serum starvation induced C-merlin dephosphorylation was completely inhibited by high concentrations of okadaic acid. Similarly, in RT4-54 cells that can over-express flag tagged full-length merlin, the dephosphorylation of full-length merlin was also inhibited only by high concentrations of okadaic acid (Figure 3.5B). Taken together, these results indicate that PP1, but not PP2A, might be the catalytic subunit of the candidate phosphatase.
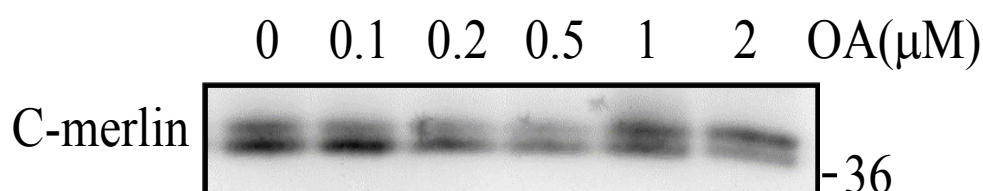
Figure 3.4. The effects of OA on the phosphorylation of C-merlin. Different amounts of okadaic acid (final concentrations range from 0 to 2 μM) were added directly to the culture medium of the RT4-C cells. The cells were incubated further for 30 minutes before being sampled for C-merlin detection with Western blotting analysis.

3.2.2 *In vitro* dephosphorylation of C-merlin

To further confirm the involvement of PP1 but not PP2A in the regulation of merlin, an *in vitro* dephosphorylation assay was performed. C-merlin was enriched upon binding to anti-flag antibodies coupled to protein G-agarose beads and then treated with different amounts of purified PP1 or PP2A. The dephosphorylation efficiencies

A

B
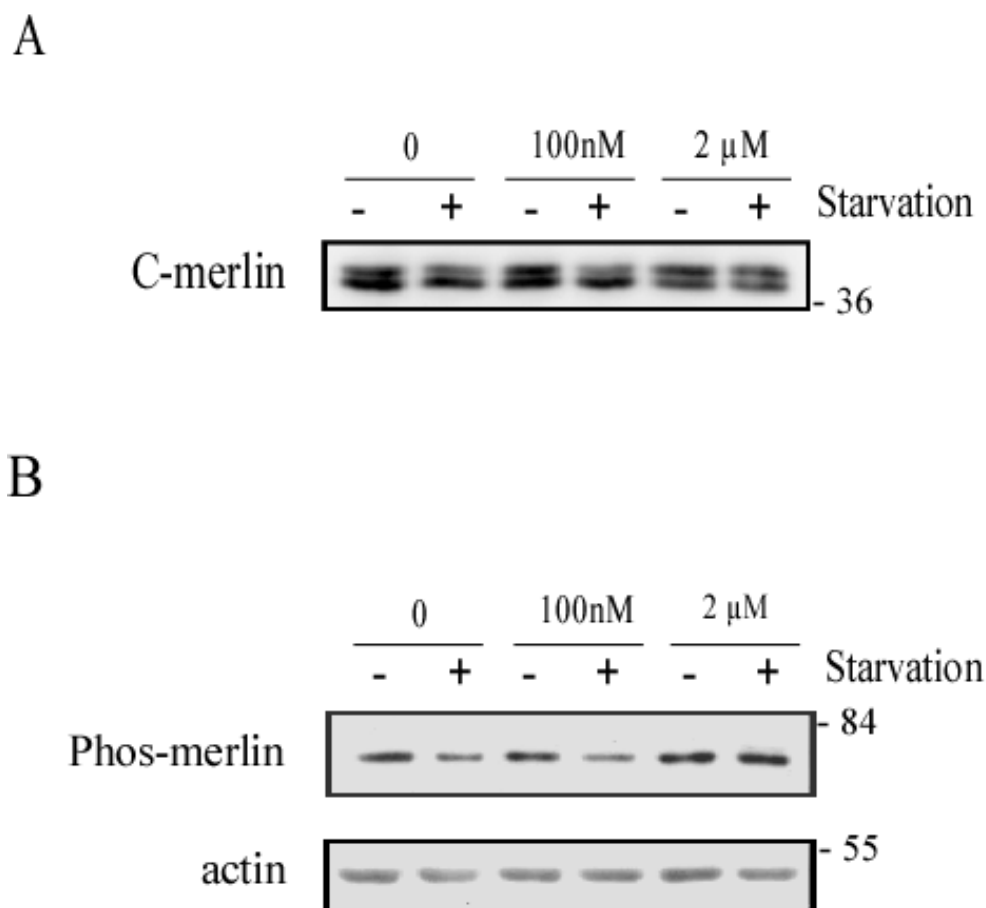
Figure 3.5.   Merlin dephosphorylation is inhibited by okadaic acid. (A) C-merlin dephosphorylation is inhibited only by high concentration of okadaic acid. RT4-C cells were pretreated with (100nM or 2 μM, respectively) or without OA for half an hour. Dephosphorylations of C-merlin were then induced by 5 minutes of starvation. The pan-merlin polyclonal antibody (C-18) was used to detect C-merlin. **(B)** The dephosphorylation of full-length merlin is inhibited only by high concentration of okadaic acid.. Experiment was done similar to A with the exception that RT4-54 cells were used and the blot was probed with the anti-phospho-Ser 518 merlin antibody. The actin blot was used as a loading control.

of these two different enzymes to C-merlin, were then determined by Western blotting analysis using the anti-merlin antibody. PP1 of 0.05 units could dephosphorylate C-merlin completely while PP2A of the same activity units had no effect. Although non-specific dephosphorylation by PP2A occurred at higher activity levels, the efficiency was still not comparable to that of PP1 (Figure 3.6). This *in vitro* dephosphorylation experiment indicated again that PP1 but not PP2A would be used by the phosphatase of merlin as its catalytic subunit.
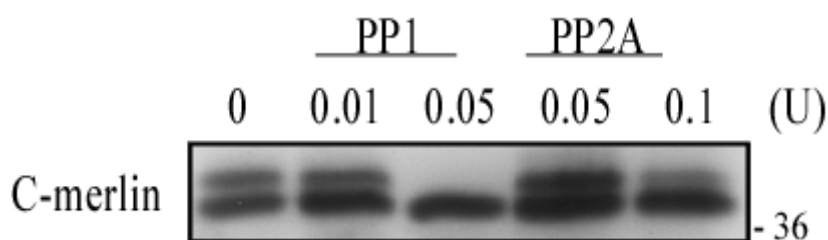
Figure 3.6. C-merlin can be dephosphorylated by PP1 *in vitro*. Immunoprecipitaed C-merlin from RT4-C cells were incubated with different amounts of purified PP1 or PP2A for 1 hour at 37°C with shaking. They were then subjected to SDS-PAGE (9.5%) separation and Western blotting detection with the merlin specific antibody (C-18).

3.2.3 Interaction of merlin with PP1

If PP1 was really involved in the regulation of merlin phosphorylation, it should form a complex with merlin *in vivo*. To test this idea, co-immunoprecipitation experiments with anti-flag monoclonal antibodies were performed using RT4-C or RT4-54 cells.

In mammalian cells there are five isoforms of PP1, designated PP1α_1, PP1α_2, PP1γ_1, PP1γ_2 and PP1ß (also termed PP1δ), respectively (Cohen, 2002b). An anti-PP1 polyclonal antibody which can recognize all five isoforms of PP1 catalytic subunit was therefore used for Western blotting to detect the presence of PP1 in the merlin associated immuno-complexes while the anti-PP2A polyclonal antibody was used to probe for PP2A. PP1, but not PP2A, could be pulled down by the anti-flag antibody from both RT4-C cells and RT4-54 cells (Figure 3.7A), indicating that both C-merlin and full-length merlin can interact with PP1 but not PP2A *in vivo*. Both C-merlin and full-length merlin in these two cell lines were over-expressed. To exclude non-specific interactions, another immunoprecipitation with the anti-merlin antibody (A-19) was carried out in the parental RT4 cells, which expresses only endogenous merlin. Again, PP1 but not PP2A was immunoprecipitated with merlin (Figure 3.7B), suggesting that PP1 can also associate with endogenous merlin *in vivo*.

Taken together, it appears that the activating phosphatase of merlin utilizes PP1 but not PP2A as its catalytic subunit.

3.3 Myosin/Moesin phosphatase is the phosphatase of merlin

The names of phosphatases are eventually defined by their target subunit because a catalytic subunit such as PP1 can be used by more than fifty target subunits to constitute different functional phosphatases. Therefore, which protein is the target subunit bringing PP1 to merlin is the next question. Myosin/moesin phosphatase is the phosphatase that also regulates moesin and probably ezrin as well (Fukata et al., 1998). This phosphatase indeed utilizes one isoform of PP1, specifically PP1δ, as its catalytic subunit. More interestingly, this phosphatase has been shown to be activated under non-permissive growth conditions such as cell-cell/ECM contact and serum starvation (Feng et al., 1999; Ito et al., 2004), correlating with the dephosphorylation of merlin. Therefore, I investigated whether this phosphatase can regulate merlin as well.

