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Le rôle de l'extrémité C-terminale de la protéine Merline dans sa fonction anti-tumorale

The role of the C-terminus Merlin in its tumor suppressor function

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ABBREVIATIONS

ABS	actin binding site
AJ	adherens junction
AKAP	A-kinase anchoring protein
AKT	AKT8 virus oncogene cellular homolog
BB	blue box= dominant negative Merlin
C-ter	carboxyl terminal
CTM	c-terminus of Merlin
CD44	cluster of differentiation 44
CRL4	cullin-RING-ligase 4
DCAF1	DDB1- and Cul4-associated factor
D-merlin	Drosophila melanogaster Merlin
Dox	Doxycycline
ECM	extracellular matrix
ERM	exrin-radixin-moesin
EGFR	epidermal growth factor
ERK	extracellular regulated kinase
F-actin	filamentous actin
FAK	focal adhesion kinase
FERM	four.1 protein, ezrin, radixin, moesin
FRET	fluorescence resonance energy transfer
GEF	guanine nucleotide exchange factor
HEI10	human enhancer of invasion clone 10
HRS	hepatocyte growth factor-regulated kinase substrate
LATS	large tumor suppressor

Merlin	moesin-ezrin-radixin-like protein
Mer	merlin
NF1	neurofibromatosis 1
NF2	neurofibromatosis 2
PAK	p21-activated kinase
Rac 1	Ras related C3 botulinum toxin substrate 1
Ras	rat sarcoma
RTK	receptor tyrosine kinase
UPP	ubiquitin proteasome pathway
VprBP	Vpr-binding protein
VS	vestibular schwannoma
WT	wild type

RESUME

La neurofibromatose de type 2 (NF2) est une maladie autosomique causée soit par l'inactivation du gène NF2, soit par la perte de la proteine issue due ce gène, Merline. Cela entraîne à son tour la formation de plusieurs tumeurs nerveuse bénignes (non invasives) comme les schwannomes, méningiomes et les épendymomes. De plus, une diminution de l'expression de Merline est observée dans les cancers du sein invasifs, toutefois le rôle de Merline dans ces tumeurs invasives est peu étudié.

Merline est la seule protéine ayant un rôle de suppresseur de tumeur dans la famille des ERM (Ezrin / Radixin / Moesin). Nous, ainsi que d'autres groupes, avons montré que la partie C-terminale de Merline est importante pour sa fonction inhibitrice de la croissance cellulaire. Par conséquent, j'ai cherché à mettre en évidence de nouveaux partenaires d'interaction non décrits à ce jour, ainsi que de nouveaux sites de phosphorylation sur l'extrémité C-terminale de Merline qui pourrait expliquer la fonction de suppresseur de tumeur de Merlin. L'utilisation d'experiences d'immunoprécipitation couplées à la spectrométrie de masse nous a permis d'identifier de nouveaux interacteurs ainsi que de nouveaux sites de phosphorylation sur ce domaine C-terminal de Merline. Nous avons analysé l'importance d'un nouvel interacteur, AmotL1, ainsi que d'un nouveau site de phosphorylation sur la threonine 581 (T581), dans la fonction suppresseur de tumeur de Merline.

La proteine AmotL1 appartient à la famille des motines, qui sont connues pour être impliquées dans la régulation de la migration cellulaire. A cet égard, nous avons montré qu'AmotL1 est un nouveau partenaire d'interaction de Merline. Nous avons étudié l'importance de cette interaction entre Merline et AmotL1 dans la migration cellulaire et nos données suggèrent fortement que Merlin pourrait inhiber la migration cellulaire médiée par AmotL1 dans les cellules du cancer du sein, via notamment la régulation de son expression et de sa localisation. Enfin, nous avons également identifié plusieurs nouveaux interacteurs de Merline, qui pourraient expliquer comment Merlin pourrait agir comme une protéine d'échafaudage à la membrane plasmique, en interagissant avec des composants essentiels de la voie Hippo, comme AmotL1, Kibra, Lats et YAP, pour réguler la prolifération et la migration cellulaire.

Dans la deuxième partie, nous avons identifié un nouveau site de phosphorylation spécifique à l'isoforme 1 de Merline, la T581, et nous avons démontré que la phosphorylation de cette threonine est importante pour la progression en mitose au moment approprié. De plus, dans cette étude, nous avons montré que Merlin est un substrat potentiel de la kinase Aurora A, un oncogène majeur, au cours de la mitose et de l'interphase, dans des lignées cellulaires de cancer du sein. Enfin, nous avons fourni des données préliminaires sur la façon dont Aurora A régule la signalisation Hippo et la fonction de DCAF1 en phosphorylant Merline.

En résumé, cette thèse met en évidence deux fonctions importantes de Merline: premièrement comment Merline régule la migration/invasion cellulaire dans des tumeurs non-nerveuses telles que les cancers du sein et deuxièmement, comment Merline est régulé au cours de la mitose et de l'interphase dans des lignées de cancer du sein, en agissant comme un substrat pour la kinase Aurora A qui est surexprimée dans plusieurs cancers comme celui du sein, du côlon et l'HCC. Prise dans son ensemble, notre étude montre le rôle potentiel de Merline dans les tumeurs invasives telles que celles rencontrées dans les cancers du sein.

ABSTRACT

Neurofibromatosis type 2 (NF2) is an autosomal disorder caused by inactivation of *NF2* gene or loss of the *NF2* product, Merlin. This in turn results in formation of multiple benign (noninvasive) nerve tumors such as schwannomas, meningiomas and ependymomas. Additionally reduced expression of Merlin is observed in invasive breast cancers however the role of Merlin in these invasive tumors is poorly investigated.

Merlin is the only tumor suppressor protein in Ezrin/Radixin/Moesin (ERM) family proteins. Previously we and others have shown that C-terminus of Merlin is important for its growth suppressive function. In this regard, I set out to investigate whether there were undiscovered interacting partners and novel phosphorylation sites on the C-terminus of Merlin that could account for tumor suppressor function of Merlin. Using immunoprecipitation coupled to mass spectrometry we have identified new interactors as well as novel phosphorylation on this C-terminus domain of Merlin. We analyzed importance of new interactor, AmotL1, as well as novel phosphorylation site on T581 in the tumor suppressor function of Merlin.

AmotL1 belongs to AMOT family proteins which are known to involve in the regulation of cell migration. In this context, we have shown that AmotL1 is novel interacting partner of Merlin. We have investigated the importance of Merlin and AmotL1 interactions in cell migration and our data strongly suggest that Merlin might inhibit AmotL1 mediated cell migration in breast cancer cells by regulating its expression and localization. Finally, we have also found several new interactors of Merlin and that could explain how Merlin might acts as scaffolding protein at the plasma membrane by interacting with Hippo core components such as AmotL1, Kibra, Lats and YAP to regulate cell proliferation and migration.

In the second part, we have identified a novel phosphorylation site at T581 which is specific to Merlin isoform 1 and demonstrated that phosphorylation of Merlin on T581 is important for the timely mitotic progression. Further in this study, we have shown that Merlin is a potential substrate for major oncogene Aurora kinase A in mitosis as well as in interphasic breast cancer cell lines. Finally we have provided initial clues how Aurora A regulates Hippo signaling and DCAF1 function by phosphorylating Merlin.

In the summary, this thesis highlights two important functions of Merlin: firstly how Merlin regulates the cell migration/invasion in non-nerve tumors such as breast cancers and secondly how Merlin is regulated in mitosis and interphasic breast cancer cells by acting as a substrate to Aurora Kinase A which is over expressed in several cancers such as breast, colon and HCC. All together our study indicates the potential role for Merlin in invasive tumors such as breast cancers.

1. INTRODUCTION

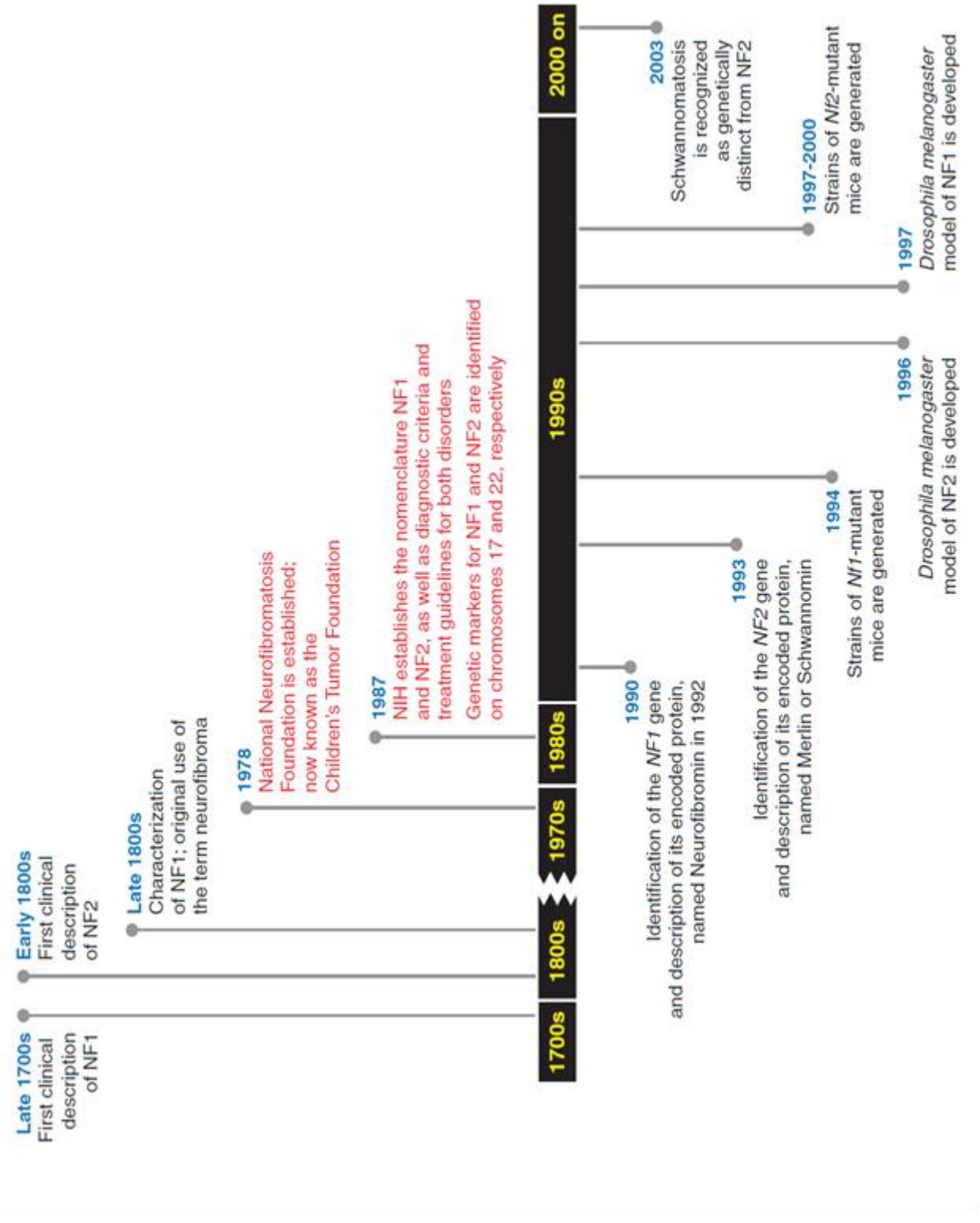


Figure1. Timeline of historical milestones in neurofibromatosis type 1 and type 2 (NF1 and NF2) research. Form (McClatchey, 2007)

1.1 Neurofibromatoses

Neurofibromatoses (NFs) are a group of familial cancer disorders characterized by the development of tumors of the nervous system, particularly of the nerve sheath (Friedman et al., 1999; Huson S et al., 1994). These disorders can cause tumors to grow on the nerves at any location and time. Based on the genetic origin and pathogenesis, there are three forms of NFs that exist. Neurofibromatosis type 1 (NF1), Neurofibromatosis type 2 (NF2) and Schwannomatosis (MacCollin et al., 2005). But the diagnostic criteria were formulated only for the two major forms of NFs: NF1 and NF2. Schwannomatosis is the latest addition to the family of the neurofibromatosis and much less is known about its genetic or molecular basis (Korf,2005). Neurofibromatosis type 1 (NF1), also known as von Recklinghausen's neurofibromatosis or peripheral neurofibromatosis has been first described in the 1700s and studied extensively in 1882 (**Fig 1**). NF1 is the most common autosomal dominant disorder due to its high incidence as 1 in 3500 individuals. NF1 gene is located on the chromosome 17q11.2 in human and it codes for the tumor suppressor protein neurofibromin. The characteristics of NF1 include plexiform neurofibroma, optic glioma and multiple benign cutaneous neurofibromas (**Fig 2A**), freckling in the axillary or inguinal region and iris hamartomas (Jouhilahti et al., 2011; 1988). Mutation in INI/SMARCB1 gene results in Schwannomatosis and is characterized by multiple schwannomas in absence of vestibular schwannomas. Schwannomatosis affects approximately 1 in 30,000 individuals (Hulsebos et al., 2007; MacCollin et al., 2005) (**Fig 2B**).

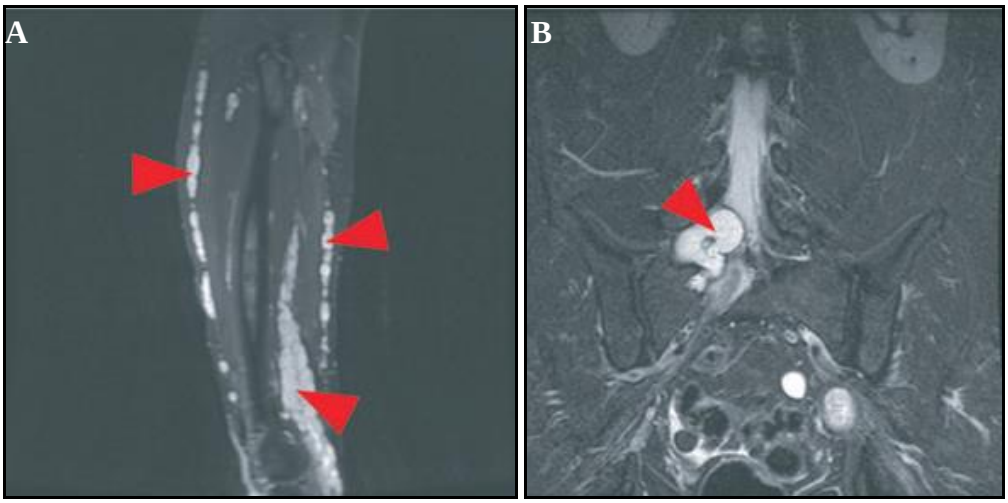


Figure 2. Coronal images of (A) a plexiform neurofibroma of the thigh in an NF1 patient. (B) Spinal schwannoma in a schwannomatosis patient. Arrowheads indicate tumor masses. From Mcclatchey et al 2007.

1.2 NF2 disease

Neurofibromatosis type 2 (NF2) is an autosomal dominantly inherited cancer syndrome characterized by the development of multiple schwannomas, meninigiomas and ependymomas. A large majority of patients develop bilateral schwannoma frequently affecting the superior vestibular branch of the eighth cranial nerve (Evans et al., 1992). NF2 was first described in the 1822 and its first report in 1920 (Wishart 1922, Feiling et al., 1920) (**Fig 1**). NF2 occurs much less frequently as its incidence is 1 in 25,000 individuals. However, NF2 prevalence has now increased to 1 in 60,000 individuals due to earlier diagnosis and better survival due to improved treatment (Evans, 2009a). The criteria for NF2 are met by an individual who has characteristics mentioned in **Table 1**.

The vital feature of NF2 is the development of multiple benign tumors of the nervous system, especially bilateral vestibular schwannomas (VS) of the eighth cranial nerve (**Fig 3**). The clinical symptoms of the VS are tinnitus (ringing in the ears), hearing loss, or imbalance or a combination of the three symptoms. Schwannomas are tumors composed only of Schwann cells. Schwann cells forms myelin sheath that surrounds axons in the peripheral nervous system. These vestibular schwannomas are present in 90% of NF2 patients. 20%-50% of NF2 patients develop other schwannomas on other cranial or spinal nerves. The other tumors associated with the NF2 are meningiomas (**Fig 3**) both intracranial (including optic nerve meningiomas) and intraspinal, and some low-grade central nervous system (CNS) malignancies (ependymomas and gliomas). Meningiomas are benign tumors developing from the meninges that surround the brain and spinal cord. These tumors are present in 20-30% of patients. Less frequently observed NF2 tumors are the ependymomas from ependymal cell of ventricular lining, and astrocytomas coming from glial star-shaped brain cells in the cerebrum called astrocytes (Evans et al., 1992; Kanter et al., 1980; Mautner et al., 1996; Parry et al., 1994).

The clinical manifestations of NF2 include benign tumors causing considerable pain and nerve dysfunction (schwannomas), or increase of the cranial pressure, causing number of symptoms including migraines, weakness, impaired vision and speech, and or memory (meningiomas). Nausea, vomiting and true vertigo are less frequent except in the late stage of the disease. Early childhood non eighth nerve tumors or an ocular presentation indicates the first sign of severe multi tumor NF2. NF2 disease, in children, is characterized by polio-like

illness with the unrecoverable wastage of muscle groups in the lower limbs. Whereas in adults, it appears to be a tendency to mononeuropathy, particularly affecting the facial nerve causing a Bell's-like palsy (inability to control facial muscles on the affected side) which does not fully recover years before the detection of VS. In addition, many NF2 patients (60-80%) develop posterior sub-capsular cataracts, a rare type of cataract that involves altered cellular organization rather than crystalline lens opacity. Also 70% of the NF2 patients have skin tumors which are mostly schwannomas. These are of three types including the most frequent type, the plaque-like lesion, followed by the more deep-seated subcutaneous nodular tumors and finally the occasional intracutaneous tumors similar to those in NF1 (Asthagiri et al., 2009; Evans, 2009b)

Table 1. Diagnostic criteria for Neurofibromatosis type 2. This also includes NIH criteria and Manchester criteria (From Evans, 2009a)

Main criteria	Additional criteria
Bilateral vestibular schwannomas (VS) or family history of NF2 plus 1. Lateral VS or 2. Any two of: meningioma, glioma, neurofibroma, schwannoma, posterior subcapsular lenticular opacities	Unilateral VS plus any two of: meningioma, glioma, neurofibroma, schwannoma, and posterior subcapsular opacities Or Multiple meningioma (two or more) plus unilateral VS or any two of: glioma, neurofibroma, schwannoma, and cataract

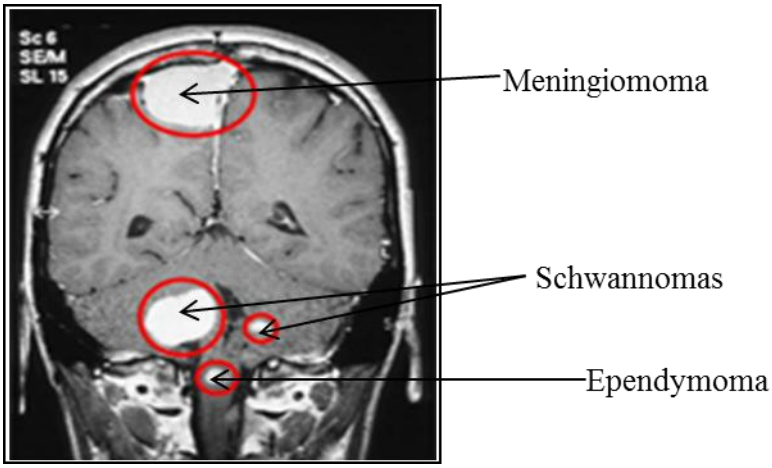


Figure 3. Tumors in NF2. MRI scan of a NF2 patient with typical NF2-associated tumors. Picture from Dr. Stephane Goutagny (Hospital Beaujon, France).