A

RT4-C   RT4-FL(54)
lys   Iso Flag   Iso Flag

PP1   - 36

PP2A   - 36

B

RT4
lys   Iso   A-19

PP1   - 36

PP2A   - 36
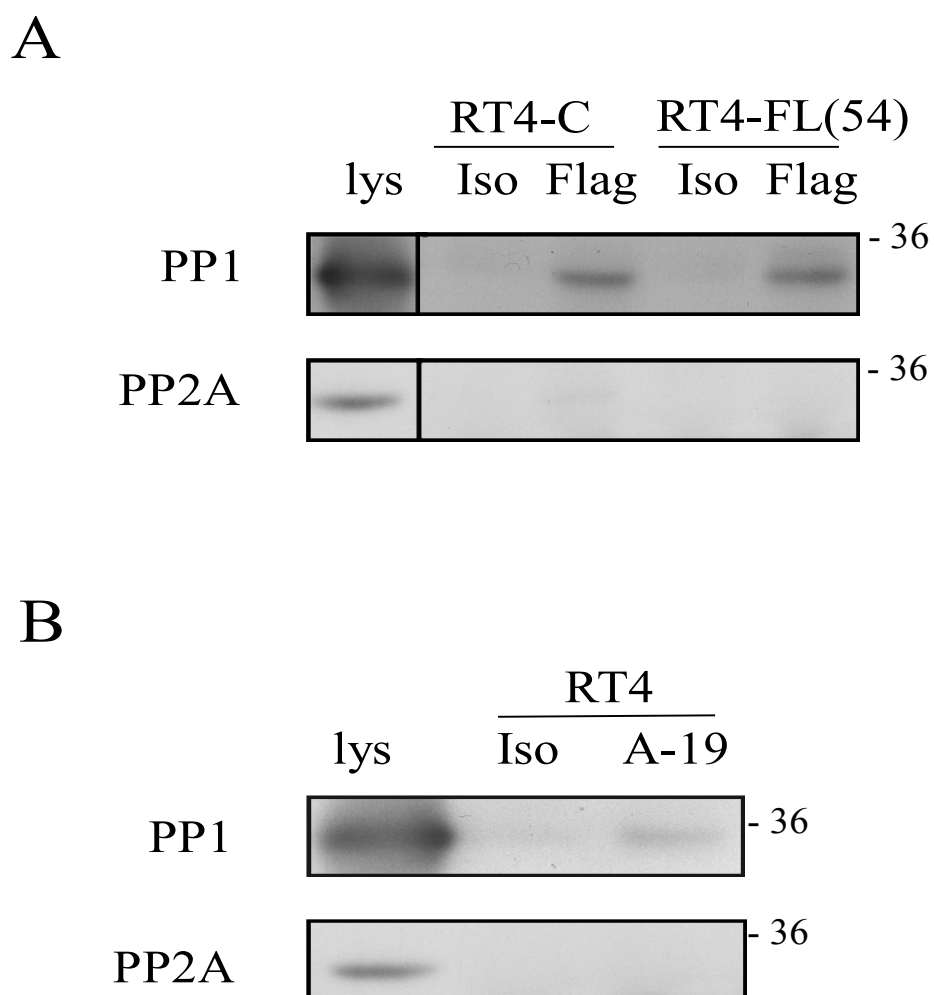
Figure 3.7. Merlin interacts with PP1 *in vivo.* **(A)** Both C-merlin and full-length merlin can interact with PP1. Co-immunoprecipitation with anti-flag antibody was performed in either RT4-C or RT4-54 cells. The blot was probed with the anti-PP1 (upper panel) and the anti-PP2A (lower panel) antibody. Immunoprecipitation with the isotope antibody was used as the negative control. **(B)** Endogenous merlin interacts with PP1 as well. Immunoprecipitation with anti-merlin antibody (A-19) or the isotope antibody was carried out in RT4 cells. The Western blot was done as same as A.

3.3.1 Interaction between merlin and myosin/moesin phosphatase

In a first approach, the *in vivo* association of merlin with myosin/moesin phosphatase was analyzed via co-immunoprecipitation. Since myosin/moesin phosphatase consists of the target subunit MYPT-1 and the catalytic subunit PP1δ, the anti-MYPT-1 and the anti-PP1δ antibody were used for Western blotting to detect the presence of the target subunit (MYPT-1) and catalytic subunit (PP1δ) of myosin/moesin phosphatase in the immunoprecipitate of merlin. MYPT-1 was pulled down by immunoprecipitation with the anti-merlin antibody (Figure 3.8). As expected, the
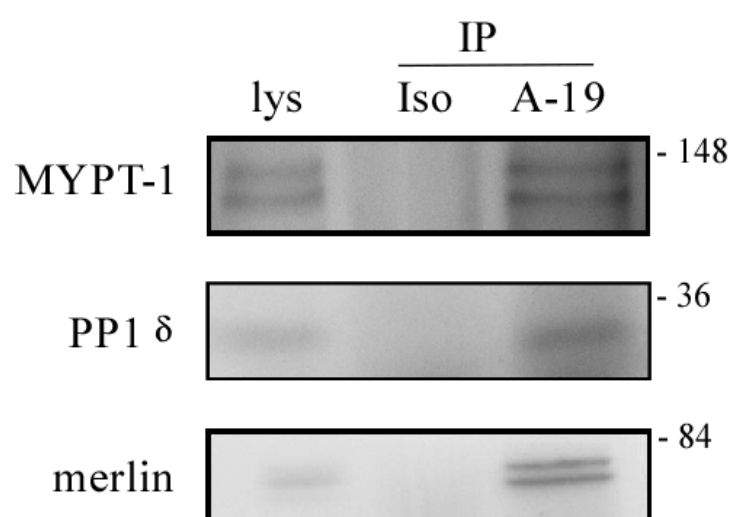

Figure 3.8. Merlin associates with myosin/moesin phosphatase *in vivo.* Immunoprecipitation with the anti-merlin antibody (A-19) was carried out in RT4 cells. The blot was probed with the anti-MYPT-1 (upper panel), anti-PP1δ (middle panel) and another anti-merlin body (C-18; low panel), respectively. The immunoprecipitation with the isotope antibody was used as the negative control.

particular catalytic subunit of myosin/moesin phosphatase PP1δ was also pulled down (Figure 3.8). This indicates that MYPT-1 can form a complex with merlin and bring the catalytic subunit PP1δ to the substrate merlin *in vivo*. However, this result does not indicate whether the interaction between MYPT-1 and merlin is direct. Therefore, an *in vitro* binding assay was performed. Recombinant C-merlin was purified *in vitro* from bacteria and then incubated for 2 hours with recombinant Glutathione-S-Transferase (GST)–tagged MYPT-1 or GST as a control. Purified C-merlin was pulled down by GST-MYPT-1 but not GST (Figure 3.9), indicating that merlin can indeed bind to MYPT-1 directly.
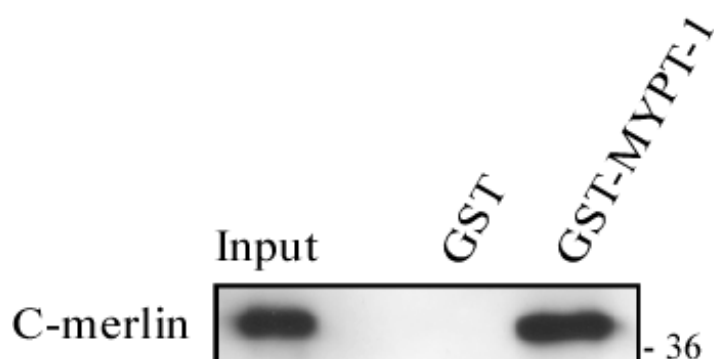
Figure 3.9. Merlin can bind to MYPT-1 *in vitro*. Recombinant C-merlin was purified from bacteria and then incubated with purified GST or GST-MYPT-1 for 2 hours. The protein pulled down was subjected to Western blotting analysis with the anti-merlin antibody (C-18).

Since merlin exists as two forms, phosphorylated and dephosphorylated, it would be interesting to elucidate which form of merlin can interact with MYPT-1. Co-immunoprecipitation with the anti-MYPT-1 antibody were therefore performed in RT4 cell lines expressing either the wild type full-length merlin, the S518A mutant or
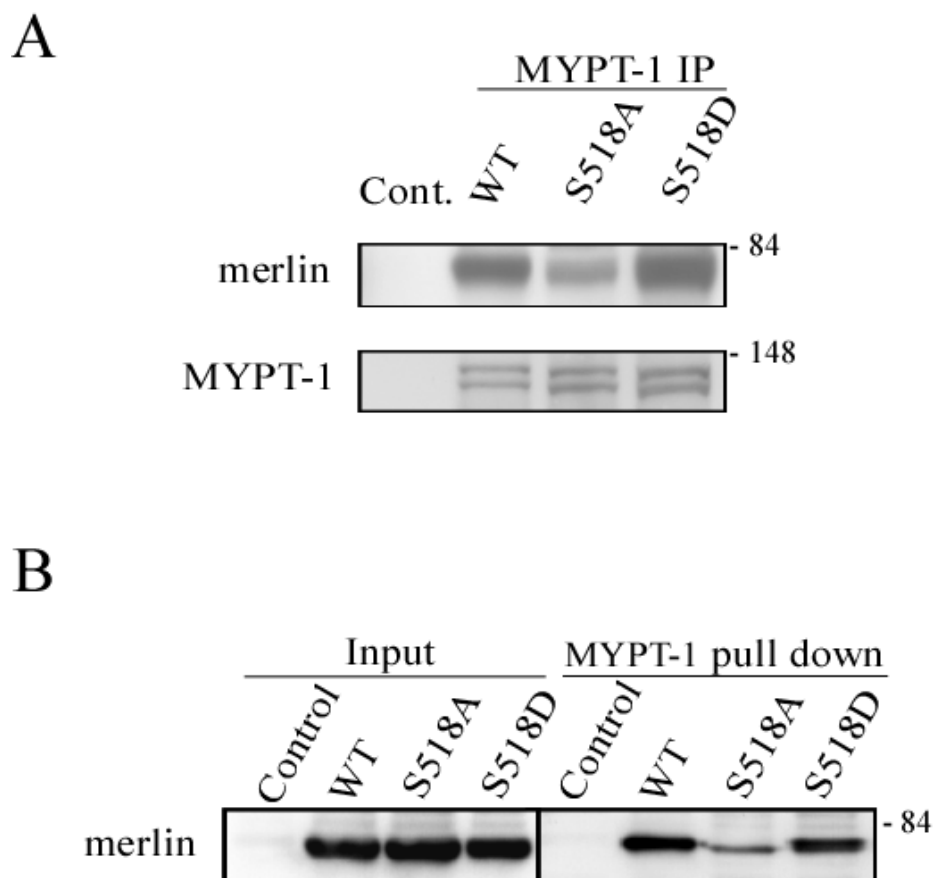
Figure 3.10. MYPT-1 interacts with different forms of full-length merlin with different affinity. **(A)** Co-immunoprecipitations with the anti-MYPT-1 antibody were performed in different strains of RT4 cells expressing wild type or mutated merlin (S518A or S518D). Imunoprecipitation with the isotope antibody was used as the control. The blot was probed with the anti-merlin (C-18, upper panel) or the anti-MYPT-1 antibody (lower panel), respectively. **(B)** GST-MYPT-1 pull down assays were carried out in the same strains of RT4 cells as A with the exception that nontransfected RT4 cells were used as a control. The blot was probed with the anti-merlin (C-18) antibody.

the S518D mutant, respectively. The serine to alanine mutation (S518A) seemed to attenuate but not abolish the association of merlin with MYPT-1 while the serine to aspartic acid mutation (S518D) had no significant effect (Figure 3.10A). A similar result was obtained when GST-MYPT-1 pull down assays were performed with these cell lines (Figure 3.10B). These results implicate that the serine 518 phosphorylation can influence the interaction of merlin with MYPT-1. However, when co-immunoprecipitation with the anti-MYPT-1 antibody was performed in RT4-C cells, MYPT-1 seemed to interact with both the dephosphorylated and phosphorylated form of C-merlin, with the same affinity (Figure 3.11). The inconsistency with the results of full-length merlin could be due to a difference in conformation between full-length merlin and C-merlin. This indicates that the phosphorylation site *per se* is not the site responsible for the interaction of merlin with MYPT-1.
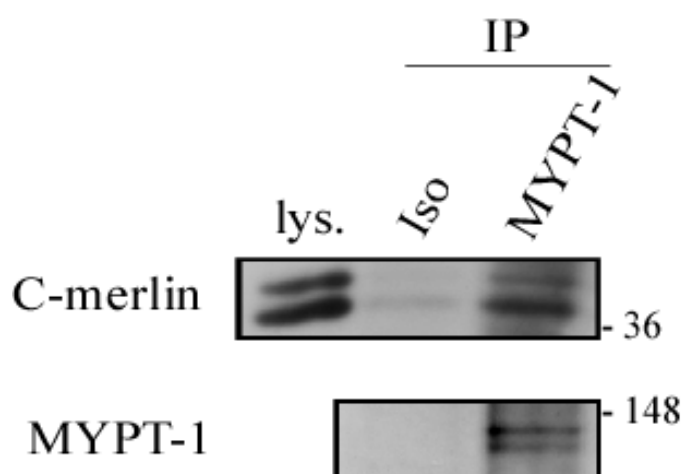
Figure 3.11. Both forms of C-merlin can interact with MYPT-1. Co-immunoprecipitation with the anti-MYPT-1 antibody was done in RT4-C cells. Immunoprecipitation with isotope antibody was used as a control. The blot was probed with the anti-merlin (C-18, upper panel) or the anti-MYPT-1 antibody (lower panel), respectively.

A

B

Figure 3.12. Interaction of MYPT-1 with merlin is dependent on its leucine zipper domain. (A) Scheme of wild type and mutated MYPT-1. There are four leucine residues in the typical leucine zipper domain of wild type MYPT-1. All of the four leucine residues were replaced by alanine residues in the mutated MYPT-1. **(B)** The interaction of MYPT-1 with merlin was abolished by the muatation of four leucine residues into alanine residues. The crude extracts of RT4 cells were incubated with GST, GST-wild type MYPT-1(GST-WT) or GST-MYPT-1 LZ mutant (GST-MT) for 3 hours at 4 °C. The pellets after centrifugation were subjected for Western blotting analysis. The upper panel is the blot probed with the anti-merlin antibody. The lower panel is the coomasie staining gel.

The next question is which site(s) or region in merlin and MYPT-1 are important for this interaction? The C-merlin contains a Coiled-coil region and a tail domain (Rouleau et al., 1993). The Coiled-coil region seems to be important for the interaction of merlin with other binding partners (Gutmann et al., 2001). MYPT-1 has a leucine zipper (LZ) domain which is a well know protein-protein interaction domain and named after the leucine residue repeats (scheme in Figure 3.12A). This domain was known to be responsible for MYPT-1 to interact with Coiled-coil domain containing proteins such as Myosin phosphatase-Rho interacting protein (M-RIP) (Surks and Mendelsohn, 2003; Surks et al., 1999; Surks et al., 2003). Therefore, MYPT-1 might interact with merlin through its leucine zipper domain. It has been shown that the substitution of all four leucine residues in the leucine zipper domain into alanine (scheme in Figure 3.12A) could specifically abolish the binding of MYPT-1 to its Coiled-coil domain containing partners (Surks and Mendelsohn, 2003; Surks et al., 2003). To test the importance of the leucine zipper domain of MYPT-1 for the interaction with merlin, I performed pull down assays with GST, GST-MYPT-1 (wild type) or the GST-MYPT-1 LZ mutant. The proteins pulled down were analyzed by Western blotting using the anti-merlin antibody. Wild type MYPT-1 pulled down merlin whereas neither GST alone nor the GST-MYPT-1 LZ mutant could pull down merlin (Figure 3.12B). This result clearly indicates that the leucine zipper domain of MYPT-1 is indeed responsible for the interaction with merlin.

The relevance of the Coiled-coild domain in merlin for the interaction with MYPT-1 was then examined by pull down assay using GST-tagged C-merlin truncation mutants. GST-C-merlin (312-595), GST-C-merlin Δ (342-595) with the deletion from residue 312 to 341 and GST-C-merlin Δ CC (506-595) with the deletion of whole Coiled-coil domain (scheme in Figure 3.13A) were purified from bacteria and incubated with crude extracts of RT4 cells. The proteins pulled down were analyzed by Western blotting using the anti-MYPT-1 antibody. Only GST-C-merlin that contains the region from residue 312 to 341, but not GST-C-merlin Δ or GST-C-merlin Δ CC, could pull down MYPT-1 (Figure 3.13B). This suggests that the region from residue 312 to 341 in merlin is important for the interaction with MYPT-1. If
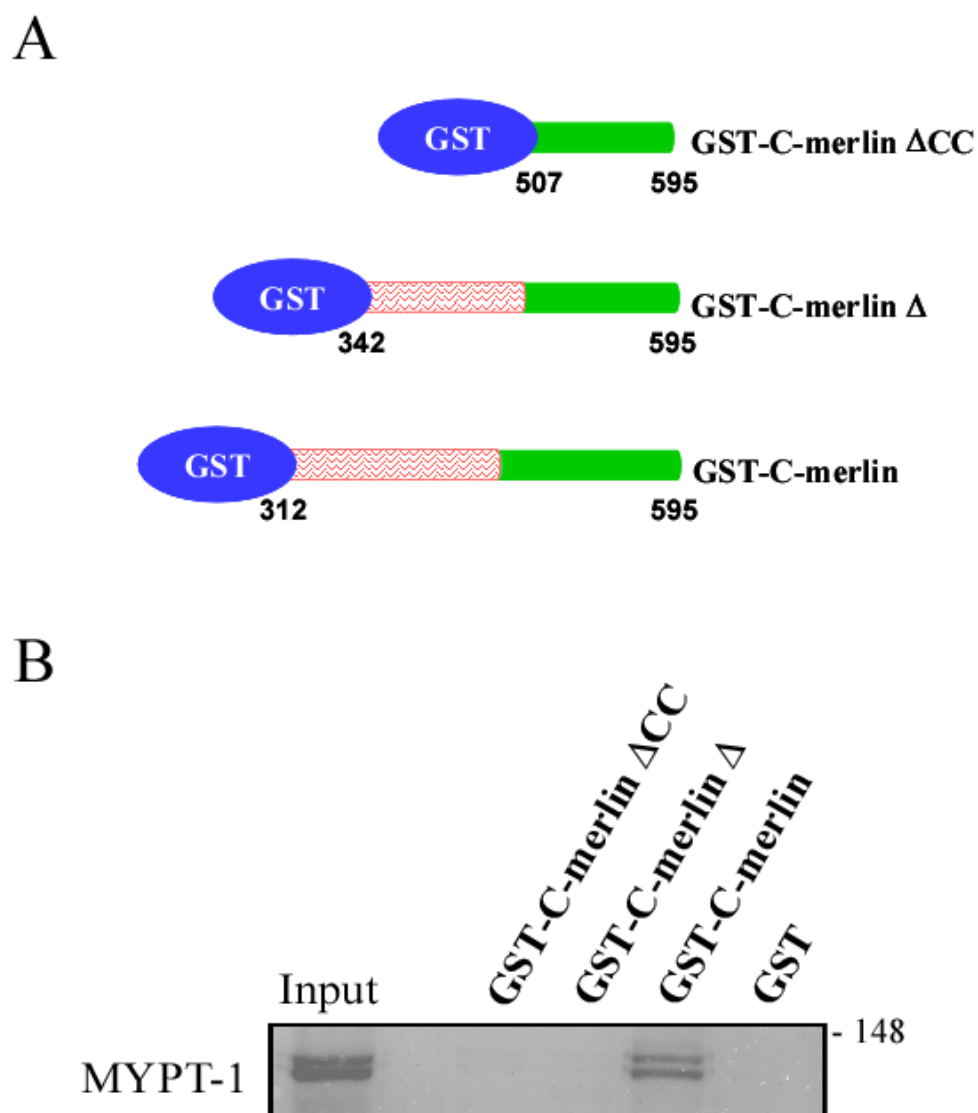
Figure 3.13. The region from residue 312 to 341 in merlin is responsible for the interaction with MYPT-1. (A) Scheme of different C-merlin truncations fused with GST. GST-C-merlin contains whole Coiled-coild domain and the C-tail domain of merlin. GST-C-merlin Δ contains the C-tail domain and a part of Coiled-coil domain with the deletion from residue 299 to 311. GST-C-merlin Δ CC contains only the C-tail domain of merlin. **(B)** Only GST-C-merlin can interact with MYPT-1. GST, GST-C-merlin, GST-C-merlin Δ or GST-C-merlin Δ CC was added into the crude extract of RT4 cells and incubated overnight at 4 °C. The blot was probed with the anti-MYPT-1 antibody.