1.3 The *NF2* gene

Genetic linkage studies and investigations of both sporadic and familial tumors suggested that NF2 is caused by inactivation of a tumor suppressor gene on chromosome 22q12 (Rouleau et al., 1987; Seizinger et al., 1987; Trofatter et al., 1993). *NF2* gene was first identified in 1993 by positional cloning and loss of heterozygosity studies, and classified as tumor suppressor genes by genetic criteria (Rouleau et al., 1993; Seizinger et al., 1986).

The presence of *NF2* homologues from other species and functional characterization of their encoded proteins provided a useful link to the better understanding of *NF2* gene. *NF2* similar genes are present in many metazoans including *Drosophila melanogaster*, *Anopheles gambiae*, *Caenorhabditis elegan*, *Xenopus laevis*, and vertebrates such as *Danio rerio*, *Oryzias latipes*, and birds (*Gallus gallus*) (Golovnina et al., 2005). No *NF2* homolog has been identified in the genomes of *Saccharomyces cerevisiae* (yeast), plants or protozoa suggesting that Merlin function is specific to the evolutionary branching of multicellular metazoans. The high degree of homology between *NF2* gene products implies that they can be used interchangeably in functional studies (Hara et al., 1994).

Although the phenotype of the NF2 disease is highly tissue specific, *NF2* gene expression has been observed in many cell types in different tissues (den Bakker et al., 1999; Bianchi et al., 1994). The *NF2* gene contains 17 exons and it has an open reading frame (ORF) of 1788 bases and produces mRNA of three different sizes; 7 kb, 4.4 kb and 2.6 kb. Analysis of these transcripts confirmed that *NF2* gene has two major splicing isoforms, isoform 1 and isoform 2 (**Fig 4**). Isoform 1 is expressed from exons 1-15 and 17. Whereas isoform 2 has 45 base pair (bp) insertion at the 1737 nucleotide, providing a termination codon that leads to a premature termination of the reading frame without translating the exon 17, but translating up to exon 16. Due to this, the C-terminus of both *NF2* gene products differs by a total of sixteen amino acids from position 579 (Bianchi et al., 1994; Rouleau et al., 1993; Trofatter et al., 1993) (**Fig 4**).

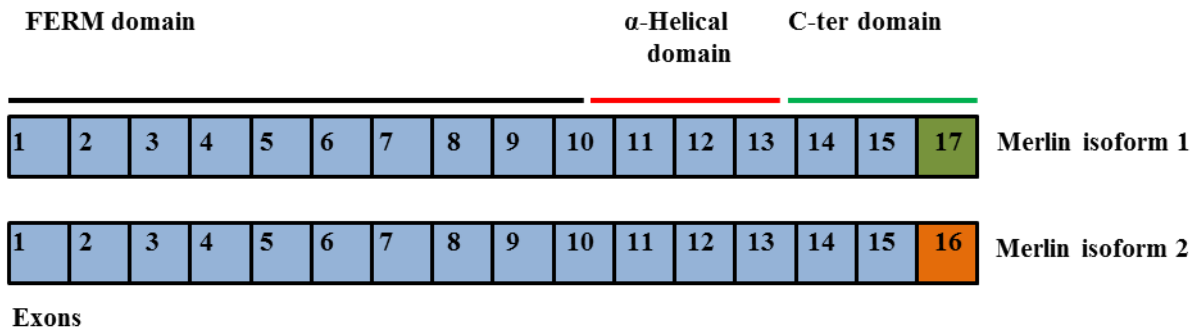


Figure 4. Exon organization of *NF2* gene: The *NF2* gene consists of 17 exons. Two most common *NF2* isoforms, isoform I and 2, and are alternatively spliced in last exons 16 or 17, respectively. Human Merlin isoform I contains 595 amino acids whereas isoform 2 has 590 amino acids with estimated molecular weights of approximately 65-70 kDa (Stamenkovic and Yu, 2010)

1.4 Mutational spectrum of *NF2* gene

NF2 gene is seen frequently mutated not only in tumors of NF2 disease but also in their sporadic counterparts, and in some malignant tumors unrelated to NF2 disease. The estimated *NF2* mutation rate is $6.5*10^{-6}$, and due to this high mutation rate, the occurrence of NF2 without family history is more common. This disorder has high penetrance and individuals who inherit a pathogenic mutation will develop the disease in an average lifetime (Evans, 2009b). In the NF2 syndrome, tumor development initiates when both alleles of the *NF2* gene are inactivated (Baser, 2006). However mathematical modeling of VS formation in NF2 patients suggests that at least one more mutational event, in addition to the biallelic inactivation of *NF2* gene, is required for the schwannoma development in humans (Woods et al., 2003). Mutations in the *NF2* gene are detected in 88% of familial and 59% of sporadic NF2 patients (Wallace et al., 2004), indicating that loss of *NF2* functional protein is the rate limiting step for the NF2 tumor development.

More than 200 different mutations in the *NF2* gene have been described. The majority of these mutations are truncating mutations with the nonsense and frame-shift mutations occurring in approximately 50% of the families and splice site mutations in 25%. In addition, single and multiple exon deletions were also detected. The point mutations with C>T transitions leading to a nonsense mutation are also the most common mutations of the *NF2* gene. However missense mutations occur at lower frequency and patients with missense mutation have statistically greater survival than nonsense and frame-shift mutations (Baser et

al., 2004). The other mutations in *NF2* gene are large scale rearrangements detected by Multiplex Ligation-dependent Probe Amplification (MLPA) and are known to account for around 15% of *NF2* germ line aberrations (Baser et al., 2005; Kluwe et al., 2005; Tsilchorozidou et al., 2004). The common MLPA includes deletion of the *NF2* promoter, exon 1 and deletions of the *NF2* gene. All these mutations are evenly distributed throughout the first 15 exons of the *NF2* gene. But exon 9 has a lower frequency rate and interestingly no mutation have been observed on exon 16 and 17(Corresponding to amino acid from 579 to the end of protein).

The different frequency of the various types of *NF2* mutations can be seen in **Fig 5**.

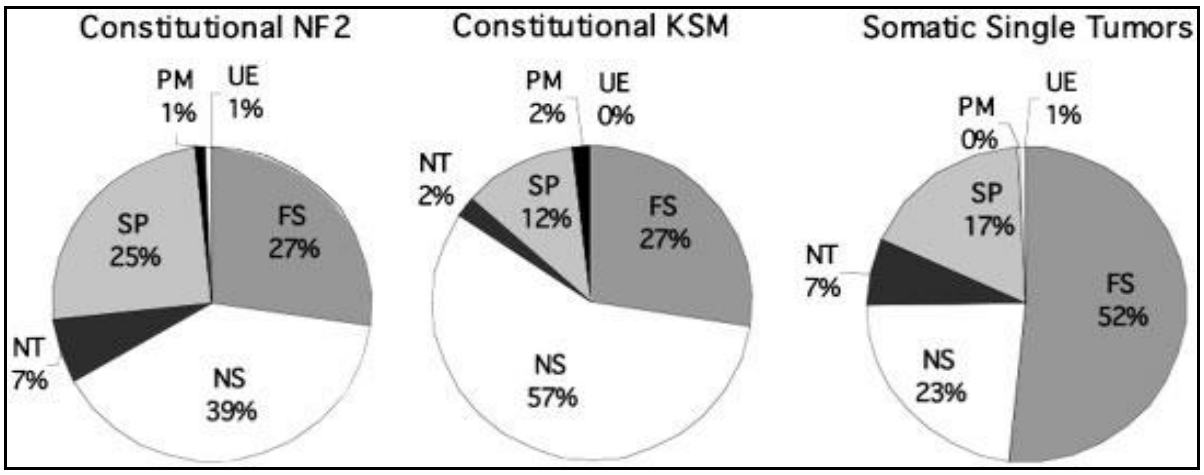


Figure 5. Comparisons of small alteration spectrum by predicted effect. SP, splice site alteration; NS, nonsense; FS, frameshift; NT, nontruncating alteration presumed to be pathogenic; UE, unclear effect; KSM, known somaticmosaic; PM, presumed polymorphism (From Ahronowitz et al., 2007).

In the NF2 syndrome, a correlation between mutation and phenotype has been observed. NF2 patients with truncating nonsense or frame-shift *NF2* gene mutations have more severe disease. However patients with missense mutations and large deletions, or somatic mosaicism caused predominantly mild phenotypes of the disease. In contrast, NF2 patients with spliced-site mutations had more variable phenotypes when compared to other mutations. Mosaicism is likely in NF2 patients particularly when tumors are predominantly on one side of the body. 20-30% of the NF2 cases without family history of the disease are mosaic (Baser et al., 2004; Parry et al., 1996).

1.5 *NF2* in other cancers

Although *NF2* has been extensively studied in tumors arising from the nervous system, it has been shown to play a vital role in development of various other cancers. For example loss or inactivation of the *NF2* gene triggers highly aggressive mesothelioma tumors, as well as breast cancers, melanomas and gliomas (Lau et al., 2008; Morrow et al., 2011; Murray et al., 2012; Sekido, 2011). Malignant mesothelioma (MM) is a rare form of cancer that develops from cells of the mesothelium, the protective layer that covers many of the internal organs of the body. Initially it has been shown that exposure to asbestos induces Mesothelioma (Pass et al., 2004; Robinson and Lake, 2005). Later reports have revealed that inactivation of *NF2* plays a crucial role in the development of mesotheliomas (Bianchi et al., 1994; Musti et al., 2006; Sekido, 2011) and the mutation rate of *NF2* gene is detected in 40–50% of MM. Additionally it has been shown recently that loss of activity of *NF2* results in inactivation of the Hippo signaling cascade which is important for the regulation of cell growth in mesothelioma (Murakami et al., 2011). Concerning the role of *NF2* gene in breast cancers, it has shown that mutations in the *NF2* gene were detected in a cohort of 60 breast cancer patients (Arakawa et al., 1994; Yaegashi et al., 1995). Additonally, mutations affecting the both isoforms of *NF2* were observed in various tumor types such as melanoma (6 out of 12 tumors) and breast carcinoma (1 out of 14) (Bianchi et al., 1994; Rouleau et al., 1993). Interestingly Murray et al., recently has been shown that expression of *NF2* was decreased in breast cancer tissues compared to normal tissue and restoring its expression in xenograft model results in reduced breast cancer features such as invasion, migration, motility and tumor growth in immunocompromised mice (Morrow et al., 2011). Similarly down regulation of *NF2* gene product was observed in melanoma and gliomas (Lau et al., 2008; Murray et al., 2012). Altogether, it appears that *NF2* is rarely mutated in non NF2 tumors but that down regulation of its expression could be involved in different types of tumor development.

The *NF2* gene codes a protein of 595 amino acids called Merlin. It belongs to the ERMs (Ezrin, Radixin, Moesin) subgroup of superfamily Band 4.1 protein. Merlin stands for **m**oesin-**e**zrin-**r**adixin **li**ke protein due to its structural similarity with ERMs. Based on homology, the study of ERMs has provided the structural and functional insights about the Merlin. So it is necessary to know about the ERMs before we enter into Merlin biology.

2. ERM proteins and Merlin

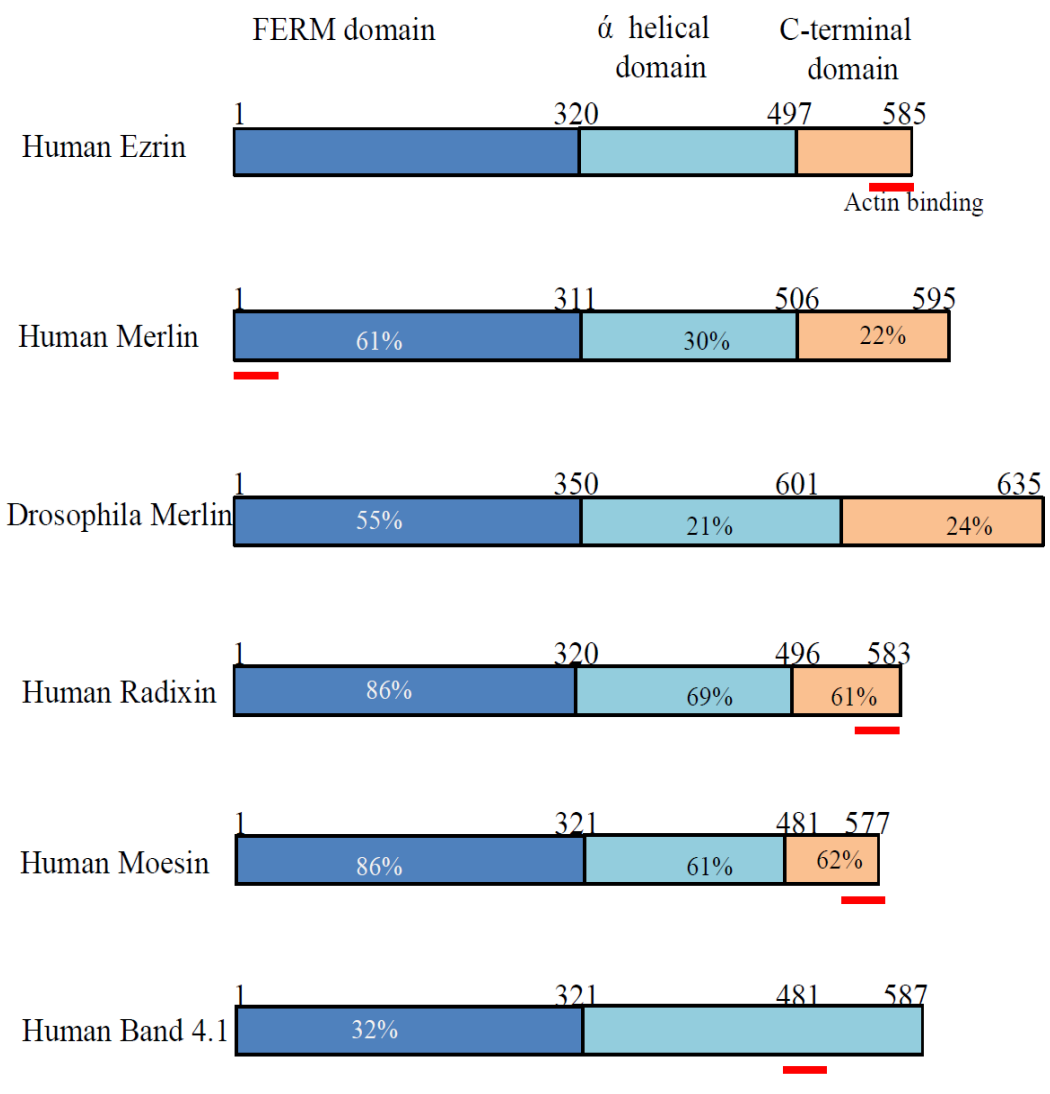
2.1 ERM proteins

2.1.1 Band 4.1 Proteins and ERMs

One of the key questions in cell biology is about the coordination of cytoskeleton and plasma membrane in forming specific domains and the participation of such domains in signal transduction pathways. This in turn allows cytoskeleton and plasma membrane to control cellular processes like cell invasion, migration, cell division and apoptosis. ERM proteins are broadly distributed plasma membrane proteins and regulate the structure and function of specific domains of cellular cortex.

The ERMs are members of the superfamily of Band 4.1 protein. Protein 4.1 members are defined based on the presence of a conserved FERM (Four.1 protein, Ezrin, Radixin, Moesin) domain at the N-terminus of the molecule and positioning on 2D SDS polyacrylamide gel electrophoresis (Chishti et al., 1998; Holzwarth et al., 1976). The first identified member is called erythrocyte band 4.1 proteins and plays a key role in maintenance of red blood cell shape by stabilizing the interactions between cytoskeleton networks and plasma membrane (Conboy J). More than 50 members have been added since its identification (McClatchey and Fehon, 2009). Based on sequence similarity, this superfamily is divided into five subgroups. Among which two of the subgroups, the ERMs and prototypic Protein 4.1 molecules, include proteins that have tumor suppressor activity.

X-ray crystallographic studies have shown that homologous FERM (Four.1 protein, Ezrin, Radixin, Moesin) domain at the N-terminus is the major characteristic feature of Band 4.1 protein family (Chishti et al., 1998**).** This FERM domain is globular in nature and is composed of ~300 amino acids (**Fig 6**). It plays an important role in targeting the protein to the plasma membrane and also in channeling the interactions between membrane and



Length	Identity
585	100
595	45
635	39
583	77
577	73
587	24

Figure 6. Sequence comparision between ERM proteins and Band 4.1. The overall homology of full length proteins compared with human Ezrin. The length and indentity (%) among all give in table. Modified from Turunen et al., 1998 and Fievet et al., 2007.

cytoskeleton network (Chishti et al., 1998; Sun et al., 2002a). Studies on FERM domain demonstrate the presence of three subdomains, F1, F2, F3, giving a cloverleaf like structure (Edwards and Keep, 2001). Structurally, F1 sub domain resembles ubiquitin, and F2 is similar to acyl-CoA-binding proteins whereas F3 is a reminiscence of three proteins which includes phosphor-tyrosine binding (PTB), pleckstrin homology (PH) and Enabled/VASP homology 1 (EVH1) domains (Pearson et al., 2000).

2.1.2 ERMs structure

ERM family comprises of three closely related proteins named Ezrin, Radixin and Moesin. The genes encoding ERM family proteins reside on different chromosomes in human as well as in mice (Sun et al., 2002a) (**Table 2**). All the multicellular metazoan organisms analyzed so far have genes encoding ERM proteins. However, depending on the species, ERM proteins varies from one to three. For example in non-vertebrates like *Drosophila melanogaster*, Moesin is the sole ERM that is expressed (Bretscher et al., 2002; McClatchey and Fehon, 2009). In all non-mammalian genomes sequenced so far, there is only one ERM gene and which in turn indicates that the genes coding ezrin, radixin and moesin are the result of gene duplication events unique to mammals (Abedin and King, 2008; King and Carroll, 2001; King et al., 2008).

Ezrin was first isolated from chicken intestinal epithelial cell brush borders as a 80 kDa protein and as cytovillin from human choriocarcinoma cells (Bretscher, 1983; Pakkanen et al., 1987). Radixin was purified as a 82-kDa adherent junction protein from rat liver (Tsukita et al., 1989) whereas Meosin was isolated from bovine uterus as a receptor protein for heparin sulfate. cDNA cloning and sequence analysis has demonstrated that Ezrin, Radixin and Moesin show significant sequence identity in their primary structure, especially at their N-terminus end (**Fig 6**) and hence categorized as ERM subfamily (Funayama et al., 1991; Lankes and Furthmayr, 1991; Sato et al., 1992).