Figure 3.14. C-merlin Δ is resistant to starvation induced dephosphorylation . RT4 cells were engineered to express MYPT-1 interacting region deleting C-merlin (C-merlin Δ). The serine 518 phosphorylations in the C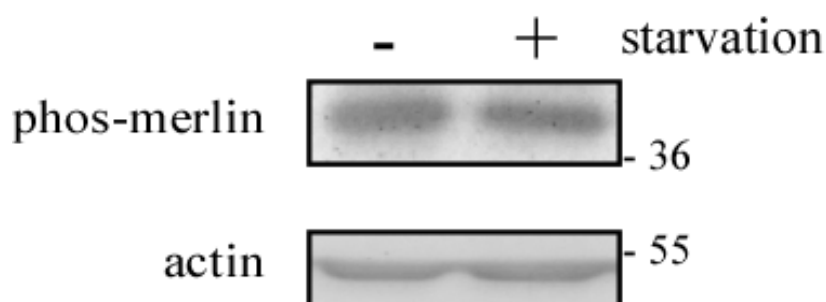-merlin from the engineered RT4 cells before and after serum starvation were determined by Western blotting analysis using the anti-serine 518-phosphorylated-merlin specific antibody. The actin blot was used as the loading control.

this is true, C-merlin with a deletion of this region should be refractory to dephosphorylation *in vivo*. To test this, RT4 cells were transfected to express C-merlin Δ (342-595). The S518 phosphorylation status of C-merlin Δ in the transfected cells before and after serum withdrawal was then monitored by Western blotting using S518 phosphorylation specific antibody. As expected, C-merlin Δ (342-595) was resistant to starvation induced dephosphorylation (Figure 3.14). This intriguing *in vivo* result confirms not only that the region from residue 312 to 341 is the region responsible for merlin to bind to MYPT-1 but also that myosin/moesin phosphatase is indeed very likely the phosphatase regulating merlin *in vivo*.

Interestingly, several missense mutations have been described in this region of merlin (Baser et al., 2004; Baser et al., 1996; De Vitis et al., 1996; Shimizu et al., 2002). To test if these mutations would affect the interaction with the phosphatase, GST-C-merlin constructs carrying one of the naturally occurring missense mutations, Leu316Phe (L316F), Gln324Leu (Q324L) or Leu339Phe (L339F), were generated by site-directed mutagenesis and expressed in bacteria. These mutants were then purified

from bacteria and used for pull down assays. Only the L339F missense mutation reduced the interaction of merlin with MYPT-1 while the other two mutations had no influence on this interaction (Figure 3.15). The disruption of the interaction of merlin with its phosphatase by the L339F mutation could confer merlin the resistance to be activated and therefore block merlins growth inhibiting function. This might be the pathogenic mechanism of this naturally occurring missense mutation.
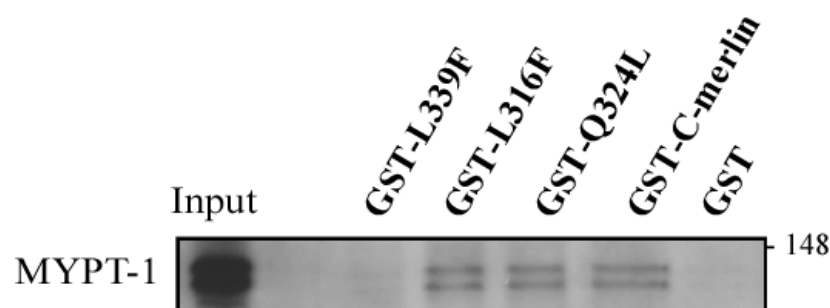
Figure 3.15. The interaction of merlin mutants with MYPT-1. The constructs encoding GST-C-merlin carrying naturally occurring missense mutations were generated by QuickChange site-directed Mutagenesis with GST-C-merlin construct as the template and then expressed in bacteria. The purified GST, GST-C-merlin or its mutants from bacteria were added into crude extracts of RT4 cells and incubated overnight at 4 °C. The complex pulled down was collected and washed with gentle centrifugation before sampled for Western blotting analysis with the anti-MYPT-1 antibody.

3.3.2 The relationship between the phosphorylation of merlin and the activity of myosin/moesin phosphatase

Based on the results above, it can be assumed that myosin/moesin phosphatase is the phosphatase of merlin. If that is true, this phosphatase should be activated but not

59

inhibited under conditions when merlin is dephosphorylated, e.g. serum withdrawal. The activity of myosin/moesin phosphatase *in vivo* is mainly inhibited by the phosphorylation of MYPT-1 on threonine 696 (Feng et al., 1999; Ito et al., 2004). This implicates that under conditions when merlin is dephosphorylated, threonine 696 in MYPT-1 has to be dephosphorylated in order to maintain myosin/moesin phosphatase in an active state. Therefore, the phosphorylation status of threonine 696 in MYPT-1 after starvation was investigated. RT4 cells cultured overnight with 10% serum containing medium were subjected to serum withdrawal for 5 minutes prior to sampling for SDS-PAGE. The phosphorylation status of both merlin and MYPT-1 was determined by Western blot analysis with the specific antibody against either
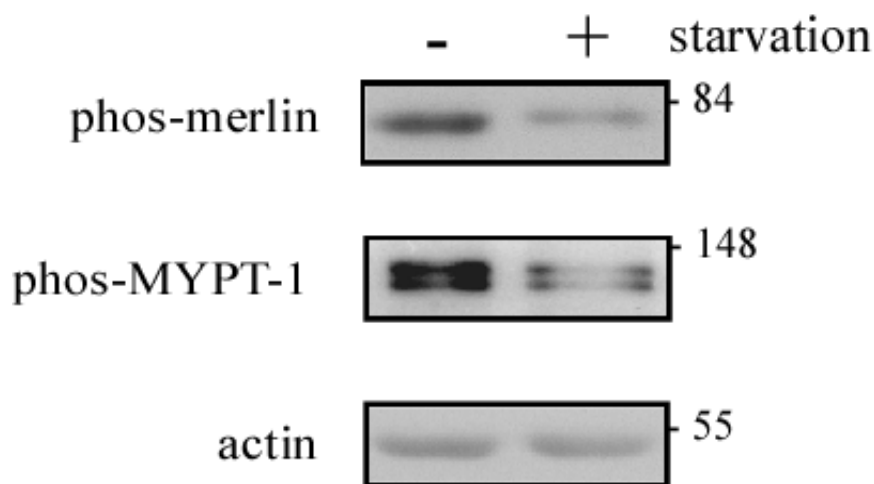
Figure 3.16. The corelationship between the C-merlin dephosphorylation and myosin/moesin activation. The phosphorylation state of serine 518 in merlin and threonine 696 in MYPT-1 in serum starved RT4-54 cell was monitored by western blotting analysis. The blot was probed with the anti-serine 518-phosphorylated-merlin specific antibody (upper panel), anti-threonine 696-phosphorylated-MYPT-1 specific antibody (middle panel) and the anti-actin antibody (lower panel, loading control), respectively.

A

B
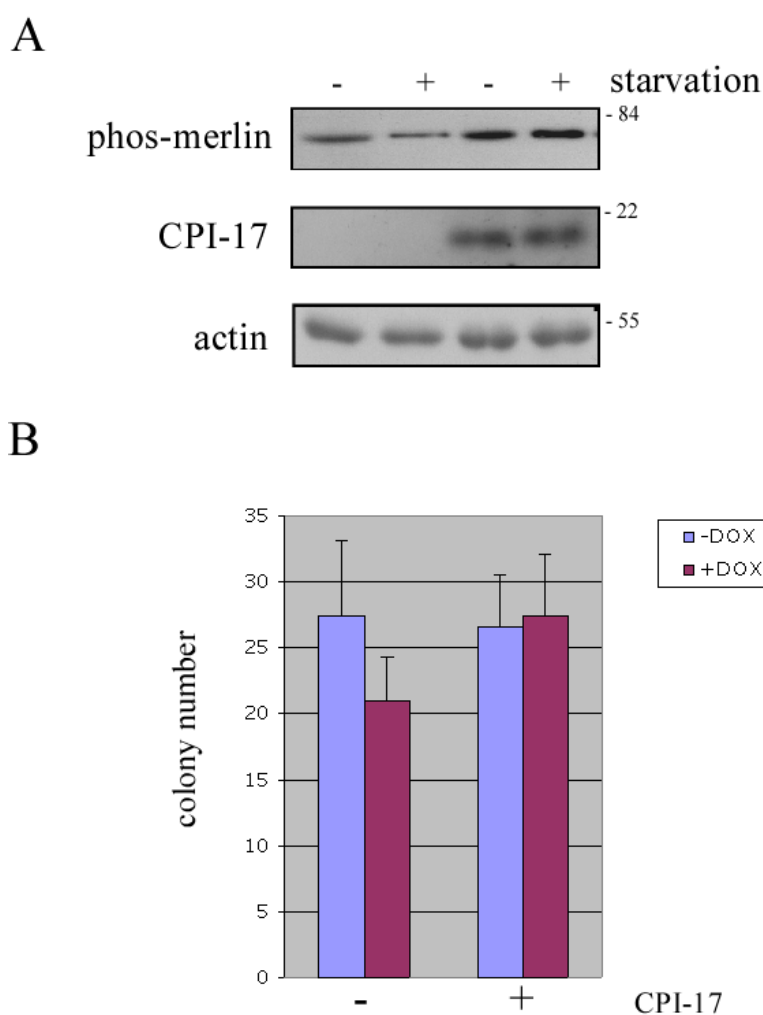
Figure 3.17. The effect of CPI-17 on merlin. **(A)** The effect of CPI-17 on the dephosphorylation of merlin. RT4-54 cells were transfected with or without CPI-17 expressing vector. The serine 518 phosphorylation of merlin in these two cell lines before and after serum starvation was determined by Western blotting analysis using the serine 518-phosphorylated-merlin specific antibody (upper panel). The anti-CPI-17 antibody was used to detect the expression of CPI-17 (middle panel). The actin blotting was served as the loading control (lower penel). **(B)** The effect of CPI-17 on the growth inhibitory function of merlin. RT4-54 cells transfected with or without CPI-17 expressing vector were cultured in 12-well-plate with 0.33% agar containing complete medium in the presence or absence of Dox. The colonies were counted 10 days later. The average numbers per well were plotted.

serine 518 phosphorylated merlin or threonine 696 phosphorylated MYPT-1. Both serine 518 phosphorylation of merlin and threonine 696 phosphorylation of MYPT-1 were strongly reduced after serum starvation (Figure 3.16). This is not only true for serum starvation. Both phosphorylations were also decreased after HA treatment (Tobias Specka, unpublished data). These results clearly indicate that myosin/moesin phosphatase is indeed activated under conditions when merlin is dephosphorylated. The correlation between the phosphorylation of merlin and activity of myosin/moesin phosphatase suggests further that myosin/moesin phosphatase is the phosphatase responsible for merlin dephosphorylation.

3.3.3 The effect of CPI-17 on the phosphorylation and function of merlin

The activity of myosin/moesin phosphatase can be significantly inhibited by CPI-17 (Eto et al., 2000; Yamawaki et al., 2001). I tested therefore whether the dephosphorylation of merlin was inhibited by CPI-17. An expression vector containing CPI-17 cDNA was transfected into RT4-54 cells. The serine 518 phosphorylation of merlin in these cells before and after serum starvation was then monitored by Western blotting analysis. The serine 518 phosphorylation of merlin from non-transfected RT4-54 cells was strongly reduced after 5 minutes of serum starvation while this dephosphorylation did not occur in the presence of CPI-17 (Figure 3.17A), indicating that the dephosphorylation of merlin could be inhibited by CPI-17. Therefore, the next prediction would be that merlin could not be activated and should lose its growth inhibition ability in the presence of CPI-17. This actually was the case. While merlin could inhibit the anchorage independent growth of RT4-54 cells in soft agar (Morrison et al., 2001), this inhibition was lost in the presence of CPI-17 (Figure 3.17B).

Both the dephosphorylation and the activation of merlin can be inhibited by the specific inhibitor of myosin/moesin phosphatase, CPI-17, strongly indicated that myosin/moesin phosphatase is the phosphatase of merlin.
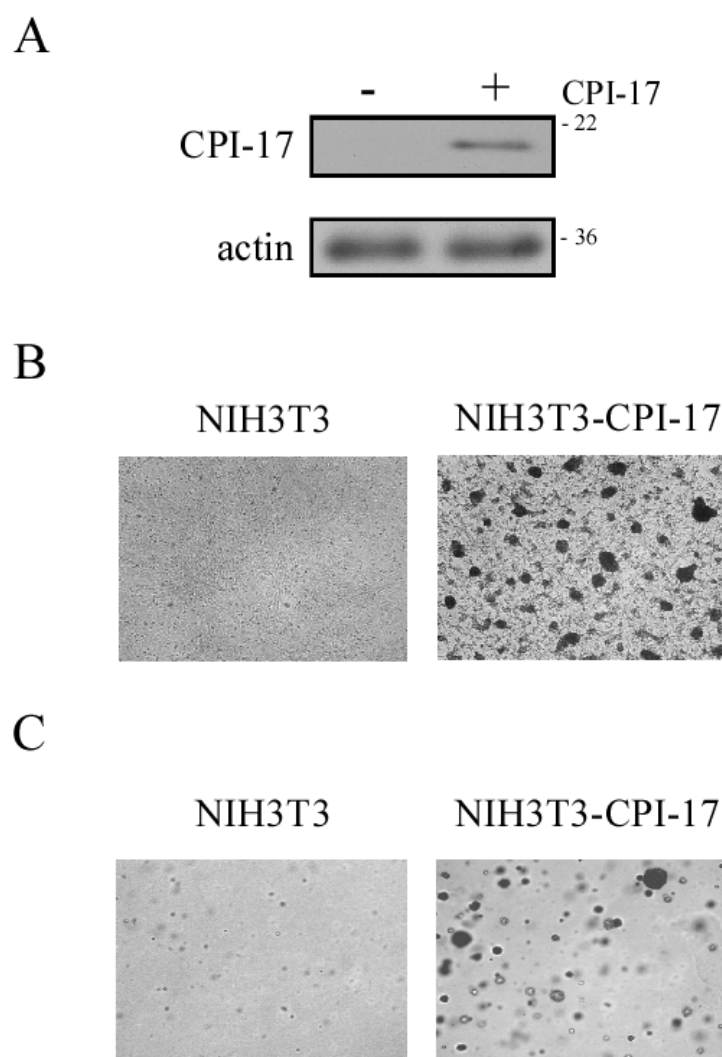
Figure 3.18. The transforming ability of CPI-17 . **(A)** The expression of CPI-17 in NIH3T3 cells. NIH3T3 cells were transfected with or without CPI-17 expressing vector. The expression was detected with Western blotting analysis. The blot was probed with the anti-CPI-17 antibody (upper panel) and the anti-actin antibody (lower panel), respectively. **(B)** The loss of contact inhibition in NIH3T3 cells expressing CPI-17. NIH3T3 cells transfected with or without CPI-17 expression vector were cultured in the confluence state for several days before the pictures were taken. **(C)** The growth of NIH3T3 cells with or without the expression of CPI-17 in soft agar. The same amount of NIH3T3 cells or NIH3T3-CPI-17 cells were placed in 0.33% agar containing medium. The pictures were taken after one-week culture.