Several studies provided the structural information about ERM protein family. Similar to band 4.1, the ERMs contain a FERM domain that is comprised of three subdomains. F1, F2 and F3, together forms a compact clover-shaped structure (Edwards and Keep, 2001; Hamada et al., 2000; Pearson et al., 2000; Smith et al., 2003). The C-terminus of the ERM proteins contains

a less conserved linker region of ~200 amino acids which forms a highly charged α-helical region, consisting of three extended helices (**Fig 6**). Finally a C-terminal tail adopts an extended, meandering structure composed of ~150 amino acids and composed of a beta-sheet, four major and two minor alpha- helices. The last 30 amino acids of the C-terminus of ERM family proteins contain an F-Actin Binding site (ABS) (Turunen et al., 1994) (**Fig 6**).

Table 2. Protein 4.1/ERM family molecules. (From Sun et al., 2002a)

Protein	Molecular mass (kDa)	chromosome	Adult tissue distribution	Subcellular localization
4.1B, type II brain 4.1	125	18p11.3	Brain, heart, lung, kidney, intestine, testis.	Plasma membrane at regions of cell-cell contact
4.1R, EPB41	80	1p36.2-p34	Erythrocytes, brain.	Plasma membrane, nucleus, spindle poles of mitotic cells, centrosomes
Merlin	69	22q12.2	Brain, lens, sciatic nerve, blood vessels, adrenal gland, Schwann cells, peripheral nerve.	Perinuclear, plasma membrane, filopodia, regions of cell/substrate adhesion
Ezrin	81	6q25-q26	Brain, kidney, intestine, lung, peripheral nerve, Schwann cell	Plasma membrane, apical microvilli, actin-containing surface structures, cell-cell adherens junctions
Radixin	82	11q23	Brain, kidney, liver, lung, thymus, peripheral nerve, Schwann cells gonads, skin	Plasma membrane, apical microvilli, actin-containing surface structures, cell-cell adherens junctions
Moesin	78	Xq11.2-q12	Brain, endothelial cells, heart, kidney muscle, lung, liver, spleen, peripheral nerve, Schwann cells	Plasma membrane, apical microvilli, actin-containing surface structures, cell-cell adherens junctions

2.1.3. Sub-cellular localization and tissue distribution of ERMs

ERM proteins are widely described as structural components of the cell membrane which connects the plasma membrane with underlying cytoskeleton network. Due to this reason, ERM proteins are known to localize at actin rich regions of membrane surface structures like filopodia, microvilli, membrane ruffles and cell adhesion structures (Berryman et al., 1993; Franck et al., 1993; Sato et al., 1992). In a developmentally regulated and cell specific manner, most of the cells express one or several of ERM proteins. For instance, the expression of the three ERM proteins is tissue-specific with Ezrin found primarily in epithelial cells, Moesin in endothelial cells, whereas Radixin is found in hepatocytes and hair cells. Coming to organs, Ezrin is mainly expressed in brain, kidney, intestine, lung, peripheral nerve and Schwann cells. Radixin in liver, lung, thymus, Schwann cells, gonads and skin. Moesin is expressed in lung, spleen and heart. The summary of ERM proteins tissue distributions, sub-cellular localizations, and chromosomal locations is shown in **Table 2** (Sun et al., 2002a)**.** Even though the ERM proteins tissue distribution seems to overlap, their subcellular distribution *in vivo* appears to be different (Scherer and Gutmann, 1996; Scherer et al., 2001), explaining their unique functions in specific tissues. ERM knockout studies in mice have provided insight into their functions and expression in specific cell types (Saotome et al., 2004). Ezrin knockout mice die soon after birth due to a defect in the gastrointestinal tract. In these mice, villus formation is abnormal suggesting a regulatory role of Ezrin in assembly of junctions. Ezrin knockdown mice show a loss of secretion of gastric acid (Tamura et al., 2005), retardation in the development of photoreceptors (Bonilha et al., 2006) and severe achlorhydria in stomach. Mice with Radixin inactivation results in hyperbilirubinemia and deafness, due to defects in liver secretion system and regeneration of cochlear stereocilia respectively (Kikuchi et al., 2002; Kitajiri et al., 2004). In contrast, Moesin knockdown mice did not shown any abnormalities (Doi et al., 1999).

2.1.4 ERM proteins and their binding partners

The N-terminal FERM domain is important for targeting the ERMs to membrane through interaction with PIP2 (Barret et al., 2000). Hence through FERM domain, ERMs interacts with transmembrane proteins which include CD43 and CD44, ICAM1, ICAM2 and ICAM3 (Yonemura et al., 1998). ERMs also indirectly bind to proteins such as ion channels,

transporters and receptors through the scaffolding factors NHERF1 (EBP50 for ERM-binding phosphoprotein 50)/ NHERF2 (Na+-H+ exchanger regulatory factor) (Murthy et al., 1998; Reczek et al., 1997). ERMs regulate signal transduction pathways by binding to the regulators of Rho GTPase signaling via FERM domain (Takahashi et al., 1997). Finally, the extreme C-terminus domain of the ERMs consists of an actin binding site (ABS) which contains a consensus actin binding motif KYTL (Pestonjamasp et al., 1995; Turunen et al., 1994) (**Fig 6**). Inhibition of ERMs function appears to alter the actin cytoskeleton network and actin containing structures indicating that ERMs have crucial role in actin network organization.

The ERMs have ability to form homo and hetero dimers. Ezrin forms dimers, tetramers and higher order oligomers through head-to-tail assembly in placental microvilli. This oligomerization does not happen *in vitro* with purified Ezrin and probably requires an activation step. These oligomers are thought to be associated with cytoskeleton and to be involved in microvillus morphogenesis. Concerning the regulation of oligomerization of Ezrin, Gautreau at al., showed that phosphorylation of Ezrin decreases the number of oligomers at the plasma membrane. In addition, this study demonstrated that phosphomimetic form of Ezrin (T567D) can associated with the cytoskeleton of actin and trigger actin-rich membrane projections. This in turn results in altered epithelial morphology and differentiation. On the other hand, T567A weakly associates with the actin cytoskeleton and promotes the oligomerization as wild type Ezrin (Gautreau et al., 2000).

2.1.5 Assimilation of ERMs into signaling pathways

Localization of ERM proteins in specialized domains at the cell cortex is important for bringing many signaling effectors together in close proximity. The assembly of these domains containing the ERMs participates to the regulation of many signal transduction cascades such as Ras signaling and receptor transport.

A. ERMs and Ras signaling

Mutational studies on Ezrin shed light onto its role in the transmission of signals elicited by HGF (hepatocyte growth factor) stimulation. HGF is a glycoprotein which has been shown to trigger cell proliferation, cell survival, motility (scattering) and invasiveness in a paracrine mode (Matsumoto and Nakamura, 1992). HGF transmits its biological effects through

activation of the receptor tyrosine kinase (RTK) c-Met (Bottaro et al., 1991; Naldini et al., 1991). The mechanisms by which HGF triggers this cellular function depends on the activation of different intracellular signaling cascades. In this regard, from our research team, it has been demonstrated that HGF phosphorylates the Ezrin at Y145 and Y353 which in turn results in HGF induced morphogenesis in LLC-PK1 cells. These results indicated that Ezrin might be a downstream target of HGF receptor signaling pathway (Crepaldi et al., 1997). Also, it has been shown that different protein kinases interact with activated HGF receptor through their SH2 domain to mediate its effects on cell proliferation and scattering. These proteins includes PI3-kinase (Royal and Park, 1995) and a small GTPase, Ras (Hartmann et al., 1994; Ridley et al., 1995). In this context, Naba et al. have shown that Fes kinase interacts with phosphorylated Ezrin at Y477 and mediates the HFG induced cell scattering (Naba et al., 2008).

On the other hand, several studies have shown that the co-receptor CD44v6, isoform of CD44, is required for the activation of the receptor tyrosine kinase c-Met by HGF (Orian-Rousseau et al., 2002). CD44 is a family of transmembrane proteins which are crucial for the survival, growth regulation, motility, and differentiation. Their elevated expressions are observed in many pathological conditions such as cancer. In this context it has been shown that ERMs binds to cytoplasmic tail of CD44v6 to transmit the signal from activated Met receptor to the downstream MAP kinases to regulate different cellular processes such as cell proliferation and motility (Orian-Rousseau et al., 2007).

Recently H. Morrison (Sperka et al., 2011) group have demonstrated that Ezrin, through its interaction with actin, plays a crucial role in coordinating the signals from co-receptors to the activation of Ras by acting as an adaptor molecule. In this study they have shown that Ezrin has separate binding sites for actin, Ras, Son of sevenless (SOS) and through this multi proteins binding capacity, Ezrin is able to forms complexes (RTK, co-receptor Grb2, SOS and Ras) necessary for the regulation of SOS and Ras activity. Mutations or inactivation of Ezrin results in the inhibition of Ezrin mediated complex formation as well as inhibition of Ras activation. Taken together these results explained how Ezrin coordinated the cell signaling and cytoskeleton machinery to regulate the Ras-Map kinase activation. In addition, this study also explained how elevated levels of ERMs results in cancer progression and metastasis by activating Ras signaling pathway (Sperka et al., 2011) (**Fig 7**).

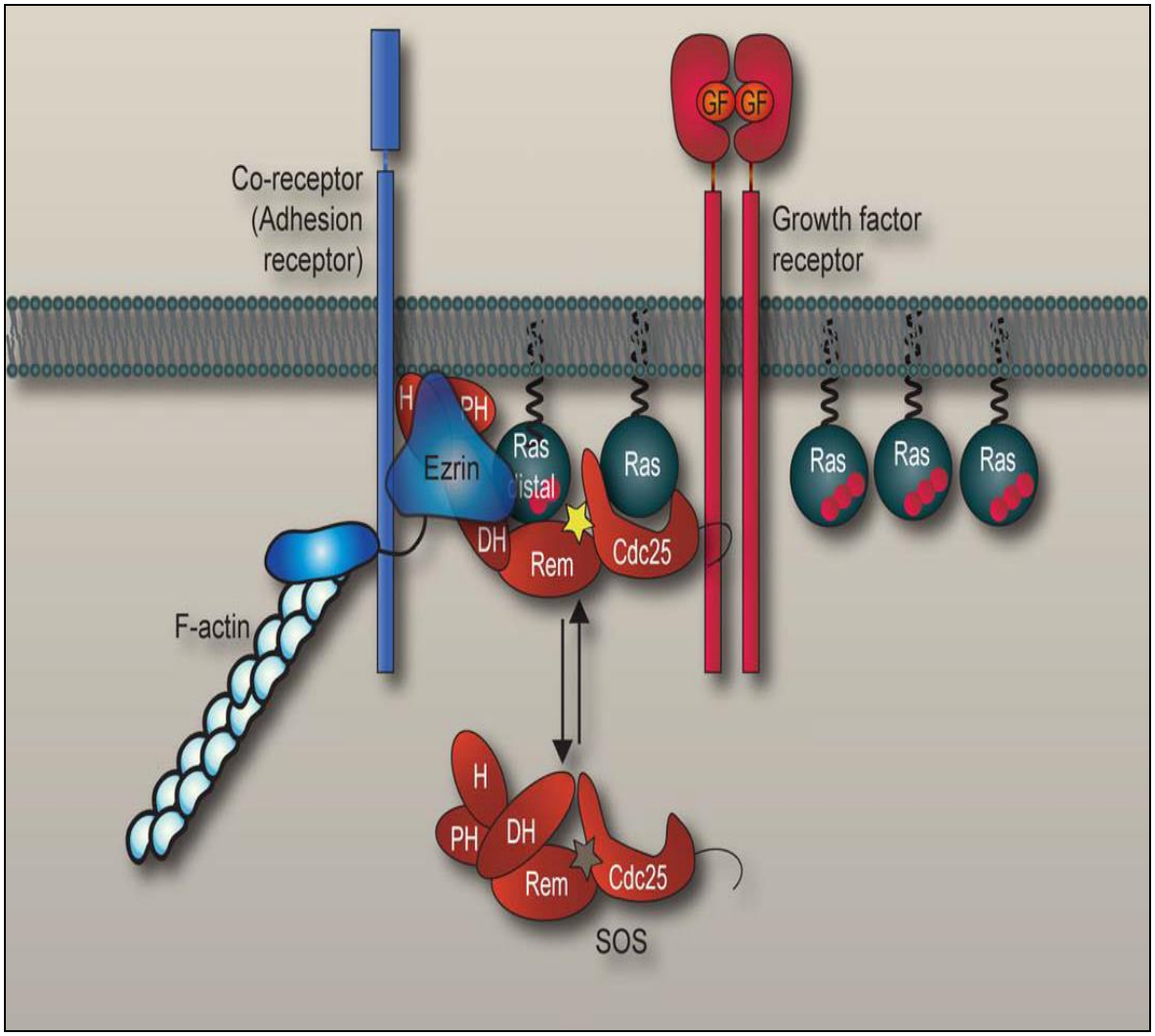


Figure 7. **Scheme showing Ezrin-mediated activation of SOS**. Binding of growth factor (GF) to its receptor resulted in Ezrin activation, which localizes to co-receptors/ adhesion receptors, while SOS couples to the activated RTK. Ezrin along with F-actin binds to the DH-PH domains of SOS and links it to plasma membrane leads to the release of SOS auto-inhibition. Binding of Ezrin to GDP (two red balls) form of Ras promotes the ability of SOS to convert GDP-Ras to GTP-Ras (three red balls) by engaging SOS allosteric site (unmasked site shown by yellow star; masked conformation in grey) (From Sperka et al., 2011).

B. ERMs in membrane transport

In addition to ERMs role in stabilizing the membrane–cytoskeleton interface, different studies described their role in regulating the function and transport of receptors. Here, we will give few examples that highlight the variety of ways in which the ERM proteins impact the cellular processes by regulating various receptors.

Several studies have demonstrated that ERMs have been associated with trafficking pathway from or to plasma membrane. Zhou et al. have shown that phosphorylation of Ezrin at Serine 66 (S66) is important for the acid secretion in parietal cells by transport of H+/K+-ATPase containing vesicles to the plasma membrane. In addition they have shown that this phosphorylation is mediated by PKA, a cAMP dependent proteins kinase, and phosphomimetic form of Ezrin (S66D) blocks the H+/K+-ATPase to the apical membrane (Zhou et al., 2003). Similarly, it has been shown that Na+/glucose transport induces the Ezrin activation through its phosphorylation by Akt and this activated Ezrin binds to Na+/H+ exchanger (NHE3) to transport it to the plasma membrane. Mutation of either of the proteins which can abolish this interaction results in inhibition of Na+/H+ exchanger transport (Cha et al., 2006; Zhao et al., 2004). In this context, interplay between Ezrin–NHERF-1 association with CFTR and NHE3 has been proposed, including roles in receptor recycling and recruitment of regulatory proteins (Lamprecht and Seidler, 2006). Further, another study has shown that interaction between Ezrin and a transmembrane protein, the α1β-adrenergic receptor, is important for receptor trafficking to the plasma membrane (Stanasila et al., 2006). Other studies have shown the involvement of ERMs in trafficking of several receptors such as transferrin receptor (Barroso-González et al., 2009).

However in spite of all these studies, the mechanism by which ERMs regulate the trafficking of receptors is not well understood. In this regard, Chirivino et al. have shown that ERMs regulate the maturation of endosomes by interacting with a subunit of HOPS complex (Hemolytic Fusion and Protein Sorting), Vps11. In this study they have demonstrated that interaction between ERMs and Vps11 is important for EGFR transport from early endosomes to lysosomes. In addition they have shown that inhibition of either Vps11or ERMs activity results in delay in recruitment of Rab7 on endosomes and it has been shown that Rab7 is important for the maturation of early to late endosomes. Collectively these results argue for a

new role for ERMs in maturation of endosomes by interacting with Vps11 which in turn helps in receptor (EGFR) trafficking (Chirivino et al., 2011).

Taken together, all the above functions demonstrate the multifunctional capacity of the ERMs in coordinating signal transduction with cytoskeleton remodeling and membrane protein transport and activity.

2.1.6 ERM functions in metastasis

The ERMs can localize to regions of cell–cell contact, indicating a role in direct intercellular communication. Altered cell-cell and cell-ECM contact is a characteristic of cancer cells and these participate in tumor invasion and metastasis. Several studies suggested a role for ERMs in metastatic progression of various human cancers. Some studies have shown that tumor promoting invasive capacity of CD44 is exclusively due to its function as a receptor for hyaluronic acid, while other have suggested that CD44 may promotes tumor invasion via its co-receptors like c-Met . In the later case, CD44 needs to associate with ERMs (Ponta et al., 2003). In addition, ERMs exerts their tumor invading capacity by indirectly activating growth-factor receptors such as c-met and EGFR family member ERBB2 **(**also known as HER2/neu) via CD44 (Bourguignon et al., 1997; Orian-Rousseau et al., 2002; Sherman et al., 2000). However, the mechanism by which ERMs promotes cancer progression in poorly described. In this context, group of studies of metastatic human carcinomas, pancreatic cancers, and head and neck squamous cell carcinoma (HNSCC) indicated that ERMs expression levels, phosphorylation status and subcellular localization are linked to invasive capacity of the tumors. In one study, Elliot et al. have shown that overexpression of Ezrin results in elevated levels of phosphorylated Akt and c-Src in AC2M2 (murine mammary carcinoma cell line) cells. In contrast, ectopic overexpression of N-terminal or dominant-negative amino-terminal Ezrin domain resulted in reduced levels of phospho Akt and c-Src which in turn lead to reduced invasion and metastasis. Concerning the interplay between c-Src and Ezrin, it has been recently shown that AC2M2 cells over-expressing a non-phosphorylatable Ezrin, Y477F, are less invasive *in vivo* and generate low number of lung metastatic lesions (Elliott et al., 2005; Mak et al., 2012). Collectively these results demonstrated that phosphorylated Ezrin at Y477 position by c-Src affects the phosphatidylinositol 3-kinase/Akt to regulate cell motility and invasion which are important

for the metastatic process. All these studies strongly support a role for ERMs in deregulation of cell-cell contacts, increased cell motility and invasion which are key steps in the metastatic cascade.

2.1.7 Regulation of ERM proteins activity

A. Conformational regulation of ERMs

ERMs contain an N-terminus FERM domain, followed by a α-helical region, and attenuates with a C-terminal domain. Biochemical and cell biological approaches demonstrated that ERMs activity is regulated in a conformation-dependent manner. Expressing either full-length Ezrin or its N-/C-terminal domains in cells triggers the intramolecular interaction between the N-ERM associated domain (N-ERMAD), residues 1-296, and the C-terminal C-ERM associated domain (C-ERMAD), residues 479-585 (Gary and Bretscher, 1995; Reczek and Bretscher, 1998). This monomeric and closed form of ERMs is considered inactive, and the F-actin binding site and potential membrane association sites are masked (Turunen et al., 1994). For the activation of ERMs, release of C-terminal domain from the FERM domain is necessary which renders binding sites in the FERM domain and the C-terminal F-actin binding site of ERMs accessible (**Fig 8E-F**).