A

B

C

D
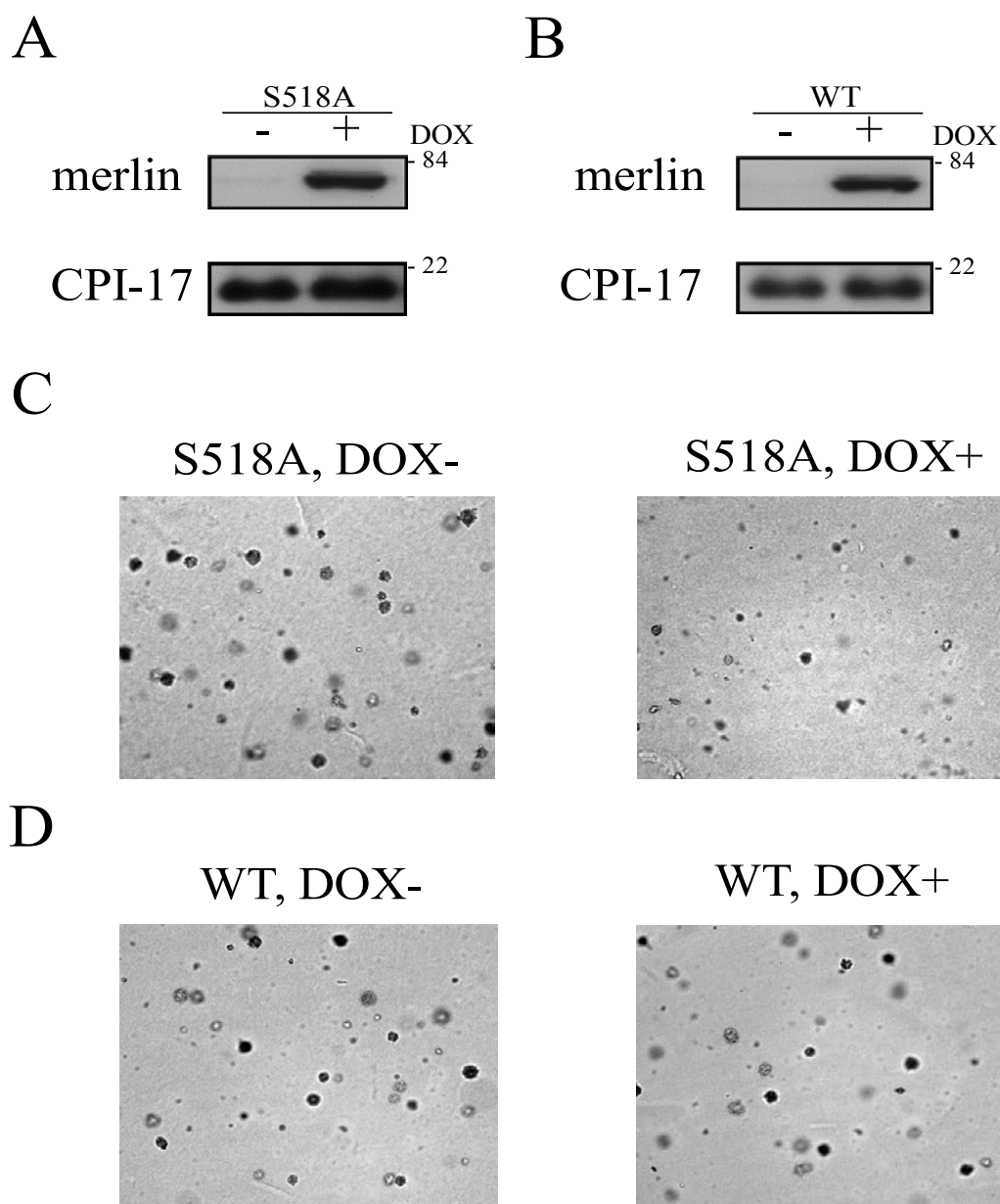
Figure 3.19. The influence of the wild type or mutated merlin on the anchorage independence growth of the CPI-17 transformed NIH3T3 cells. NIH3T3-CPI-17 cells were engineered to express the S518A mutated **(A)** or wild type **(B)** merlin after Dox induction. The expression were checked by anti-merlin antibody (C-18, upper panel). The actin blot was used as the loading control (lower panel). The engineered NIH3T3-CPI-17-S518A **(C)** or NIH3T3-CPI-17-WT **(D)** cells were placed in 0.33% agar containing medium in the absence or presence of Dox. The pictures were taken after one-week culture.

3.3.4 The effect of CPI-17 on cell growth

Merlin is a tumor suppressor and can inhibit tumorigenesis. However, its growth inhibitory function could be impaired by CPI-17. Therefore, CPI-17 was predicted to promote tumorigenesis. To explore this, CPI-17 was introduced into the non-transformed but immortalized fibroblast cell line NIH3T3 that does not express endogenous CPI-17 (Figure 3.18A). Non-transfected NIH3T3 cells stopped growing after reaching confluency in culture dishes and could not grow in semi-solid soft agar medium (Figure 3.18B and C, left panel), demonstrating their contact inhibition and anchorage dependence of growth. However, CPI-17 expressing cells continued to grow and piled up to form foci in the culture dishes (Figure 3.18B, right panel), indicting the loss of contact inhibition in the presence of CPI-17. In addition, large colonies appeared when these cells were placed in soft agar, indicating the gain of anchorage independence (Figure 3.18C, right). In a word, as expected, CPI-17 indeed has transformation ability.

To confirm that in fact, the inactivation of merlin in these cells is the trigger for the transformation induced by CPI-17. I introduced the constitutively active S518A mutant of full-length merlin into NIH3T3-CPI-17 cells (Figure 3.19A). The S518A mutant of merlin is an active form of merlin since its growth inhibitory function was independent of the activation by its phosphatase. Intriguingly, both the number and size of the colonies growing in soft agar were dramatically reduced after the expression of the S518A mutant (Figure 3.19C). In contrast, the expression of wild type merlin had no significant influence (Figure 3.19B and D). Thus, only the S518A mutant, but not the wild type merlin, could reverse the transformation phenotype initiated by myosin/moesin phosphatase specific inhibitor CPI-17. This indicates that CPI-17 induces the transformation mainly, if not completely, by inhibiting the activation of tumor suppressor merlin.

As a summary, I found that, the myosin/moesin phosphatase composed of a PP1δ

catalytic subunit, the target subunit MYPT-1 and a small subunit M20, is the phosphatase that accounts for the dephosphorylation and activation of merlin. The interaction of merlin with this phosphatase is mediated by the binding between a region from residue 312 to 342 in merlin and the leucine zipper domain in MYPT-1. A pathogenic mutation L339F occurred in this region can attenuate the interaction of merlin with its phosphatase. A specific inhibitor of this phosphatase, CPI-17 induces transformation in that it inhibits the activation of merlin.

4. Discussion

4.1 Growth control and contact inhibition

Cell proliferation in the body has to be strictly regulated to maintain both the number and spatial organization of cells. This growth regulation largely depends on cell-cell interactions and cell-extracellular matrix interactions. This was actually termed 'contact inhibition' based on the *in vitro* observation that normal cells inhibit their growths by mutual physical contacts (Abercrombie and Heaysman, 1954). Most transformed cells have lost contact inhibition and do not stop replicating when they come into contact (Abercrombie and Ambrose, 1962). Loss of contact inhibition is therefore one of the most important characteristics of cancer cells. How contact inhibition occurs or how cancer cells can escape from contact inhibition is poorly understood. It was well accepted however, that the mediator of contact inhibition must be associated with certain cell surface receptors which can sense extracellular signals. The activity of this mediator has to be regulated in a dynamic and delicate way so that it can switch promptly from one activity state to another in response to the changes of growth conditions (Abercrombie, 1970; Fagotto and Gumbiner, 1996; Weiss, 1970). Since such a dynamic regulation in mammalian cells is principally achieved by reversible protein phosphorylation (Krebs, 1985), the activities of these mediators are controlled most likely by reversible protein phosphorylation. Reversible protein phosphorylation, the covalent attachment of a phosphate group to the hydroxyl group on a serine, threonine, or tyrosine side chain of a protein, is the predominant strategy employed in eukaryotic cells to control the activity of proteins. Nearly all aspects of cell life, such as gene expression regulations, cell cycle controls, metabolic processes and protein transports, are regulated by reversible protein phosphorylation. More than 10% of the total proteins in a typical mammalian cell are thought to be regulated by phosphorylation. Each phosphate group carries two negative charges. The attachment and removal of a phosphate group from a protein can therefore have profound effects on its activity and property (Anderson, 1992; Cohen, 2002a; Fischer, 1997; Hunter, 1995; Krebs, 1985).

The tumor suppressor merlin was recently proposed to be a candidate mediator of contact inhibition since it fulfills all of the predicted requirements mentioned above (Morrison et al., 2001). Merlin can interact with membrane associated adhesion molecules such as CD44 proteins and has a high homology with ERM proteins that are thought to act as membrane-cytoskeleton linkers (Bretscher et al., 2002; Gautreau et al., 2002; Gusella et al., 1999; McClatchey, 2003). As a tumor suppressor, merlin can inhibit cell growth but only when it is dephosphorylated under certain non-permissive growth conditions, while in growing cells merlin is phosphorylated and lacks the growth inhibitory function.

The interplay between the kinase and phosphatase of merlin is therefore proposed to play a critical role in the regulation of contact inhibition. The phosphatase of merlin can dephosphorylate and activate merlin and finally inhibit the inappropriate cell proliferation. The proper regulation of this phosphatase is hence important for cell growth control, and its deregulation would promote disorders such as tumor growth. The identification of merlin activating phosphatase is therefore beneficial to the understanding of not only how merlin is regulated by cell-cell/ECM contacts and how merlin functions as a contact inhibition mediator but also how cancer cells progress to escape from contact inhibition. Furthermore, some specific therapeutic approaches could be developed on the basis of this identification to restore contact inhibition in cancer cells. This cytostatic strategy is more rational and promising than traditional cytotoxic interventions.

4.2 The system of RT4-C cell line

The ENU induced rat schwannoma cell line RT4-D6P2T has been used to investigate the regulation and function of merlin (Morrison et al., 2001). Full-length merlin in this cell line is regulated by phosphorylation in response to cell density, serum status and HA incubation. In this work, serine 518 containing C-merlin (299-595) was expressed in this same cell line. C-merlin was found to be regulated in the same way as full-length merlin. The gel migration ability of C-merlin was influenced by the

phosphorylation of serine 518, indicating that the phosphorylation of serine 518 could have a significant effect on the conformation of merlin. This is consistent with the importance of this site for regulating the function of merlin. The difference in gel motility of C-merlin either phosphorylated or dephosphorylated at serine 518 made it possible to monitor the activity of the merlin activating phosphatase simply by Western blotting analysis using the anti-merlin antibody. Alternatively, a phospho-merlin specific antibody that became commercially available after the establishment of this system was also used.

Although serine 518 was well known to be the most critical site to regulate the function of merlin, there are several other potential phosphorylation sites in merlin with unknown functional importance, e.g. threonine 576 (Surace et al., 2004). Are they also the targets of the phosphatase identified by this system? There are no antibodies available to detect the phosphorylation of other sites and either S518A or S518D mutant of C-merlin expressed in RT4 cells migrates as a single band, making it impossible with this system to analyze other phosphorylation sites. Therefore, it cannot be excluded that the phosphatase found here might regulate other sites besides serine 518.