The X-ray crystallographic structure of Moesin N-ERMAD complexed with the C-ERMAD revealed that C-ERMAD adopts an extended structure that covers the large segments of domains F2 and F3 masking the F-actin and membrane binding sites (Edwards and Keep, 2001; Hamada et al., 2000; Pearson et al., 2000; Smith et al., 2003) (**Fig 8 C-D**). In contrast, nuclear magnetic resonance (NMR) study has revealed that C-ERMAD helices bind the FERM domain independently, implying that individual secondary structures alone can make a binding contribution to attain the high affinity of the N-ERMAD and C-ERMAD interaction (Li et al., 2007)). In addition, the structure of *Spodoptera frugiperda* moesin showed that α-helical region contributes with the C-ERMAD to the masking of a large area of the FERM domain and it indicated that this α-helical segment is important for the activation of ERMs (**Fig 8E**). Based on previous studies a hypothetical model has been proposed for the activation of ERMs which is described in **Fig 8F.**

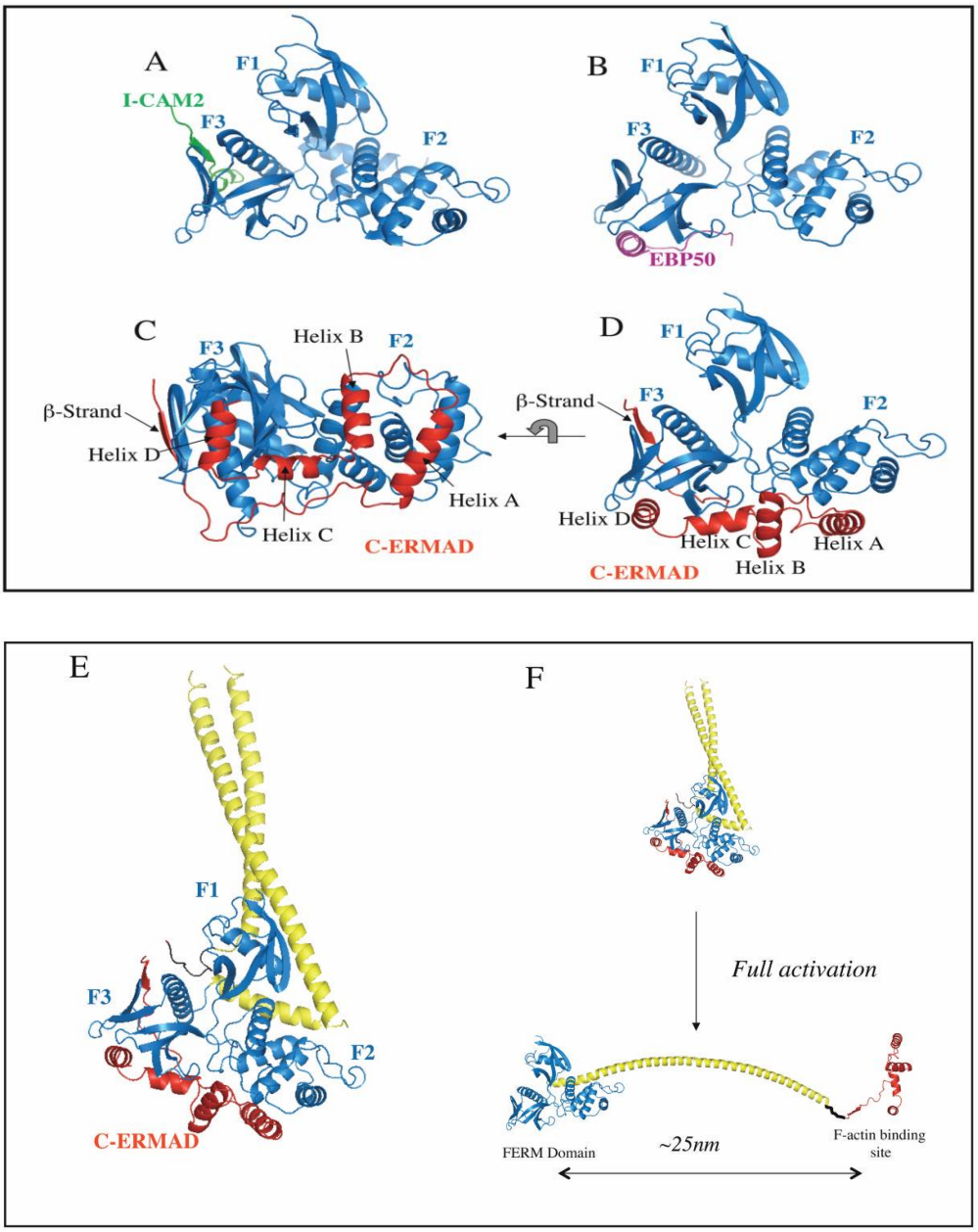


Figure 8. Structures of FERM domains with bound ligands. A. The complex of FERM domain of Radixin and a peptide from the cytoplasmic tail of ICAM2. The β-strand binds to a groove on the F3 subdomain. B. The complex of FERM domain of Moesin with the C-terminal peptide of EBP50. The tail of EBP50 forms a α-helix that binds to the surface of the F3 subdomain. C-D: Two views of the C-ERMAD of Moesin complexed with the FERM domain of Moesin. The C-ERMAD binds the F2 and F3 subdomains through a β-strand followed by four helices (helix A, B, C and D). E. Domain structure of full length *Spodoptera frugiperda* Moesin revealing the structure of the central α-helical region. F. A hypothetical model of ERMs activation showing the complete dissociation of the C-ERMAD from the N-ERMAD domain and allowing the central α-helical region to unknot and potentially extent up to 25nm (From Fehon et al., 2010)

B. ERMs regulation by phosphorylation

Proteins can be either activated or inactivated by phosphorylation. There is now significant confirmation that serine and/or threonine phosphorylation plays an important role in ERM activation. Initial studies showed that Moesin is phosphorylated on threonine 558 during platelet activation (Nakamura et al., 1995) and later studies then demonstrated that the corresponding residue in Ezrin, Radixin and Moesin (T576, T564, T558, respectively) can lead to ERMs activation via phosphorylation. In all ERMS threonine is masked in the N-/C-ERMAD interface and its phosphorylation triggers steric and electrostatic hindrance inhibiting the N-/C-ERMAD association. ERMs are phosphorylated by numerous kinases on this threonine residue such as Rho Kinase (ROCK), PKCα, LOK (Lymphocyte-Oriented Kinase) and AKt (Belkina et al., 2009; Matsui et al., 1998; Ng et al., 2001; Shiue et al., 2005)**.** For the activation of ERMs, it has been proposed that they are recruited to membrane regions rich in phosphatidylinositol, 4,5-bisphosphate (PIP2) and binding of PIP2 to FERM domain of ERM proteins results in the conserved threonine amino acid residue being more accessible to phosphorylation (Fievet et al., 2004; Hamada et al., 2000; Niggli et al., 1995)**.** In this fashion, closed from of ERMs is activated by the opening of their conformation which will allow them to bind various proteins (Actin, ICAM and EBP50), to regulate cell adhesion and migration (**Fig 8A**-**B**).

The ERMs are also phosphorylated at other sites including S66 by PKA (Zhou et al., 2003), Y145 by Lck (Autero et al., 2003), Src (Srivastava et al., 2005), T235 by cdk (Yang and Hinds, 2003), Y353 by Akt (Gautreau et al., 1999), Y477 by Src (Heiska and Carpén, 2005), suggesting that phosphorylation of ERM proteins may be triggered by different pathways making their regulation more complex. In addition, *Drosophila* has a single ERM called Moesin known to be phosphorylated at conserved threonine by Slik. It is a Ste20 kinase involved in the regulation of mitosis and its knockdown results in various mitotic defects as well as the inhibition of phosphorylation of Moesin. Similarly, loss of Moesin leads to delay in mitotic progression and cytokinesis. These results strongly suggest a role for ERMs in mitosis (Carreno et al., 2008; Hipfner et al., 2004; Hughes and Fehon, 2006a).

2.2 Merlin

2.2.1 Merlin: structure

Merlin (or schwannomin), the product of the *Neurofibromatosis type 2* (*NF2*) tumor suppressor gene, belongs to the Ezrin-Radixin-Moesin (ERM) subgroup. Merlin is well conserved in the evolution across the species (Golovnina et al., 2005), and in mouse, Merlin shares 98% homology with human Merlin. Its orthologs, present in *Drosophila*, (called D-Merlin) shares 55% identity with human protein. Merlin exists in alternatively spliced forms in human and mouse but not in *Drosophila* (Claudio et al., 1994; Haase et al., 1994; Hara et al., 1994).

Like the ERM proteins, Merlin is composed of three functional domains. A tri-lobe globular N-terminal FERM domain covering the first 314 (1-314) amino acids, followed by a central alpha-helical region (315-491) and a charged hydrophilic COOH terminal tail (492-495). The N-terminus is subdivided into F1, F2 and F3 lobes (**FIG 6**).The crystallographic studies suggested that the three dimensional structure of the FERM domain of Merlin resembles the FERM domain of ERMs (Kang et al., 2002, 2002; Shimizu et al., 2002). ERMs and Merlin have at least some common binding partners, especially CD44, which is well studied. In addition to homo dimerization, Merlin and ERMs can interact hetero-typically (Grönholm et al., 1999; Meng et al., 2000), and interestingly it was shown that Merlin forms more stable dimers with ERMs than itself (Lallemand et al., 2009a; Nguyen et al., 2001). In this context, recent study using fluorescence resonance energy transfer (FRET) suggested that Merlin exists predominantly as a monomer in a stable, closed conformation (Hennigan et al., 2010). All these observations indicate that Merlin shares many similarities with ERMs in its structure especially in the FERM domain.

However Merlin and ERMs also show clear differences in their structure. Unlike ERMs, Merlin does not have actin binding domain at its C-terminus. In contrast, Merlin binds to actin via its FERM domain (Brault et al., 2001; Huang et al., 1998; Xu and Gutmann, 1998). More recently it has been shown that the F1 subdomain of Merlin is essential for cortical actin association and formation of cortical actin structures (Lallemand et al., 2009a). Although ERMs and Merlin share 43% similarity in FERM domains of *Drosophila* and humans, there are regions which are distinct in ERMs and Merlin (Kang et al., 2002; Shimizu et al., 2002).

In particular, a seven amino acid stretch is conserved exclusively and perfectly in *Drosophila* and human, and several other species of Merlin, but not in ERMs. This conserved sequence is called **'blue box'**(LaJeunesse et al., 1998), and was shown to be necessary for Merlin growth suppressive function. Moreover, Merlin has an extended 17 amino acid sequence at N-terminus which is not present in ERMs and that was proposed to be necessary for actin binding (Cole et al., 2008)**.**

2.2.2 Isoforms of Merlin

The *NF2* gene consists of 17 exons and is known to encode at least 10 isoforms. Out of which isoform I and II are the most extensively investigated ones. These two isoforms differ at their extreme C-terminus due to alternative splicing in *NF2* gene (**Fig 9**). The two isoforms are identical up to amino acid 579, but differ in the last 16 amino acids (Arakawa et al., 1994; Huynh et al., 1994; Pykett et al., 1994; Bianchi et al., 1994; Schmucker et al., 1999). The structure analysis of these isoforms reveals that Isoform I has hydrophobic helical C-terminus whereas isoform II has non helical C-terminus with hydrophilic amino acids. Due to hydrophilicity at C-terminus, it is believed that isoform II does not self-interact (Sherman and Gutmann, 2001; Sherman et al., 1997) and also that self-association within the molecule (closed conformation) is important for the tumor suppressor function of Merlin. In contrast, lack of intermolecular interactions results in open conformation which would be inactive. The two isoforms bind differently to their interacting partners such as HRS (Scoles et al., 2000a) and F-actin (James et al., 2001), RhoGDI (Maeda et al., 1999). Initially, it was believed that only isoform I functions as a tumor suppressor. However, recent reports suggest that isoform II alone inhibits the growth in SC (Bretscher et al., 2002; Lallemand et al., 2009a). Interestingly, isoform II has the capacity to rescue the phenotype resulting from the lack of isoform I in mice (Bretscher et al., 2002), suggesting that the two isoforms have redundant functions during embryogenesis and development. Also, *NF2* gene inactivating mutations were observed in first 15 exons, but not in exons 16 and 17 that are different between the two isoforms, suggesting that the two isoforms are not different from each other at functional level (Ahronowitz et al., 2007; Baser, 2006). This in turn led to believe that disruption of the isoforms alternative C-termini would not be enough to eliminate the Merlin's growth suppressor function (Jacoby et al., 1996).

Merlin isoforms differ in their capacity to regulate the actin reorganization. For example, overexpression of Merlin isoform I effect the cell motility, whereas Isoform II doesn't (Gutmann et al., 1999). In addition, Merlin isoforms have different binding capacities due to masking of N-terminal actin binding site in closed conformation of isoform I (Sivakumar et al., 2009). This might explain the effect of isoform II on focal adhesion stability mediated by strong interaction with cytoskeleton components, which in turn effects the detachment of cellular protrusions. However, both isoforms are able to inhibit the Ras dependent cell growth in soft agar colony formation assay and as well as cell proliferation in an conformation independent manner (Laulajainen et al., 2012). This in turn supports the recent study which suggests that both isoforms of Merlin inhibit the Schwann cell proliferation (Lallemand et al., 2009a). Finally, the group of H. Morrison has recently demonstrated another isoform specific function of Merlin. In this study for the first time they have shown that Merlin isoform 2 has a specific function in maintaining axonal integrity and propose that reduced axonal *NF2* gene dosage leads to NF2-associated polyneuropathy (Schulz et al., 2013).

Collectively these results suggest that both isoforms might have the same role in growth inhibition but different functions in regulating the cell morphology and migration effecting differently the cytoskeleton organization and development.

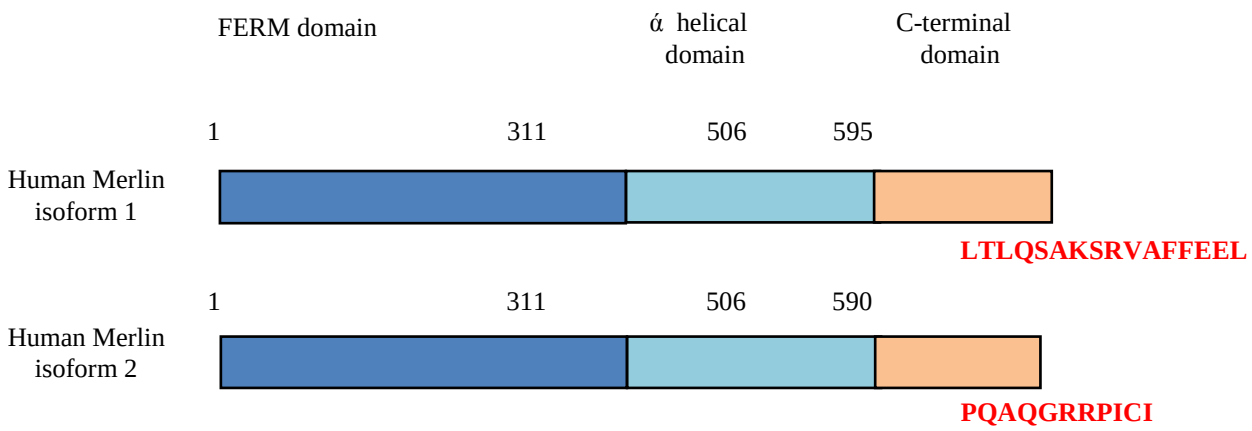


Figure 9. Domain organization of Merlin isoform 1 and 2. The most common isoforms of Merlin present in human and mouse. Human Merlin isoform 1 contains 595 amino acids whereas isoform 2 has 590 amino acids with estimated molecular weights of approximately 65-70 kDa (Modified Stamenkovic and Yu, 2010). They are different at their extreme C-terminus end from amino acid 579 to 595 (difference in amino acid sequence between two isoforms is shown in red)

2.2.3 Tissue distribution and sub cellular localization of Merlin

As described in Table 4**,** Merlin is expressed in wide a range of human tissues such as brain, sciatic nerve, peripheral nerves and gonads. Merlin presence has been observed in smooth muscle cells, neurons and Schwann cells (den Bakker et al., 1995; Grönholm et al., 2005; Stemmer-Rachamimov et al., 1997a). Although ERMs and Merlin are present in Schwann cells, only the loss of function of Merlin alone leads to the development of schwannomas (Stemmer-Rachamimov et al., 1997b). At subcellular level, a major fraction of Merlin is known to be in a soluble cytosolic fraction and a small portion in detergent insoluble fraction. However Merlin localization changes depending on the cell-cell contact. Merlin associates with Moesin in *Drosophila* at sub-apical junctional region and also it is concentrated in actin rich regions known as membrane ruffles, and along cell-cell boundaries (Gonzalez-Agosti et al., 1996; Lallemand et al., 2003, 2009a). In addition, Merlin was shown to localize to membrane vesicles or membrane lipid rafts suggesting role for Merlin in receptor trafficking (Grönholm et al., 2005; Stickney et al., 2004; Lallemand et al., 2009b). All these results show that Merlin functions at or near the plasma membrane. In this context, recent studies have demonstrated that Merlin also localizes to the nucleus in various cell types (Li et al., 2010; Muranen et al., 2005). These studies together support the idea that Merlin is a novel type of tumor suppressor protein which might exerts its function in the different cellular compartments, from cell-cell contacts to the nucleus.

2.2.4 Functional analysis of Merlin in different model organisms

NF2 gene has been found in many other metazoans, including invertebrates. On the contrary, its homologues were not found in fungi, plants or protozoa. Mutational studies of Merlin in the *Drosophila* and mice have given important details of Merlin function (Fehon et al., 1997). Several mouse models have been generated to study the NF2 tumorigenesis. Targeted inactivation approach was used to generate the germline *Nf2* homozygous (*Nf 2*$^{-/-}$) mutants, and these mice died at E.7 without gastrulation demonstrating the role of Merlin in early embryogenesis (McClatchey et al., 1997). However, unlike humans, *Nf2*+/− mice failed to develop the schwannomas and other tumours that are clinical features of human NF2. Instead these mice develop other high grade tumors such as osteosarcomas and liver tumors (both hepatocellular carcinomas and cholangiocarcinomas) (McClatchey et al., 1998) and also

chondrosarcomas, fibrosarcomas, and rhabdomyosarcomas with lower frequency. In addition, these *Nf2*+/− mice showed an increased sensitivity to asbestos fibers and developed mesotheliomas at a higher rate compared to wild-type mice (Fleury-Feith et al., 2003). More importantly, these mice high metastasis rate indicated that Merlin loss can lead to metastatic progression. Though *Nf2*+/− mice don't develop schwannomas spontaneously, biallelic inactivation of *Nf2* in Schwann cells (SCs) readily generated Schwann cell hyperplasia and schwannomas (Giovannini et al., 2000). Similarly, it has been shown that Merlin inactivation in arachnoid cells using conditional knock-out results in the formation of meningiomas (Kalamarides et al., 2002). These studies indicated that the absence of schwannoma and meningioma development in *Nf2* heterozygote mice is not due to functional difference between mice and human, but is due to the difference in the probability of loss of the wild-type allele in the cells from which schwannomas and meningiomas originate. In order to determine the genetic co-operativity of *Nf2* with other tumor suppressor genes, *Nf2*-mutant mice were crossed with mice carrying *p53* tumor suppressor gene mutations. Unlike in humans, *Nf2* and *p53* tumor suppressor loci are all genetically linked on the long arm of mouse chromosome 11. In these studies, striking cooperation was observed between *Nf2* and *p53* tumor suppressor gene mutations when they are in *cis* position (*Nf2*+/−;*p53*+/− *cis* mice) and these mice develop osteosarcomas early in life (McClatchey et al., 1998). Recent studies in mice have shown that biallelic loss of Merlin in the liver result in hepatomegaly and formation of malignant tumors. This study suggests that Merlin is a potent regulator of liver size and tumor suppression (Zhang et al., 2010a).

Studies have shown that Merlin is also a tumor suppressor in flies (LaJeunesse et al., 2001). Interestingly mutational studies on *Drosophila* Merlin have provided many clues about the Merlin function (LaJeunesse et al., 1998). This study has identified a unique sequence in *Drosophila* FERM domain of Merlin called "Blue Box" mutant (Mer BB- mutation of seven amino-terminal amino acids) and upon ectopic overexpression Mer BB acts as dominant negative by interfering with the activity of the wild-type protein (Johnson et al., 2002; LaJeunesse et al., 1998). In a similar way, to understand the structure and function of *Nf2* gene, the group of M. Giovannini has generated transgenic mice expressing, either a mutant Merlin modeled from a naturally occurring mutation, *Nf2*-▲ exon 2-3 where exon 2 and 3 are deleted (▲39–121), or a Merlin prototypic for carboxyl-terminal deletion mutants, *Nf2*-▲ Cter, under the control of the Schwann cell-specific P0 promoter. By doing so, they found that

Nf2-▲ exon 2-3 promotes the Schwann cell (SC) hyperplasia and tumors; in contrast NF2-▲ C-ter did not promote the tumor formation. These results argues that the growth promoting phenotype of Mer-▲(39–121) 2-3 is due to its dominant negative nature which in turn results from altered binding properties to membrane and intracellular components (Giovannini et al., 1999). However important differences exist between *Drosophila* and mammalian Merlin. For example, the sequence of the FERM domain of Merlin rescues development in *Drosophila* in which NF2 is knocked out but does not rescue proliferation defects in mammalian Nf2 deficient SCs (Lallemand et al., 2009a).

3. Merlin functions and regulations

Overexpression of Merlin in NIH3T3 fibroblasts revealed that it can restore the loss of contact inhibition of cell growth. Also it has been shown that wild type, but not mutant, Merlin inhibits cell proliferation and causes morphological changes (Lutchman and Rouleau, 1995; Tikoo et al., 1994). Likewise overexpression of Merlin in meningioma cells, rat and human Schwann cells inhibits cell proliferation (Fraenzer et al., 2003; Schulze et al., 2002). In NF2-/- schwannoma and mesothelioma cells, reintroduction of Merlin inhibits cell proliferation and increases apoptosis (Lallemand et al., 2009a; Xiao et al., 2005). Collectively these results suggest that Merlin inhibits cell proliferation and hence acts as tumor suppressor.

More detailed studies have pinpointed the specific aspects of proliferation that are regulated by Merlin such as contact dependent inhibition and apoptosis/survival.

3.1 Contact dependent inhibition

Normal cells stop to proliferate after coming into contact with each other and assembling intercellular junctions, which is important for the tissue organization. Loss of contact inhibition is one characteristic of tumor cells and participates in tumor formation. Several studies suggested the key role for Merlin in this process.

With respect to Merlin role in contact inhibition of proliferation, initial studies have shown that Merlin mediates contact inhibition of proliferation by interacting with cytoplasmic tail of CD44. According to this model, phosphorylated Merlin (known to be inactive) and ERM proteins form complexes in sub-confluent cells which might activate mitogenic signaling. In confluent cells and in presence of hyaluronic acid, Merlin undergoes dephosphorylation (active) and inhibits cell proliferation by blocking the mitogenic signals through its interaction with CD44 (Bai et al., 2007; Morrison et al., 2001a) (**Fig 10**). CD44v6, an isoform of CD44, is known to be involved in receptor tyrosine kinase activation such as met to stimulate cell proliferation (Rosen and Jordan, 2009). All together this data indicate that Merlin negatively regulates CD44 function in cell proliferation and metastasis, and loss of Merlin results in cancer development. However in confluent cells, deletion of CD44 does not show the same phenotype as loss of Merlin. This suggests that CD44 is not essential for contact inhibition at least in endothelial cells and fibroblasts (Lallemand et al., 2003; Okada et al., 2005; Grazia Lampugnani et al., 2003). Alternatively, it has been observed a considerable defect in recruitment of Rac to the plasma membrane in confluent endothelial cells leading to down-regulation of mitogenic signaling pathway. Silencing Merlin restores recruitment of Rac to the membrane, suggesting that Merlin inhibits the recruitment of Rac to the membrane for proper contact inhibition of cell proliferation (Okada et al., 2005) (**Fig 11**). Finally it has been demonstrated that in D*rosophila,* Merlin mediates contact dependent inhibition of proliferation in association with another FERM domain containing protein called Expanded by regulating the transport of receptors at the plasma membrane (Hamaratoglu et al., 2006; Maitra et al., 2006). Altogether strongly support the ideat that Merlin is involved in the contact dependent inhibition of proliferation.

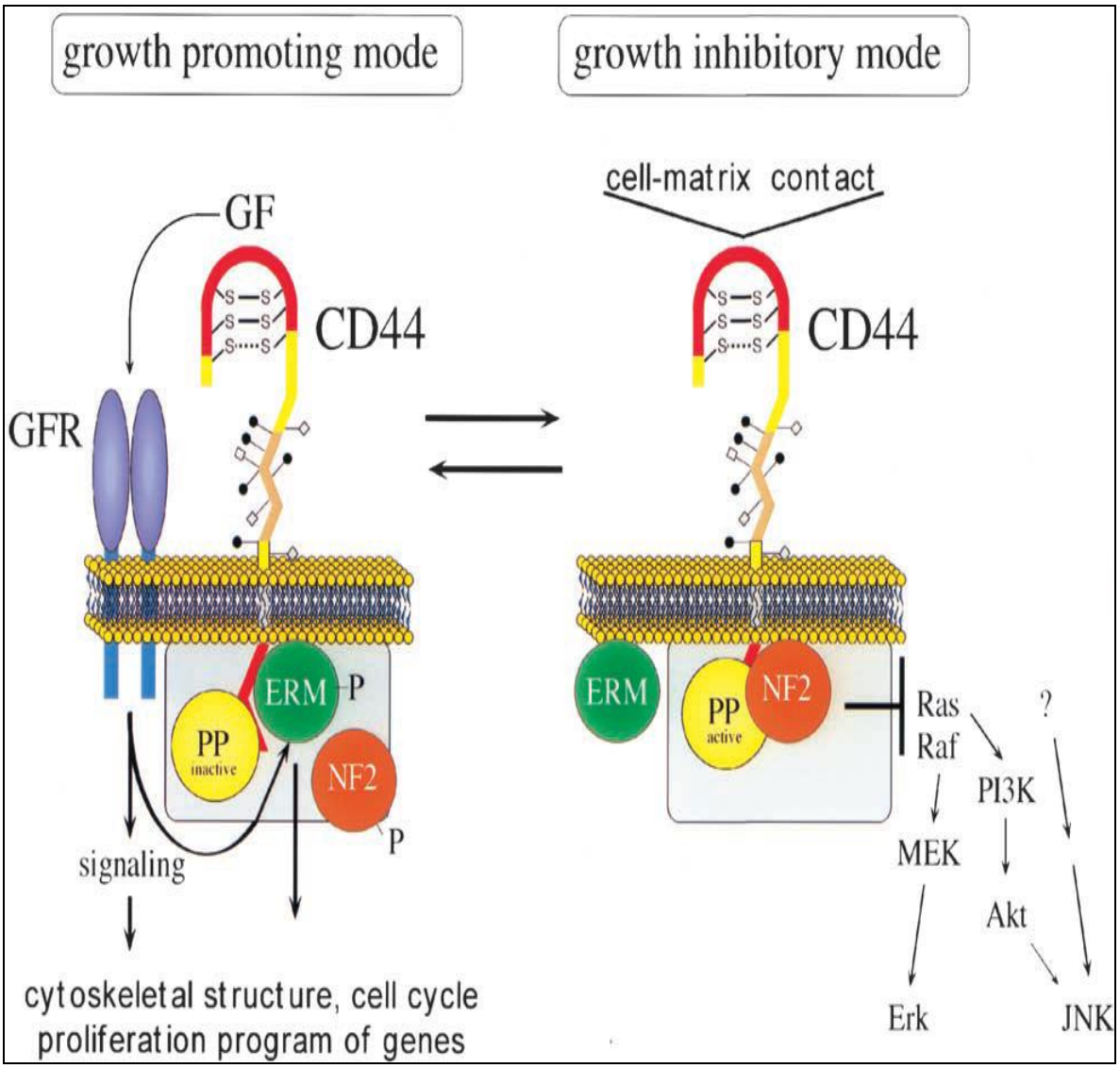


Figure 10. Interplay betwwen CD44 and Merlin in confluent growth conditions. Binding of ligand influences the two functional states of CD44 that in turn affects the cytoplasmic complexes. CD44 is proposed to serves as platform for the activation of growth factors (GF) even though the ligands of the growth mode have not been identified. (GFR) growth-factor receptor; (PP) protein phosphatase. Additional components are likely associated with the CD44-bound complexes (gray boxes) (From Morrison et al., 2001).

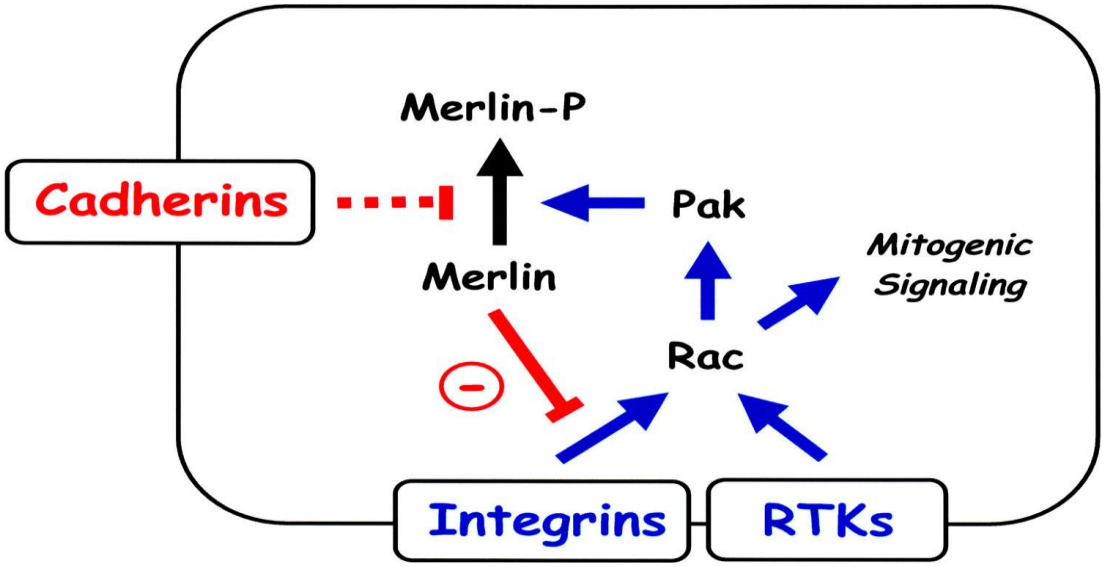


Figure 11. Proposed model of Merlin mediated Rac inhibition. Up on contact inhibition Merlin is activated and Pak inactivated. This active form of Merlin suppresses integrin-mediated recruitment of Rac, and hence mitogenic signaling. Upon release from contact inhibition, Pak is activated and phosphorylates Merlin (in active), allowing recruitment of Rac to the membrane and mitogenic signaling (From Okada et al., 2005).

3.2 Apoptosis/survival

It has been shown that Merlin is a regulator of apoptosis through different mechanisms. For example mutational studies in *Drosophila* Merlin (*mer*) and Expanded (*ex*) indicated that Merlin negatively regulates apoptosis and proliferation. In this study it has been shown that mutation in *mer* and *ex* results in inactivation of canonical Hippo signaling which in turn leads to increases cyclin E and *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1) levels and results in decreased apoptosis, increased proliferation and survival (Huang et al., 2005; Pellock et al., 2007). Similar mechanisms are conserved in mammals where the inactivation of Merlin results in elevated levels of cyclin E and cIAP1/2, respectively (Chan et al., 2008; Hergovich and Hemmings, 2009; Kanai et al., 2000). Finally it has been shown that over expression of Merlin can also provoke the apoptosis by targeting Mdm2 to degradation which in turn leads to increased stability and tumor suppressor function of p53, cell cycle exit and induction of apoptosis. Merlin can also trigger apoptosis by inhibiting the cell progression through suppression of PAK1 mediated expression of cyclin D1 (Kim et al., 2004; Xiao et al., 2005) indicating strong role for Merlin in apoptosis.

3.3 Regulation of Merlin activity

3.3.1 Conformational regulation of Merlin activity

Merlin and ERMs have significant sequence homologies especially at their N-terminus FERM domain. From the crystallographic studies on Merlin FERM domain, it is predicted that Merlin is regulated by conformational changes as it exist in open and closed forms (Kang et al., 2002; Shimizu et al., 2002). Like ERMs, Merlin forms head-to-tail self-association, which results in closed conformation mediated by binding of its C-terminal domain to the N-terminus (Grönholm et al., 1999; Hennigan et al., 2010; Sherman et al., 1997). However due to low sequence identity between the C-terminal domains of Merlin and of ERMs, it's not clear whether Merlin self-association is identical to ERMs or not. Merlin can form oligomers by homodimerizing and can heterodimerizes with ERMs (Deguen et al., 1998; Gonzalez-Agosti et al., 1999). The interaction between N- to C-ERMAD of Merlin is weaker and more dynamic compared to ERMs.

Although Merlin C-terminus sequence is unique when compared to ERMs, 81% of the conserved residues are located on the predicted N-/C-ERMAD interface, making it almost certain that Merlin can make intramolecular association similar to ERMs. Similarly to ERMs, Merlin C-ERMAD folds to bind with F2 and F3 sub domains of FERM, and along with the central α-helical domain interactions, regulates the closed active conformation (Hennigan et al., 2010; Sivakumar et al., 2009). There are two separate intramolecular associations essential for closed conformation in Merlin (**Fig 12**). 1. Folding within the N-terminal domain between amino acids 8-121 and 200-320. 2. N- to C-terminal association requires residues 302-308 and residues 580-595 (Gutmann et al., 1998; Sherman et al., 1997). However due to variant C-terminus, which lacks exon 17, Merlin isoform 2 form only weakly intramolecular complexes and is thought to exist in constitutively open form. This difference in conformation of isoforms indicates that they have differential ability to bind their interacting partners (Sivakumar et al., 2009).

The closed conformation of Merlin is believed to be active and growth suppressive (**Fig 12**). Moreover recent crystallographic study indicated that closed conformation of Merlin is indeed an open dimer of two Merlin molecules, essential for Merlin growth suppressive activity (Sherman et al., 1997; Yogesha et al., 2011) (**Fig 12**). In contrast to Merlin isoform 2, Merlin

with mutations at N- or C-terminal domain, exhibit open conformations and are unable to acts as growth regulators. However in *Drosophila*, it has been observed that intramolecular associations does not seem essential for growth suppressive activity as both C-ter truncated D-Merlin and the FERM domain alone are fully functional. In this context, recent FRET studies demonstrated that the conformational regulation of Merlin is more complex than just inter conversion of open and closed states (Hennigan et al., 2010). In fact the interaction between FERM and C-terminus domain seem to be less important than the previously expected. In contrast, through phosphorylation or by interacting with other proteins, Merlin undergoes subtle conformational change suggesting a new mechanism for the regulation of interaction between FERM domain and C-terminus domain.

However, a recent study from the group of A. Bretscher clearly demonstrated that in fact Merlin controls the growth in its open conformation and that phosphorylation converts it into a more close conformation. In this study authors have generated the more stable closed or open form of C-ter Merlin by mutation analysis and they have shown that the open form of mutant Merlin bind better to FERM domain compared to closed mutants. They have also shown that phospho-mimetic mutant of C-ter Merlin (S518D) bound better to FERM domain compared wild type. Finally they have demonstrated that more open conformations of Merlin have higher growth suppressive capacity and bind better to EBP50. Altogether, now it is clear that open conformation (not fully) of Merlin is active and growth suppressive even though Merlin may exist in intermediary conformations (Sher et al., 2012).

3.3.2 Merlin regulation by Phosphorylation

Merlin molecular interactions and activity are controlled by a combination of phosphorylation and phospholipid binding. Like ERMs, Merlin interacts with phosphoinositide-(4, 5)-bisphosphate (PIP2) through a conserved motif in its FERM domain. This interaction targets Merlin to the membrane. For the growth suppressive function of Merlin, both PIP2 binding and membrane localization are essential. Interestingly, deletion or mutation of PIP2 binding domain in Merlin does not seems to affect self-association and phosphorylation of Merlin (Mani et al., 2011; Okada et al., 2009).

The phosphorylation of Merlin by various kinases is involved in the inter-conversion of closed form of Merlin to open conformation (**Fig 12**). Merlin is phosphorylated in response to growth factors, low cell density, or cell/ substrate attachment. Merlin is phosphorylated on

both serine and threonine residues. Tyrosine phosphorylation has not been observed. Merlin seems to exist in three differentially phosphorylated forms as it observed three distinct bands around 70kDa in electrophoretic mobility assays (Alfthan et al., 2004; Shaw et al., 1998a, 2001). The band with increased mobility is hypophosphorylated, while the form with the decreased mobility is hyperphosphorylated. Merlin is phosphorylated at serine 518 (S518), (Kissil et al., 2002; Shaw et al., 2001; Xiao et al., 2002), threonine 230 (T230), serine 315 (S315) and S10 (Laulajainen et al., 2008; Tang et al., 2007), but no other phosphorylation sites have been reported. In *Drosophila* slik has been shown to phosphorylate Merlin at T616 and this phosphorylation is important for microtubule-dependent transport for Merlin function (Benseñor et al., 2010; Hughes and Fehon, 2006a).