4.3 The interaction of merlin with myosin/moesin phosphatase

Myosin/moesin phosphatase was primarily found to be a trimeric holoenzyme consisting of a larger subunit MYPT-1, a smaller subunit M20 and the catalytic subunit PP1δ (Alessi et al., 1992). Purified PP1 alone can also dephosphorylate C-merlin *in vitro*, indicating that C-merlin contains a target site specific for the catalytic subunit PP1. This implicates that C-merlin and PP1δ can interact with each other independent of the target subunit MYPT-1 in certain situations, e.g. in the presence of a large amount of PP1δ or C-merlin. This could explain that the over-expressed C-merlin Δ which lacks the MYPT-1 interaction region could still be dephosphorylated *in vivo* although its dephosphorylation was much less efficient than the dephosphorylation of C-merlin (Figure 3.14).

No antibody so far is available for the detection of the subunit M20 and its function is completely unknown. Most of the characteristics of the phosphatase are actually contributed by MYPT-1. M20 has no affect on the activity of the phosphatase at all. Furthermore, M20 could not be detected in some tissues such as brain and skeletal muscles (Hartshorne, 1998; Hartshorne et al., 1998; Ito et al., 2004). It thus seems possible that myosin/moesin phosphatase is a trimeric holoenzyme only in certain tissues such as smooth muscle cells but exists as a dimer in other tissues such as skeletal muscles.

The interaction of merlin with the myosin/moesin phosphatase therefore can be simplified as the interaction between merlin and MYPT-1. Here it is shown that this interaction is due to the direct binding of a region from residue 312 to 341 in merlin to the leucine zipper domain in MYPT-1. In agreement with this finding, the MYPT-1 interaction region in merlin was indeed found to be exposed on the surface of merlin and was proposed to be involved in protein-protein interactions by the crystal structure analysis of N-merlin (1-342) (Shimizu et al., 2002). The interaction region does not contain the phosphorylation site. This could explain the difference in interactions of MYPT-1 with the full-length merlin and C-merlin. MYPT-1 binds to the S518D mutant of full-length merlin with higher affinity than to the S518A mutant whereas it binds to both forms of C-merlin with a similar affinity (Figure 3.10 and 3.11 respectively). In case that serine 518 is dephosphorylated or substituted by phosphorylation defective alanine residue, the full-length merlin is in a closed conformation because of the interaction between its N- and C-terminus (Sherman et al., 1997). This closed conformation could lead to the masking of the interaction region and thus inhibit the interaction of merlin with MYPT-1. When serine 518 is phosphorylated or substituted by phosphorylation mimicked aspartic acid (S518D), merlin is in an open conformation due to the loss of intra-molecular interaction and the interaction region will hence be accessible to MYPT-1. Therefore, S518D mutant of merlin could bind more strongly to MYPT-1 than the S518A mutant (Figure 3.10 A and B). However, either phosphorylated or dephosphorylated C-merlin seems to be in

a constitutively open conformation because of the lack of N-terminal merlin and hence the interaction region is always accessible to MYPT-1. Therefore, both forms of C-merlin could bind to MYPT-1 with the similar affinity (Figure 3.11).

Most *nf2* germline and somatic mutations result in truncated proteins and only few are missense mutations (Baser et al., 2004; De Vitis et al., 1996; Faudoa et al., 2000; Hung et al., 2000; Jacoby et al., 1994). How these missense mutations can confer the host cells a certain kind of growth advantage and thus be kept during tumor development remains largely unknown. The L339F (Leucine 339 Phenylalanine) mutation, resulting from the transition of a C to T at position of 1015, affects the interaction of merlin with MYPT-1. This mutant merlin is therefore proposed to be refractory to dephosphorylation and hence unable to inhibit cell growth. This might be the molecular mechanism of this pathogenic mutation. This missense mutation, however, can only attenuate but not completely abolish the interaction with MYPT-1. This is consistent with the clinical observation that missense mutations of *nf-2* gene are in general associated with mild phenotypes. The other two missense mutations, Q324L (Glutamine 324 Leucine) and L316F (Leucine 316 Phenylalanine), seem to have no influence on the interaction of merlin with MYPT-1. Interestingly, leucine 339 is encoded by exon 11 which is one of the most mutation-prone exons (De Vitis et al., 1996; Gutmann, 1997) while Glutamine 324 and Leucine 316, which have no effects on the interaction of merlin with MYPT-1, are encoded by exon 10. This implicates that the exon 11 rather than exon 10 coded sequences could play an important role in the regulation of merlin activity through mediating the interaction with MYPT-1.

Unfortunately, the MYPT-1 antibody available is not suitable for immuno-histochemical staining. Therefore, the subcellular localization of MYPT-1 was not investigated here. However, MYPT-1 has been found to localize on cell-cell contact sites and sub-membrane cortex areas such as ruffles in a variety of cells (Fukata et al., 1998; Hirano et al., 1999). This indicates that MYPT-1 has a similar localization with merlin. Importantly, both phosphorylated and dephosphorylated merlin can interact

with MYPT-1. This indicates that the colocalization of MYPT-1 with merlin is not the critical step for the myosin/moesin phosphatase to dephosphorylate and activate merlin but rather the regulation of the myosin/moesin phosphatase activity seems to be more important.

4.4 The regulation of the myosin/moesin phosphatase activity

The regulation of the myosin/moesin phosphatase activity has been investigated mainly by studying the phosphorylation of one of its well-known substrates, myosin (Somlyo and Somlyo, 2003). The increase of myosin phosphorylation can be achieved by the activation of its kinase, which is activated in response to the increase of intra-cellular Ca^{2+} concentration. However, the phosphorylation of myosin can also be increased at fixed suboptimal Ca^{2+} concentration when its kinases is not activated, resulting in Ca^{2+} sensitization. This is actually due to the inhibition of the myosin/moesin phosphatase activity (Kitazawa et al., 1991; Somlyo and Somlyo, 1993). This inhibition of myosin/moesin phosphatase is mainly due to the phosphorylation of MYPT-1 at threonine 696 (Ichikawa et al., 1996). The small GTPase Rho is involved in this phosphorylation. Over-expression of RhoA increased the phosphorylation of MYPT-1 and inhibited the activity of myosin/moesin phosphatase (Kimura et al., 1996). Both Ca^{2+} sensitization and MYPT-1 phosphorylation could be specifically inhibited by exotoxin C3 which can ADP-ribosylate and inactivate Rho (Pfitzer and Arner, 1998). The kinase responsible for this phosphorylation of MYPT-1 was then identified as ROK (Rho-associated kinase), one of the downstream effectors of the active Rho (Feng et al., 1999). Interestingly, several other kinases, such as integrin-linked kinase (ILK) and Raf, was also found to phosphorylate MYPT-1 at threonine 696 and thereby inhibit the activity of myosin/moesin phosphatase (Broustas et al., 2002; Kiss et al., 2002). The convergence of different signaling pathways on MYPT-1 indicates the importance of this phosphatase for the regulation of fundamental physiological processes in cells, such as cell migration and contraction. Now, the identification of tumor suppressor

merlin as a novel substrate puts this phosphatase to the central stage of cell growth control.

The involvement of Rho on the regulation of the myosin/moesin phosphatase activity puts merlin downstream of the Rho pathway. Rho is well known to be involved in tumorigenesis although the detailed molecular mechanism is not completely clear (Aznar and Lacal, 2001; Frame and Brunton, 2002; Olson, 1996; Pruitt and Der, 2001). The inactivation of the tumor suppressor merlin via the inhibition of its activating phosphatase could be a novel mechanism of Rho to promote tumor growth.

The molecular basis for the inhibition of the myosin/moesin phosphatase activity as a consequence of MYPT-1 phosphorylation is not yet understood except that the dissociation of MYPT-1 with the catalytic subunit and the subsequent disassembly of the phosphatase seems to be unlikely (Feng et al., 1999; Ito et al., 2004). The phosphatase responsible for the dephosphorylation of MYPT-1 and the subsequent activation of myosin/moesin phosphatase is also unknown.

In agreement with published results, the antibody against MYPT-1 (anti-MYPT-1, N-15) can recognize a doublet of MYPT-1 (Boudrez et al., 1999). This could be due to the alternative splicing or post-translational modifications. However, phosphorylated MYPT-1 recognized by the phospho-MYPT-1 specific antibody is also a doublet (Figure 3.16) while the exogenous myc tagged MYPT-1 expressed in the same cell line actually displayed only one single band (data not shown). Therefore, the doublet is due to alternative splicing rather than post-translational modifications. Several alternative spliced products of MYPT have been reported. Most of the isoforms of MYPT-1 are differing by the presence or absence of a central insert and the C-terminal LZ domain (Dirksen et al., 2000; Ito et al., 2004). Both isoforms in the doublet can be pulled down by C-merlin, indicating that these two isoforms of MYPT-1 might differ in the central insert but not the LZ domain that is responsible for the interaction with merlin.