Merlin can be phosphorylated on multiple residues by various kinases which are summarized in **Table 3.** Phosphorylation of Merlin on serine 518 (S518) at its C-terminal tail is shown to be most important for the regulation of the activity of Merlin. As a consequence of this event, Merlin is proposed to convert into an open, inactive conformation, with no tumor-suppressor activity (Alfthan et al., 2004; Kissil et al., 2002). In addition, two well-characterized phosphorylation mutants of S518 have suggested the importance of this site: one is Merlin S518A, which cannot be phosphorylated and is stabilized in the closed conformation and functionally active. The other is phospmimetic mutant of Merlin (518D), which prevents N-terminus to C-terminus interactions and is functionally inactive. The phosphomimetic, open form of Merlin (S518D) cannot inhibits cell growth or motility in RT4 rat schwannoma cells, but triggers changes in cell morphology and actin cytoskeleton organization (Rong et al., 2004a; Surace et al., 2004).

The best studied example for the phosphorylation of Merlin is Rac induced phosphorylation of Merlin at S518 position, which is mediated by the major Rac effector p21-activated kinase (Kissil et al., 2002; Xiao et al., 2002). In response to various stimuli, Merlin is phosphorylated by PAK1/2, which in turn interrupts the intramolecular association between the FERM domain and the C-terminal domain that retains the protein in the closed form, thereby inactivating Merlin. Merlin is also phosphorylated at S518 by c-AMP dependent protein kinase-A (Alfthan et al., 2004) (PKA). When PAK activity is suppressed, Merlin can still be phosphorylated by PKA in cells suggesting that these two kinases function independently. The phosphorylation of Merlin at S518 not only impairs the association of N- and C-terminus association but also inhibits the binding of Merlin to interacting partners including CD44 and

Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (Rong et al., 2004a). In addition to S518, PKA phosphorylates Merlin at S66 and it has been recently shown that PKA also phosphorylates Merlin at S10, leading to altered actin cytoskeleton organization (Laulajainen et al., 2008) without effecting the Merlin-Ezrin interaction. In this study authors also demonstrated that dephosphorylation of Merlin at S10 results in defective migration capacities of cell in a wound healing assay.

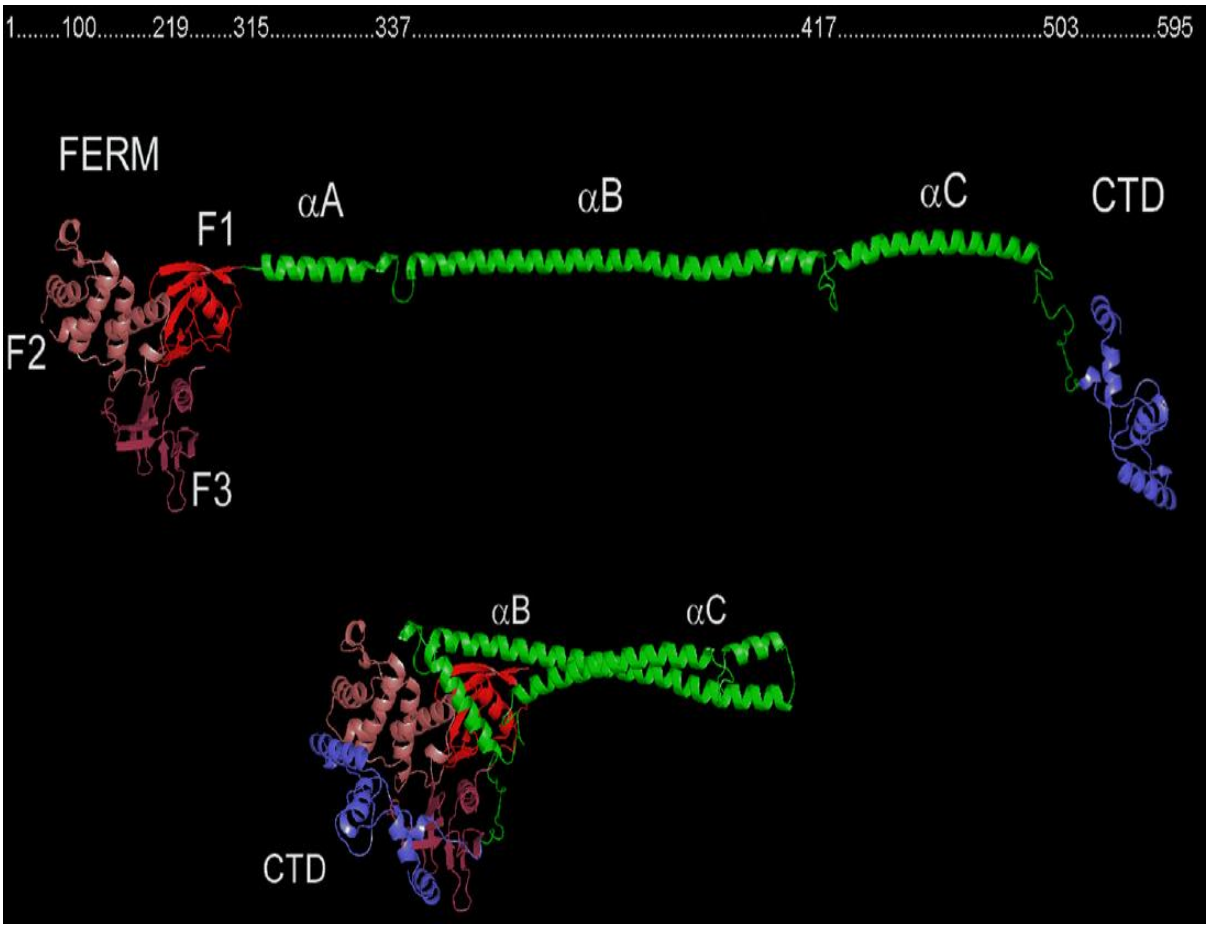


Figure12. Tertiary structure of full length Merlin. Subdomains of the Merlin are assigned in a different color. This tertiary structure consists of an N-terminal FERM domain folded into three lobes, F1, F2, and F3. This is followed by a central α-helical domain containing three sub-helices (αA, αB, and αC) and a CTD with four short helices. The open conformation is an extended structure with the FERM domain and the CTD separated by the α-helical domain that is more than 240 Å long. Whereas in the closed conformation, the α-helical domain bends at the αA-αB junction and again at the αB-αC junction, causing the CTD to be positioned over F2 and F3 of the FERM domain. More than half of the surface of the FERM domain is masked by interaction with the CTD, αA, and parts of αB and αC (Hennigan et al., 2010).

3.3.3 Merlin phosphorylation by Akt

In response to growth factors that are abundant in the tumor environment, such as EGF (epidermal growth factor) or IGF (insulin growth factor), Akt phosphorylates Merlin at threonine 230 (T203) and serine 315 (S315) (Tang et al., 2007). The presence of two motifs includes 225–231 (RNKKGTE) and 310–316 (RRKADSL) in Merlin indicated that it is a good substrate for Akt mediated phosphorylation and numerous studies suggest a crosstalk between Merlin and Akt. The serine/threonine kinase Akt (protein kinase B) controls many cellular functions such as motility, growth, and apoptosis (Jacob et al., 2008; Li et al., 2001). The activation of Akt pathway is observed in both human schwannomas (Ammoun et al., 2008; Jacob et al., 2008) and malignant mesotheliomas from heterozygous *Nf2+/-* mice (Altomare et al., 2005). Merlin regulates the activation of phosphoinositide 3-kinase (PI3K)/Akt pathway by suppressing the Akt signaling (Fraenzer et al., 2003; Rong et al., 2004b). It inhibits the PI3K activity by binding to PI3K enhancer long isoform (PIKE-L), preventing its association with PI3K. Interestingly, wild‐type Merlin, but not patient‐derived mutant (L64P), binds PIKE‐L and inhibits PI3‐kinase activity. Unlike Merlin, Ezrin activates Akt signaling which in turn leads to increased cell survival (Gautreau et al., 1999). Merlin phosphorylation at T230 and S315 mediated by Akt suppresses the self-association of Merlin, which in turn inhibits its association with PIK-L, CD44 and Ezrin. In addition, Akt mediated phosphorylation results in Merlin degradation via proteasomal pathway. Expression of phosphomimetic Merlin mutant (T230DS315D) increases cell motility and proliferation, whereas the expression of non-phosphorylatable mutant (T230A/S315A) has the opposite effects (Tang et al., 2007). Akt mediated phosphorylation of Merlin inhibits its pro-apoptotic activity. Recently one study demonstrated that Ser10 is a new Akt phosphorylation site that targets Merlin for proteasomal degradation too (Laulajainen et al., 2011). Also, this study revealed that S518 phosphorylation facilitates the S10 phosphorylation. The interplay between all these kinases to regulate the Merlin tumor growth suppressive function is summarized in **Figure 13**.

Table 3. Known phosphorylation sites and their effect on Merlin activity/stability. (From Morrow and Shevde, 2012)

Phosphorylation Site	Kinase/phosphatase	Result	Functional effect
Serine 10	PKA/AKT	May target Merlin for degradation	Altered cell morphology, growth permissive
Threonine 230/ Serine 315	PAK1/2, AKT	Targets Merlin for proteasomal degradation	Growth permissive
Serine 518	PKA, PAK1, Myosin phosphatase (MYPT1-PP1δ)	Switches Merlin to open conformation	Growth permissive

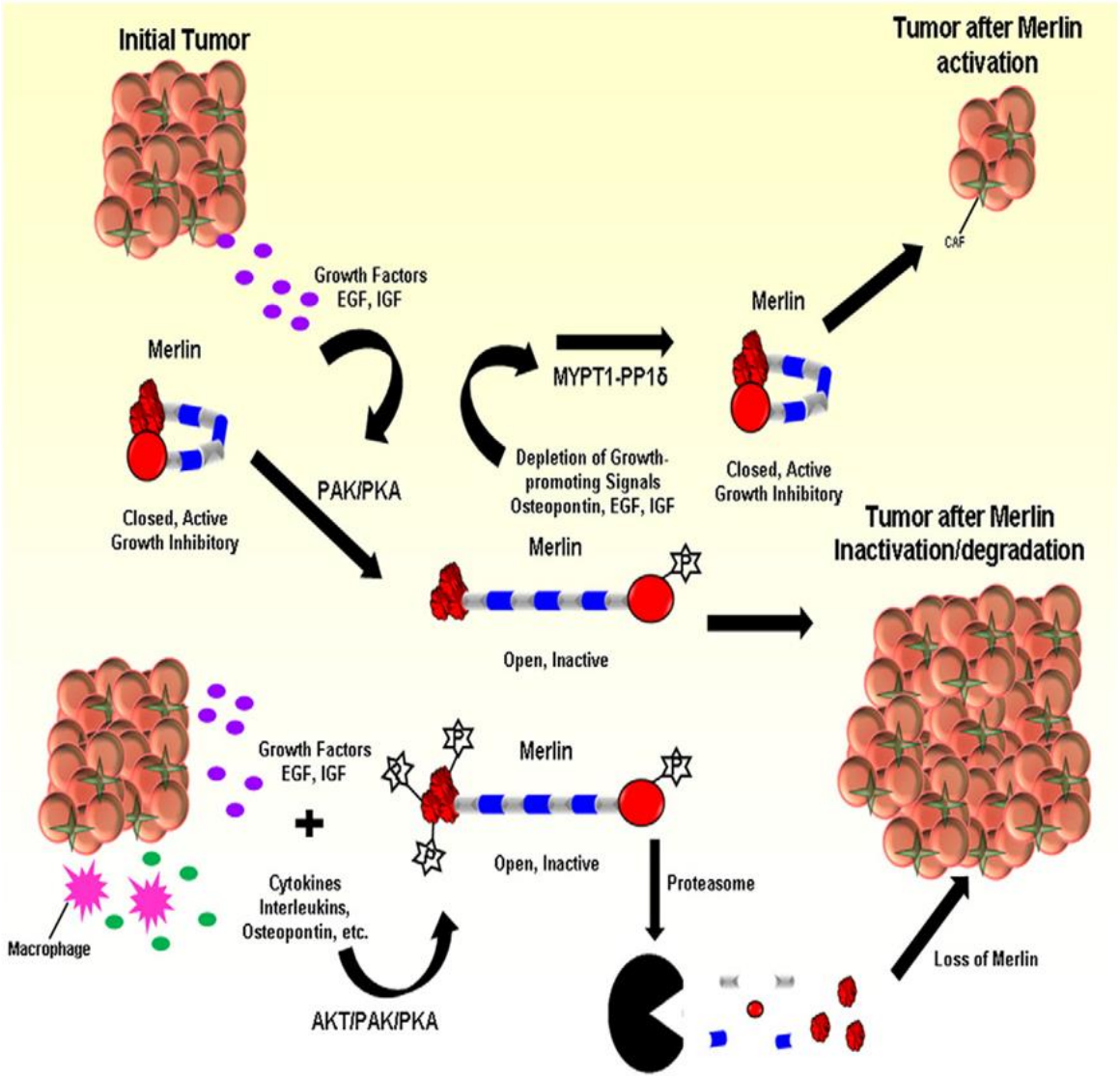


Figure 13. Scheme showing the effect of phosphorylation on Merlin activity/stability and tumorigenesis. The availability of growth factors (EGF, IGF, osteopontin etc.) from tumors or from its microenvironment proposed to inactivate Merlin by phosphorylation at S518. This phosphorylation mediated by PKA/PAK/AKT leads to opening of Merlin conformation and makes it in active. In addition, Merlin phosphorylation at Threonine 230/ Serine 315 targets Merlin to proteasomal degradation, leading to the loss of availability of Merlin to function as a tumor suppressor, promoting subsequent malignant progression. In contrast, MYPT1-PP1δ dephosphorylation results in activation of Merlin. This in turn permits Merlin to perform its tumor suppressor function (CAF represents cancer associated fibroblasts) (Morrow and Shevde, 2012)

3.3.4 Phosphatases and Merlin

In response to high cell density or serum withdrawal, Myosin phosphatase MYPT1-PP1δ dephosphorylates Merlin at Ser518 and thereby activates Merlin. Also it has been demonstrated that the oncogene protein kinase C-potentiated phosphatase inhibitor of 17 kDa (CPI-17) inhibits Merlin activity in mesothelioma by inhibiting the targeting subunit of MYPT1-PP1δ (Jin et al., 2006; Morrison et al., 2001a; Thurneysen et al., 2009).

3.4 Proteasomal mediated degradation of Merlin.

In addition to phosphorylation, other post translational modifications like ubiquitination play a crucial role in regulating Merlin activity. Two major pathways control the protein turnover in Eukaryotic cells. The first one is the proteasomal pathway and the second one is the lysosomal pathway. The lysosomal pathway is mainly involved in degradation of membrane-associated or endocytosed extracellular proteins. In this pathway lysosome with proteolytic enzymes plays an important role in turnover of cytosolic proteins. Most of the intracellular proteins are degraded by the ubiquitin-proteasome pathway (UPP). This plays important role in eliminating the misfolded and abnormal molecules by proteolysis, but also functions in regular proteins turnover. Depending upon cell requirements, UPP pathway quickly adjusts the concentration of selected proteins by targeting them to degradation. The selective and controlled proteolysis of proteins by UPP has an important role in a variety of cellular processes, including cell signaling, transcriptional regulation, translational regulation, cell cycle progression and proliferation, angiogenesis, protein quality control and apoptosis.

Emerging reports suggest that Merlin is degraded by UPP pathway. Both full length missense mutants and N-terminally deleted Merlin show an increase ubiquitination and are degraded by UPP. These results suggest that UPP is important for turnover of defective Merlin products whereas wild type protein is more stable. Therefore loss of function of Merlin in NF2 disease is related to decrease in half-life of mutant protein as a result from increased degradation rather than their abnormal function. To support this, it has been shown that mRNA expression levels of missense Merlin mutants are unchanged in NF2 associated tumors even though their reduced protein levels have been observed (Gautreau et al., 2002a; Yang et al., 2011).

Concerning alternative pathways in Merlin degradation, initial studies have shown that calpain, a calcium dependent cysteine proteinase, cleaves Merlin. Calpain activity is elevated in NF2 related tumors such as schwannomas and meningiomas and results in increased turnover of Merlin. In other studies it has been observed that oxidative stress triggers the activation of calpain pathway which in turn results in Merlin turn over in meningioma cell leading to loss of contact dependent inhibition. In contrast, later studies have shown that no correlation found between calpain status and the abundance of Merlin suggesting the activation of calpain may not results in NF2 (Kaneko et al., 2001; Kimura et al., 1998, 2000).

Several studies observed that Merlin degradation is mediated by Akt pathway. Phosphorylation of Merlin at T230 and S315 targets it for proteasomal degradation (Tang et al., 2007). Similarly, recently it has been demonstrated that coordinated phosphorylation of N- and C-terminus of Merlin by different oncogenic kinases increases Merlin degradation via proteasomal pathway. Also, this study observed that phosphorylation of Merlin at S10 was increased by C-terminus S518 phosphorylation which in turn alters the binding capacity of Merlin to DCAF1 (DDB1-and Cul4-Associated Factor 1) (Laulajainen et al., 2011). This supports the previous observation that DCAF1 is essential for the degradation of Merlin by targeting it to the Roc1-Cullin4A-DDB1 E4 ligase complex upon serum stimulation (Huang and Chen, 2008). In this process, Merlin binds to E3 ligase complex mediated by direct interaction with the WD40-containing adaptor protein VprBP/DCAF1, which in turn leads to polyubiquitination of Merlin followed by proteasomal degradation. In contrast to this observation, another study demonstrated that Merlin inhibit the DCAF1 function, ubiquitination of various target proteins, when hypophosphorylated form of Merlin enters the nucleus (Li et al., 2010). In addition, it has been recently shown that Akt mediated degradation of Merlin results in breast malignancy, a novel mechanism for the loss of Merlin protein in breast cancer (Morrow et al., 2011). Other studies suggested that Merlin can be degraded by interacting with proteins which has E2 or E3 ubiquitin ligase activity including Spectrin (Grönholm et al., 2006; Scoles et al., 1998) and human enhancer of invasion clone 10 (HE10), a cell cycle regulator. Collectively these results suggest that Merlin targeted to proteasomal degradation by various proteins potentially resulting in tumor development.

4. Merlin controls the cell proliferation by regulating various signaling pathways at the plasma membrane and in the nucleus

4.1 Regulation of mitogenic signaling pathways by Merlin

Growth inhibitory activity of Merlin is partly mediated by regulating the signaling of Rho GTPases at the plasma membrane surface. Rho GTPases, which include Rho, Rac, and Cdc42, regulate both cell– cell and cell–matrix adhesions, and can influence the motile and invasive properties of tumor cells *in vitro*. In addition, Rho GTPases plays an important role in the regulation of cytoskeletal organization as well as intracellular pathways involved in cell proliferation, transformation, and transcriptional activation (Bosco et al., 2009; Price and Collard, 2001). These small GTPases, Rac1 and Cdc42, are recruited to the membrane upon integrin activation, and increased adhesion to the extracellular matrix activates the effectors p21 activated kinases (PAKs) and then downstream protein such as c-Jun N-terminal kinase (JNK) (Rong et al., 2004a; Xiao et al., 2002). There are six PAK serine/ threonine kinases that function immediately downstream of Rac. Has we discussed previously, PAK 1 and 2 phosphorylate Merlin at serine 518 (S518) (PAK1/2) and results in inhibition of growth suppressive activity of Merlin (Kissil et al., 2002; Shaw et al., 2001). However, several studies have suggested that Merlin is not only regulated by the Rac-PAK but also functions as an inhibitor of Rac-PAK signaling by direct interaction with PBD domain (Rac/Cdc42 binding domain of PAK) which interferes with PAK1 recruitment to focal adhesions (Flaiz et al., 2009; Kissil et al., 2003; Morrison et al., 2007). This feedback loop between Merlin and PAKs indicates that loss of Merlin might trigger the activation of the small GTPases at the membrane (Flaiz et al., 2007, 2009; Kaempchen et al., 2003). In support of this idea, loss of Merlin function was shown enhance Rac activity. In contrast, over expression of Merlin suppresses the Rac-mediated growth and cell transformation (Tikoo et al., 1994). Not surprisingly, it has recently been shown that loss of Merlin leads to increase in levels of PAKs activity, suggesting the role of PAKs in Schwann cell tumorigenesis. Altogether, these studies suggest that Merlin regulates cell proliferation by inhibiting Rac in two ways. First Merlin blocks the recruitment of Rac to the plasma membrane and it also suppresses Rac activation (**Fig 14**). In addition, a recent study suggested that Merlin can regulate Rac1 and PAK signaling by forming a tight-junction-associated protein complex with Angiomotin (Amot),

Patj and Pals1. In this complex, binding of Merlin to Amot releases Rich1, a small GTPase-activating protein (GAP) for Rac1, hence inhibiting Rac1 activity (Yi et al., 2011) (**Fig 15**).

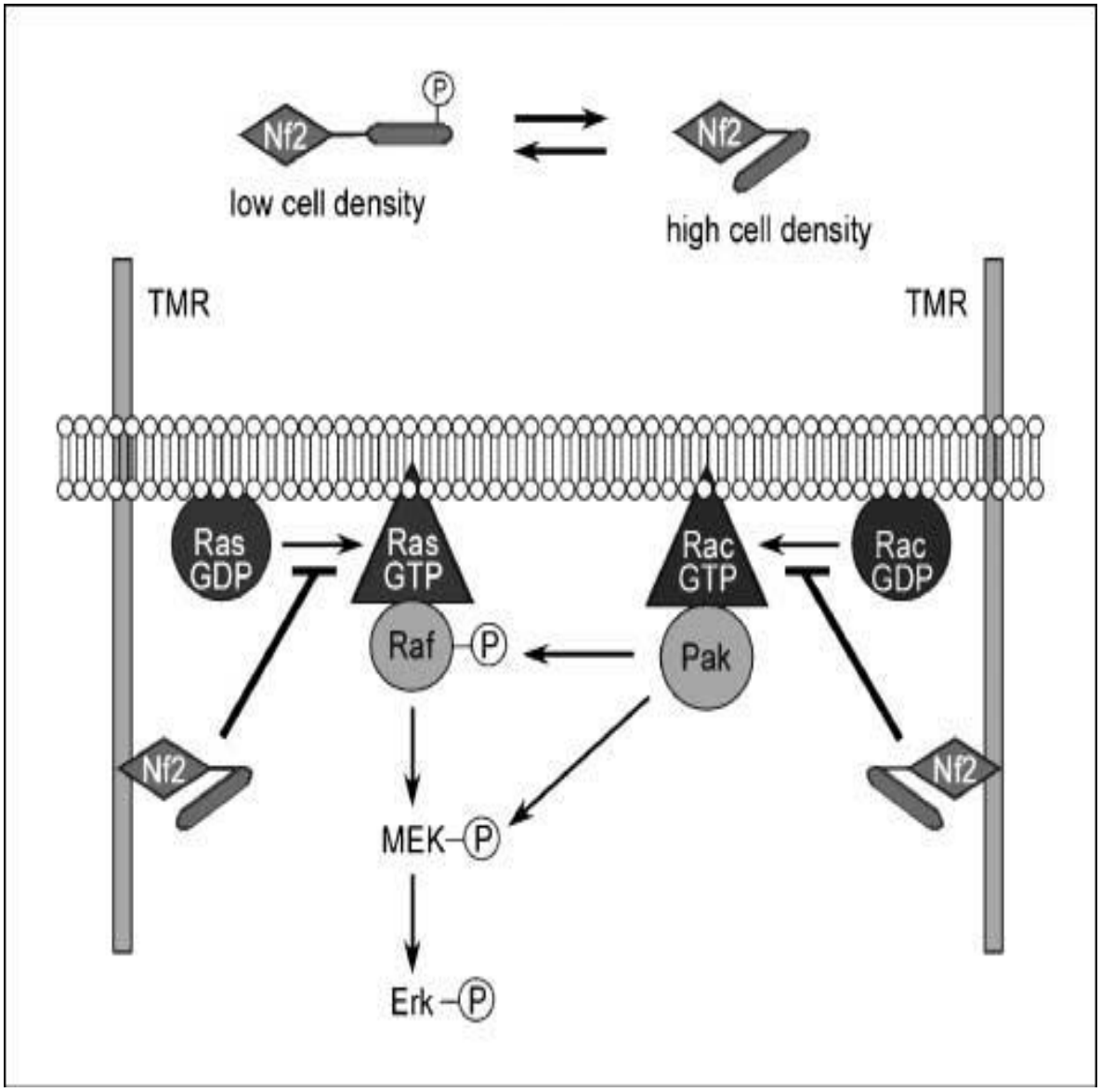


Figure14. Model of Merlin action in regulating the Ras-Rac pathway. Dephosphorylation of Merlin leads to its activation and which in turn allow Merlin to bind to plasma membrane protein (e.g., TMR, transmembrane receptor). This activated Merlin inhibits the activation of Ras and Rac. In the absence of Rac activation, no activation of Raf and MEK by PAK, which is a crucial step in Ras-dependent signal transduction. This model indicates that Merlin also inhibits signaling from constitutively active Ras (From Morrison et al., 2007).

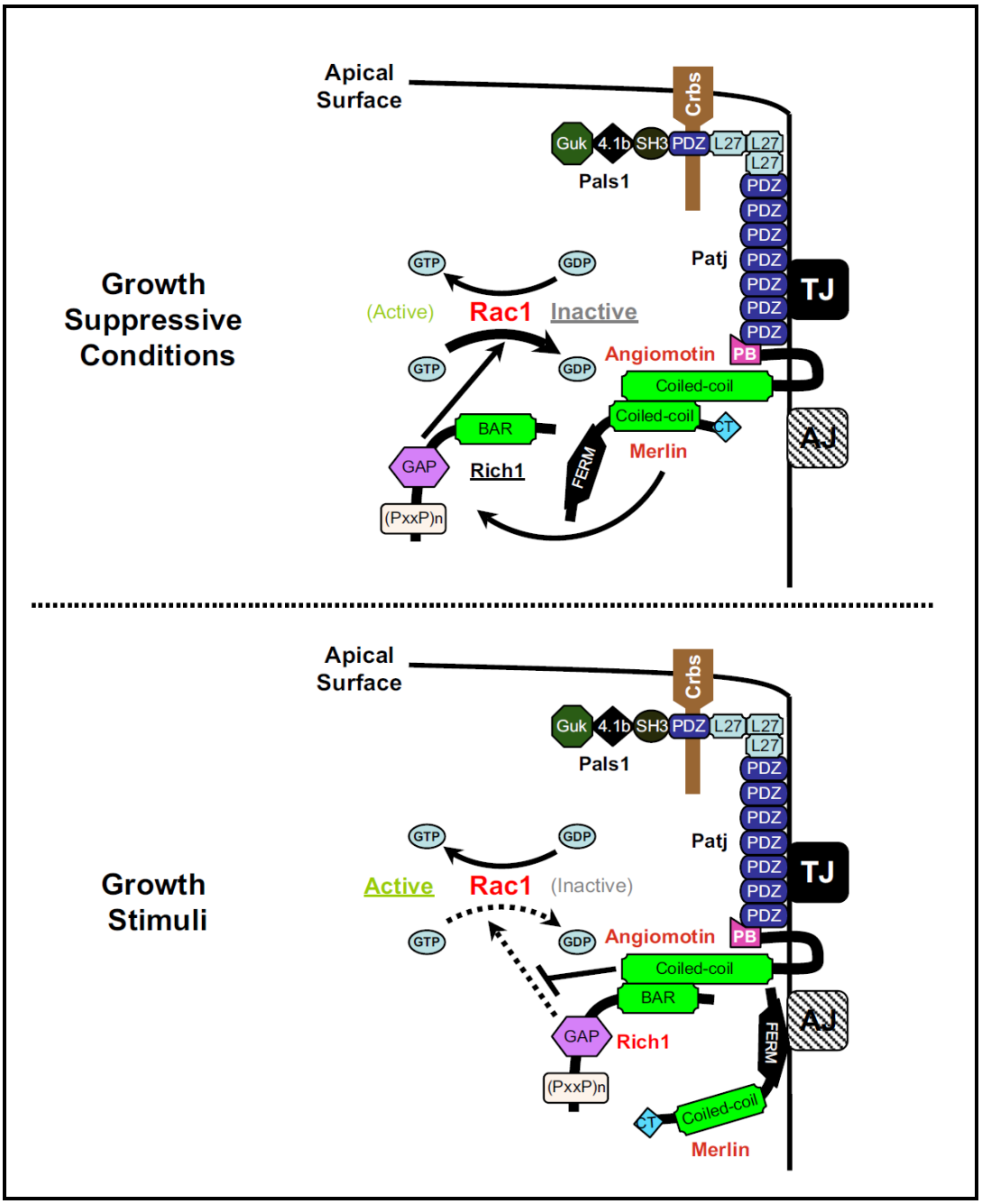


Figure 15. **Proposed model of Merlin function in inhibiting the Angiomotin.** Model showing the interplay between Merlin, Angiomotin and Rich1to modulate Rac1 activity. Under growth suppressive conditions, binding of Merlin to Angiomotin allows Rich1 to release from Angiomotin to inactivate Rac1 by converting Rac1-GTP to Rac1-GDP. Upon growth stimuli, Merlin dissociates from Angiomotin. This allows Angiomotin binds to and blocks Rich1's GAP activity, leading to increased levels of Rac1-GTP (From Yi et al., 2011).

In addition to PAK activation, integrins can also control focal adhesion kinase (FAK) activity and its interacting partner Src (Ammoun et al., 2008; Mitra et al., 2005; Poulikakos et al., 2006). A few studies observed that in Merlin null cells, FAK functions upstream of PI3K/AKT and Raf/MEK/ERK signaling cascades to potentiate schwannoma proliferation, migration and cell survival. Interestingly it has been shown that Merlin inhibits FAK activity by inhibiting the RAC-PAK signaling cascade. All these results indicate that Merlin might inhibit Rac-PAK signaling by multiple, potentially synergistic mechanisms. These might be important for the maturation and stability of cell-cell junction which in turn allows contact inhibition of proliferation.

Merlin dephosphorylation is one of the regulatory events to reactivate Merlin tumor suppressor function. As we discussed previously MYPT-1-PP1d dephosphorylates Merlin at Ser518, which results in Merlin activation and MYPT-1-PP1d inhibitor, CPI-17, inhibits Merlin activation to promote Ras activation and induce cellular transformation (Jin et al., 2006; Okada et al., 2005). Consistent with this notion, in RT4 schwannoma cells and NIH3T3 fibroblasts, forced over expression of CPI-17 suppresses Merlin and activates Ras signaling to ERK, enabling these cells to grow in soft agar and form tumors in nude mice. Abnormal activation of PAK can cause similar effects and moreover, PAK can phosphorylate and activate CPI-17 *in vitro*. According to other studies constitutive activation of PAK4 is sufficient to transform fibroblasts *in vitro* and in addition PAK4 levels are increased in various tumors (Callow et al., 2002; Takizawa et al., 2002). All these data together indicate that PAK4 can contribute to tumorigenesis by activating CP1-17, Ras and Rac which in turn leads to inactivation of Merlin (**Fig 13**).

4.2 PKA pathway

The cAMP-PKA signaling cascade was shown to regulate many cellular processes like proliferation, cytoskeleton remodeling, apoptosis and differentiation. In vivo (mice), inactivating mutation of the gene encoding RIα regulatory subunit, PRKAR1A, triggers the development of schwannomas due to elevated levels of PKA leading to phosphorylation and inactivation of Merlin at S518 position (Jones et al., 2008; Kirschner et al., 2005; Naviglio et al., 2009).

Cyclic AMP (cAMP), which is produced following activation of G protein coupled receptors acts as a second messenger to trigger the activation of downstream effectors essentially Protein Kinase-A (PKA). PKA is a holoenzyme which exists as a hetero-tetramer. In its inactive state, two catalytic subunits are bound to two regulatory subunits. When cAMP binds to the regulatory subunits, it triggers a conformational change that releases the active catalytic subunits from the tetramer complex. There are four regulatory subunit isoforms; RIα, RIβ, RIIα, and RIIβ, and three catalytic isoforms; Cα, Cβ, and Cγ. These can be assembled in various combinations. In order to become fully active, PKA needs further phosphorylation of its motif, either by auto phosphorylation or by 3-phosphoinositide-dependent kinase 1(PDK1). Then, fully activated PKA phosphorylates its substrates both in cytoplasm and nucleus (Kannan et al., 2007; Pearce et al., 2010). In addition, PKA needs to bind A-Kinase Anchoring Proteins (AKAPs) to confine it near downstream targets for efficient signaling. More than 50 different types of AKAPs have been identified which bind to regulatory subunits of PKA and helps in targeting the PKA to particular sub-cellular location (Wong and Scott, 2004). In this context, Merlin via its α-helical domain was shown to bind to regulatory subunit (RIβ) of PKA and being phosphorylated on S518. This phosphorylated form of Merlin has been suggested to act as an AKAP in the central nervous system (CNS) and in cultured neuronal cells (Grönholm et al., 2003). Similarly, Ezrin is phosphorylated by PKA and functions as an AKAP by binding to PKA regulatory subunit in gastric parietal cells (Dransfield et al., 1997; Sun et al., 2000).

Neuregulin or laminin trigger the phosphorylation of Merlin at S518 by two independent mechanisms in Schwann cells. Merlin is phosphorylated on S518 by PAK following laminin-1 binding to β1 integrin, and by protein kinase A following neuregulin-1b (NRG1b) binding to ErbB2/ErbB3 receptors (Thaxton et al., 2008) (**Fig 16**). Finally as previously mentioned, PKA also phosphorylates Merlin at Serine 10 (S10) position which is shown to modulate the organization of actin cytoskeleton. (Laulajainen et al., 2008). All these results indicate that PKA phosphorylates the Merlin at S10 and S518 depending on the signaling and cell confluency as described in **Fig 16**.

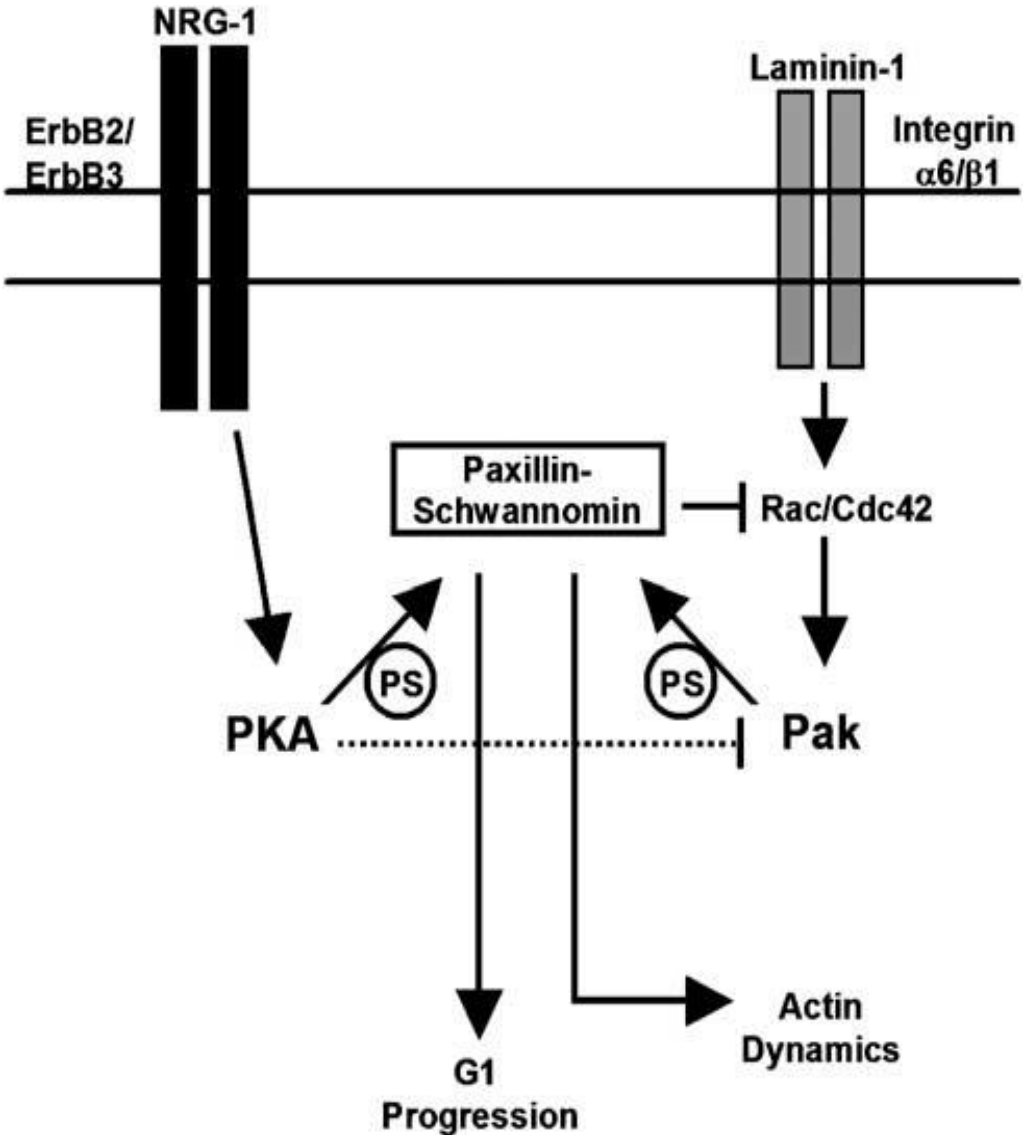


Figure16. Proposed mechanism of Merlin phosphorylation by ErbB and b1 integrin induction in sub confluent SCs. Active form of Merlin restricts cell proliferation by suppressing the activation of the Rac–Pak pathway in confluent cells. In sub confluent cells, Pak phosphorylates Merlin in response to activation of ErbB2/ErbB3 and a6/β1 integrin receptors by neuregulin-1b (NRG1β) and laminin-1, respectively. This in turn results in inactivation of Merlin to inhibit the growth. Simultaneous co-activation of both receptors does not synergistically up-regulate Merlin phosphorylation, instead ErbB2 antagonizes Pak phosphorylation of Merlin, possibly through its activation of PKA (dashed line). Upon Phosphorylation, Merlin is unable to inhibit Rac- Pak and allows transduction of ErbB and a6β1 integrin signals that increase G1 progression. In addition, the presence of this complex at the motile distal tip of SC processes coordinates motility along axons and other cytoskeletal changes in response NRG and laminin, present in basal lamina, which is involved in the myelination of peripheral nerves during development (From Thaxton et al., 2008).