4.5 The function of merlin

The antagonistic interplay between merlin and ERM proteins is important to control the organization of the cortical actin cytoskeleton beneath the cytoplasm membrane and the activation and propagation of the Ras-Erk signaling pathway, the most important growth controlling signaling pathways addressed by many mitogenic stimuli (Helen Morrison, unpublished data). In response to the activation of RTKs (Receptor Tyrosine Kinases) upon the binding of their corresponding growth factors (GFs), a complex around Ras will form in order to bring the activator of Ras, SOS, into proximity of Ras and the downstream effector MEK (Mitogen and Erk Kinase) close to the activated Ras. This process is dependent on ezrin, probably moesin and radixin as well, by linking F-actin to the membrane associated adhesion molecules that can act as co-receptors for growth factors, e.g. CD44 proteins (Orian-Rousseau et al., 2002). Merlin could interfere with this process by the replacement of ERM proteins to these trans-membrane co-receptors and prevent the assembly of the SOS/Ras/MEK complex. This novel finding could largely explain how merlin inhibits cell growth. However, the over-expression of the N-terminal half of merlin that can still bind to trans-membrane co-receptors, is not sufficient to reverse the malignant phenotypes of transformed cells (Morrison et al., 2001; Sherman et al., 1997; Tikoo et al., 1994), indicating that additional mechanisms of how merlin functions as a tumor suppressor might exist.

A variety of malignant tumors with unusual metastasis potential were developed in *nf-2* heterozygous mice, although NF2 associated tumors are normally benign (McClatchey et al., 1998). In addition, high frequency of *nf-2* mutations could be detected in patients with malignant mesothelioma (Bianchi et al., 1995; Lechner et al., 1997; Lee and Testa, 1999). All of these facts indicate that merlin could be involved in the prevention of tumor metastasis, the most catastrophic process of cancer. The deregulation of cell migration is crucial during the insidious process of tumor metastasis (Friedl and Wolf, 2003; Quaranta, 2002; Ridley et al., 2003; Wells, 2000). Cell migration is a highly coordinated process on which the myosin/moesin

phosphatase plays an important role via the regulation of myosin phosphorylation (Somlyo and Somlyo, 2003; Trybus, 1994). The dysregulation of this phosphatase could therefore be one of the most important events during tumor metastasis. S518A mutant, the active form of merlin, could bind to MYPT-1 as well, raising a question if merlin acts as a tumor metastasis suppressor through enhancing the activity of myosin/moesin phosphatase. A role of merlin in the inhibition of cell migration is supported by *in vitro* studies in which the ectopically expression of merlin could impair the motilities of some tumor cells and chicken muscle precursor cells (Chen et al., 2004; Gutmann et al., 1999b). Furthermore, merlin was recently found to be able to inhibit the activation of Rho, the most important activator of other ERM proteins (Tobias Specka, unpublished data). To make the interplay between merlin and ERM proteins more significant and efficient, it seems reasonable for merlin to prevent the activation of ERM proteins by increasing the activity of myosin/moesin phosphatase, the inhibitor of ERM proteins, together with inhibiting the activation of Rho, the activator of ERM proteins. Importantly, ERM proteins was found to be a key determinant in tumor metastasis although the underlying mechanism is unknown (Curto and McClatchey, 2004; Hunter, 2004; Khanna et al., 2004). Thus, this potential feedback regulation on the myosin/moesin activity could present a novel molecular mechanism of merlin to act as a tumor suppressor especially a metastasis suppressor.

4.6 The growth promoting function of CPI-17

Apart from the inhibition by MYPT-1 phosphorylation, the activity of the myosin/moesin phosphatase can also be blocked by the presence of CPI-17. CPI-17 has been known to be implicated in the pathogenesis of hypertension since it can increase the phosphorylation of myosin and leads to the dysregulation of vascular smooth muscle's contractility (Seko et al., 2003; Somlyo and Somlyo, 2003). Strikingly, CPI-17 is shown here for the first time to have transformation ability. This implicates that CPI-17 could act as a novel oncoprotein. Support for this prediction is the finding that the expression of CPI-17 is up-regulated in some tumor cells, e.g. pancreatic adenocarcinoma cells and ovary carcinoma cells (SAGE database).

Furthermore, the level of phosphorylated Erk was increased in CPI-17 expressing NIH3T3 cells (data not shown), indicating the upregulated activity of Ras dependent signaling pathways in these cells.

The phosphorylation of ERM proteins is also increased in CPI-17 expressing cells (data not shown). This could theoretically contribute to the transformation induced by CPI-17. However, the knock down of merlin expression alone in NIH3T3 cells with siRNA (small interference RNA) technique could indeed lead to the loss of contact inhibition (Tobias Specka, unpublished data) and the growth of NIH3T3-CPI-17 cells in soft agar can be inhibited by the expression of dominant active form of merlin (S518A mutant) (Figure 3.19C). Thus, the transformation ability of CPI-17 might be mainly, if not completely, due to the functional inhibition of tumor suppressor merlin.

Interestingly, the inhibitory potential of CPI-17 for the myosin/moesin phosphatase could be enhanced more than 1000 folds by PKC (Protein Kinase C), which is known to be involved in the pathogenesis of many cancers (Blobe et al., 1994; Li et al., 1998; O'Brian and Ward, 1989; Ohki et al., 2001). CPI-17 can act as an oncoprotein could therefore be a novel mechanism of PKC to promote tumorigenesis. This might also explain the puzzle how merlin can be phosphorylated and unable to prevent the assembly of Grb2-SOS/Ras/MEK complex after the activation of RTKs upon the binding of their corresponding growth factors. PKC can be activated in response to PLC (Phospholipase C) activation induced by activated RTKs. The activation of PKC can then enhance the inhibitory activity of CPI-17 to myosin/moesin phosphatase and finally lead to the phosphorylation and inactivation of merlin, permitting the association of Ras to Grb2-SOS and its effectors (model in Figure 4.1).

Additionally, the inhibition of CPI-17 to myosin/moesin phosphatase could also be potentiated independent of PKC (Erdodi et al., 2003; MacDonald et al., 2001), consistent with certain PKC-independent tumorigeneses. CPI-17 could therefore be a novel oncoprotein involved in tumorigenesis more generally.
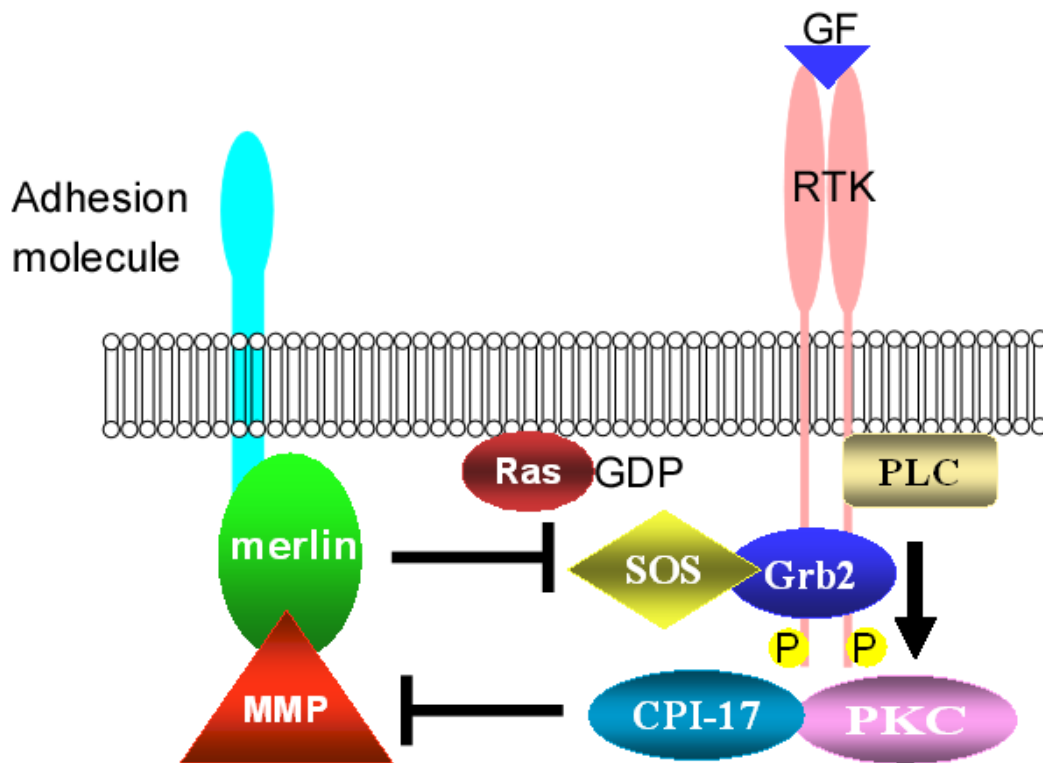
Figure 4.1. Model of the regulation and function of merlin. Upon the binding of GFs to RTKs, PKC can be activated by the activated RTKs through the activation of PLC. This leads to the activation of CPI-17 and the subsequent inhibition of MMP (Myosin/Moesin Phosphatase), finally results in the phosphorylation of merlin that is pre-requisite for the assembly of SOS/Ras/MEK complex. While merlin is dephosphorylated and associated with adhesion molecules, it can prevent the assembly of this growth promoting signaling complex.

In summary, this thesis work revealed that the myosin/moesin phosphatase is the activating phosphatase of the tumor suppressor merlin via the dephosphorylation at the serine 518 site. The interaction of merlin with the phosphatase is mediated by the

direct binding of the region from residue 312 to 342 in merlin to the leucine zipper domain in MYPT-1, the target subunit of the myosin/moesin phosphatase. The molecular basis of a pathogenic mutation in merlin (L339F) is to attenuate the interaction of merlin with its phosphatase. CPI-17, the potential inhibitor of the myosin/moesin phosphatase, exploits transformation capability and acts like an oncoprotein by interfering with the activation of merlin.

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