4.3 Merlin role in receptor tyrosine kinase (RTK) regulation

Endocytosis mediated internalization of membrane receptors frequently commits them to degradation in lysosomes, and leads to signal attenuation. Alteration in this process can lead to tumorogenesis. Merlin is frequently found in lipid rafts and it has been shown that it is essential for receptor internalization from the cell surface and controlling of their downstream signaling cascades (Polo et al., 2004; Sigismund et al., 2008; Stickney et al., 2004). Recently several studies have shown that Merlin is important for regulating the distribution, aggregation, and availability of several transmembrane growth factor receptors at the plasma membrane, especially receptor tyrosine kinases (RTKs) (Curto and McClatchey, 2008; Lallemand et al., 2009b; Maitra et al., 2006). Loss of Merlin and Expanded, a FERM domain protein, in *Drosophila* leads to elevated levels of several receptors including Notch, Patched, and epidermal growth factor receptor (EGFR) at the cell surface due to defect in receptor endocytosis and degradation. This receptor accumulation at cell surface in turn triggers the activation of downstream signaling such as Ras-ERK, Rac and Pak that leads to increased cell proliferation (Kissil et al., 2003; Lallemand et al., 2009b; Morrison et al., 2007; Okada et al., 2007). The same phenotype, accumulation of receptors at the cell surface, has been observed in *Drosophila* by depleting the components of Hippo pathway. This suggests that regulation of receptor trafficking might occurs via canonical Hippo signaling cascade in *Drosophila* (Genevet et al., 2009).

Concerning the role of Merlin in mammals, A.McClatchey group observed that Merlin inhibits the ligand induced EGFR internalization and signaling in various cell types. In this study authors have proposed that Merlin binds to EGFR through NHE-RF1 which in turn leads to inhibition of ligand induced EGFR internalization and its downstream mitogenic signaling cascades required for cell proliferation (Curto et al., 2007). However, there is no unanimity as to whether these mechanisms are central to specific NF2-related tumors. In this context for the first time our laboratory has demonstrated that loss of Merlin up regulates the levels of ErbB2, ErbB3, IGF1R and PDGFRβ at plasma membrane in peripheral nerves of Nf2-null mice as well as in human NF2 schwannomas (Lallemand et al., 2009b). Additionally, we also proposed that Merlin inhibit the transport of RTKs to the plasma membrane and which in turn blocks the downstream signaling cascades important for the cell proliferation and tumor growth. Taken together our results have shown how tumor suppressor Merlin regulate the protein transport and hence tumorogenesis. These studies in *Drosophila*,

MEFs and Schwann cells indicate that Merlin function is conserved in controlling the subcellular trafficking of multiple transmembrane receptors such as Erb2, Erb3 in mammals and Notch, EGFR in *Drosophila*. However the mechanisms look different. This is due to the fact that in Drosophila, Merlin and Expanded modulate growth factor receptor internalization, but single D-Merlin or Expanded inactivation did not affect internalization. In contrast Merlin alone inhibits the delivery of proteins to the plasma membrane in SCs. In addition, the differences across the species might also come from the tissue-specific factors as in MEFs and liver-derived cells Merlin suppress the EGFR internalization upon cell-cell contact preventing the activation of downstream signaling pathways. From these studies, it is clear that growth factor receptor kinases at cell surface are regulated by Merlin in different ways. The proposed mechanisms may reveal the prevalence of a specific function in a particular organism or cell type. However our results provide the solid proof that ErbB-mediated activation of downstream mitogenic signaling cascades is important for schwannoma development.

4.4 The Hippo pathway

Regulation of development and tissue homeostasis is mediated by controlling the cell number. Organ growth is a combination of increase cell number and cell size. Any disturbance in this process leads to tumor formation or organ defects. The Hippo pathway plays a crucial role in regulating cell number by modulating cell proliferation, cell death, and cell differentiation in both *Drosophila* and mammals.

4.4.1 Core components of the Hippo pathway

In *Drosophila*, the canonical Hippo pathway contains of number of tumor suppressor genes including Warts (wts), Salvador (sav), Hippo (hpo), and Mob as tumor suppressor (mats) (**Fig 17**). Mutations of any one of these components results in massive tissue overgrowth in various epithelial structures such as the wings, the legs, and the eyes in flies(Harvey et al., 2003; Tapon et al., 2002; Udan et al., 2003). In addition, it has been shown that loss of function of Warts or Salvador results in increased proliferation and reduced apoptosis, suggesting that both regulate cellular processes to control cell number. Hippo, from which the pathway name was derived, encodes a Ste20 family protein kinase. Loss of Hippo also shows a similar type

of phenotype shown by loss of Salvador or Warts. During the activation of the pathway, Hippo first auto-phosphorylates and then phosphorylates Warts, Sav, and Mats. Sav couples the phosphorylation from Hpo to Warts, and Mats binds to Warts to enhance its catalytic activity. Warts then negatively regulate a transcriptional coactivator, Yorkie, mainly by phosphorylating it to promote cytoplasmic retention through 14-3-3 proteins binding (Dong et al., 2007; Oh and Irvine, 2009). In response to growth signal or stimuli the Hippo signaling is attenuated or inhibited, Yorkie then translocates into the nucleus and binds to different DNA-binding proteins to mediate transcription of target genes such as cyclin E and diap1 (Harvey et al., 2003; Udan et al., 2003; Wu et al., 2003). These Yorkie-targeted genes are often involved in cell proliferation and tissue growth (**Fig 17**).

Hippo pathway is highly conserved in mammal and it has been shown that some of the human homologs can rescue the overgrowth phenotype of mutants of their orthologs in *Drosophila* (Tumaneng et al., 2012; Zhao et al., 2011a). Each of the core components of the *Drosophila* Hippo pathway has at least one ortholog in mammals. Hippo core components in mammals includes 1) Hpo: mammalian sterile 20-like kinases 1 and 2 (MST1 and MST2, referred as MST1/2), 2) Warts: large tumor suppressor 1 and 2 (LATS1 and LATS2, referred as LATS1/2), 3) Mats: Mps Once Binder kinase activator-like 1(MOB1), 4) Sav: SAV1, also known as WW45, 5) Yorkie: Yes-associated protein (YAP). TAZ, also known as WWTR1, is a paralog of YAP. The regulation of YAP/TAZ proceeds in a similar fashion as in Drosophila, in which LATS1/2 directly phosphorylates YAP/TAZ, promoting its cytoplasmic retention (Dong et al., 2007) (**Fig 17**).

The phosphorylated form of YAP is sequestered in the cytoplasm via a 14-3-3 interaction and is in turn targeted for degradation (**Fig 17**), resulting in the inhibition of target gene transcription (Zhao et al., 2007). Also, TAZ and YAP are phosphorylated by Lats1/2 on multiple HXRXXS sites. In contrast, when upstream kinases are inactive, YAP/TAZ will not be phosphorylated and translocate into the nucleus to exert their functions on gene expression (Dong et al., 2007; Kanai et al., 2000; Oh and Irvine, 2009; Ren et al., 2010; Zhao et al., 2007). YAP/TAZ does not contain intrinsic DNA-binding domains but instead bind to the promoters of target genes by interacting with DNA-binding proteins. YAP/TAZ mainly bind to the DNA binding proteins TEAD1–4 to regulate genes involved in cell proliferation and cell death (Zhao et al., 2008).

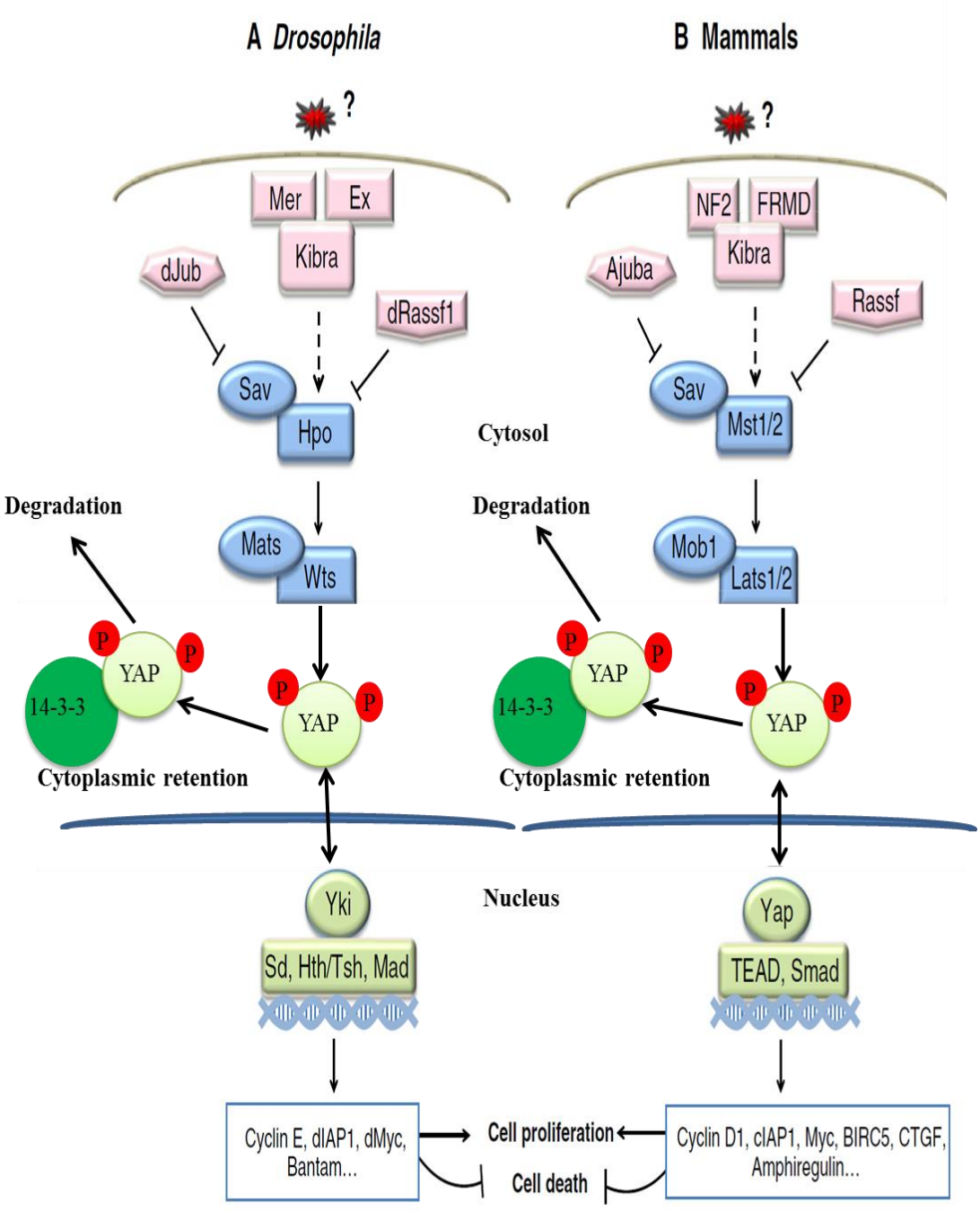


Figure17. Proposed model of Hippo pathway in Drosophila and Mammals. (A) Scheme showing how Drosophila Hippo core kinases sequentially activated to phosphorylate the co-transcription factor, Yki. This in turn leads to either cytoplasmic retention of Yki or target it for degradation. (B) Schematic representation of the Hippo pathway which is conserved in mammals. Core components, upstream regulators, and downstream targets of the Hippo pathway are labeled in blue, pink, and green colors, respectively (Modified Tumaneng et al., 2012).

4.4.2 Merlin, an upstream regulator of Hippo pathway

To date, many regulatory proteins upstream of the canonical Hippo pathway have been identified but their mechanism of action is unclear in most cases. Several upstream regulators of Hippo pathway are better characterized in *Drosophila* than in mammal. Earlier studies in *Drosophila* suggested that the atypical cadherin Fat (Ft) interacts with another atypical cadherin Dachsous (Ds) to control Hippo activity and then affecting target gene transcription via Yki (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2008). The molecular mechanism of this regulation is still poorly described, however it is believed that Ft and Ds exert their function by interacting with FERM domain protein Expanded (Ex) and Merlin (Hamaratoglu et al., 2006; Silva et al., 2006; Tyler and Baker, 2007). Merlin (Mer) and Expanded (Ex) in turn binds to Kibra, Hpo and Sav to mediate their tumor suppressor function (Baumgartner et al., 2010; Yu et al., 2010). The mammalian homologs of Ft, Merlin, and Kibra are FAT4, Merlin (*NF2*), and KIBRA, respectively. However, in contrast to *Drosophila*, mammalian Ds and FAT4 do not seem to affect the YAP or LATS1 activity indicating that regulation of Hippo pathway by FAT4 is limited in the mammalian context (Tumaneng et al., 2012).

Concerning the role of Merlin in Hippo pathway regulation, one study has provided a number of remarkable evidences demonstrating a functional link between *NF2* and the Hippo signaling cascade. In this study, conditional knockout of *Nf2* in mice liver triggers the formation of hepatocellular carcinomas and bile duct hamartomas in combination with hyperplasia of hepatocytes and biliary epithelial cells. In contrast, Yap inactivation results in loss of hepatocytes and biliary epithelial cells, suggesting that *Nf2* and YAP act antagonistically to regulate liver development. In the context of hippo pathway core component regulation, inactivation of *Nf2* results in decrease in phosphorylation of YAP and LATS1/2, suggesting that YAP and LATS could be downstream targets of Merlin to regulate the Hippo pathway. Strikingly, the phenotypes induced by *Nf2* deficiency are suppressed by heterozygous deletion of YAP. These results strongly implicates that Yap is a major effector of *Nf2* tumor suppressor in mammalian growth regulation. Finally, this study has shown that *Nf2* binds to KIBRA and WW45, known core components of the Hippo pathway, and triggers the activation of Hippo signaling cascade in a WW45-dependent manner (Zhang et al., 2010a). Collectively, these studies indicate that *NF2*/Merlin function is conserved in

Drosophila and mammals. However, how Merlin regulates the Hippo core pathway is still hypothetical and its understanding represents an exciting challenge in the field.

4.4.3 Regulation of the Hippo pathway in physiological and pathological conditions.

It is well documented that the canonical signaling cascade Hippo pathway is involved in organ size regulation as mutants of the hippo pathway results in overgrowth in the imaginal discs in *Drosophila* and in adult organs in mammals (Edgar, 2006; Pan, 2007; Saucedo and Edgar, 2007). The mutants of the Hippo pathway show resistance to contact inhibition of proliferation and to apoptosis. Inactivation of Hippo core components, MST1/2 and LATS 1/2, or activation of YAP is observed in many cancers (Li et al., 2012; Xu et al., 2009; Zender et al., 2006). In addition, Yap overexpression triggers intestinal tissue overgrowth, and, in contrast, inactivation of Yap severely impairs intestinal regeneration. These studies suggest the important function of Yap in growth control and regeneration mediated by Hippo pathway (Camargo et al., 2007; Lee et al., 2010). In addition to all these mechanisms, recently it has been proposed that the hippo pathway also interconnect and cooperate with other signaling cascades involved in cell proliferation and apoptosis/cell survival such as Wnt, TGFβ etc. (Yu and Guan, 2013; Zhao et al., 2010a)**.** The functions of Hippo core component pathway, by regulating the YAP, are summarized in **figure 18**.

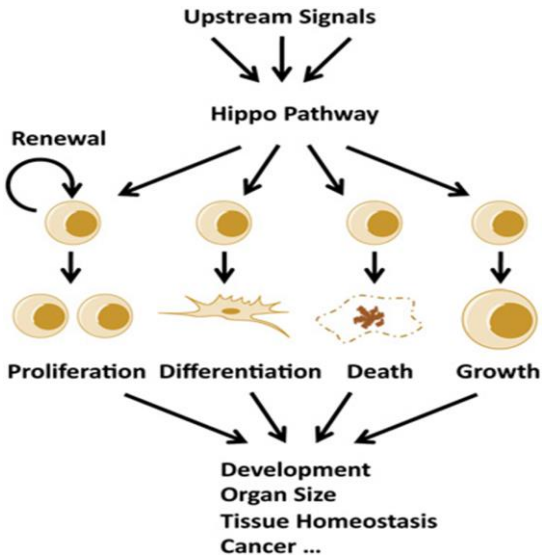


Figure 18. Functions of the Hippo pathway in cell biology. The Hippo pathway regulates cell proliferation, differentiation, growth, and death. The coordination of various cellular processes by the Hippo pathway may contribute to different physiological and pathological conditions such as development, tissue homeostasis, and tumorogenesis (From Yu and Guan, 2013).

4.5 Merlin inhibits the E3 ubiquitin ligase, CRL4-DCAF1, in the nucleus

Although Merlin performs most of its tumor suppressive function by inhibiting the mitogen signaling cascades from or near the membrane, the hypo-phosphorylated form of Merlin has been shown to translocate into nucleus. Ubiquitin ligase CRL4-DCAF1 has more recently gained appreciation for its interaction with Merlin in the nucleus. Ubiquitin ligase CRL4DCAF1 is a member of the cullin-ring family of E3 ligases. In the multi-subunit complex, the adaptor (DDB1) and scaffolding (Cullin-4) subunits interact with the substrate acceptor (DCAF1). Activation of the CRL4DCAF1 complex also permits cell cycle progression by regulating expression of ubiquitin ligases that target histones, or by recruiting chromatin remodeling enzymes (Higa and Zhang, 2007; O'Connell and Harper, 2007). In a recent study, it has been demonstrated that DCAF1 was a nuclear binding partner for Merlin. In the nucleus, Merlin inhibits the ubiquitination of target proteins suggesting that it negatively regulates the CRL4DCAF1 function (**Fig 19**). In the *NF2* null Schwannoma cells, it has been shown that re-expression of Merlin or depletion of DCAF1 concordantly caused a decreased expression of genes that uphold cell-cycle progression and elevated expression of genes that are involved in growth arrest and apoptosis. This study demonstrated that Merlin mediated suppression of cell proliferation is going through the inhibiting of DCAF1 function. Interestingly, hyper-phosphorylated form of Merlin does not bind to DCAF1, suggesting that only the hypophosphorylated form of Merlin localizes to the nucleus and bind to DACF1 to inhibit its function. In addition, this study also demonstrated that Merlin-deficient cells were more sensitive to inactivation of CRL4DCAF1 than their normal counterparts. Finally, among several tumor-derived Merlin mutants, some exhibited defective nuclear translocation whereas others were impaired in binding to DCAF1, and few that bound to DCAF1 did not inhibit the DCAF1 E3 ligase activity. These results strongly suggest the tumor suppressor function of Merlin is mediated, at least in part, by suppressing CRL4DCAF1 ubiquitin ligase activity (Li et al., 2010).

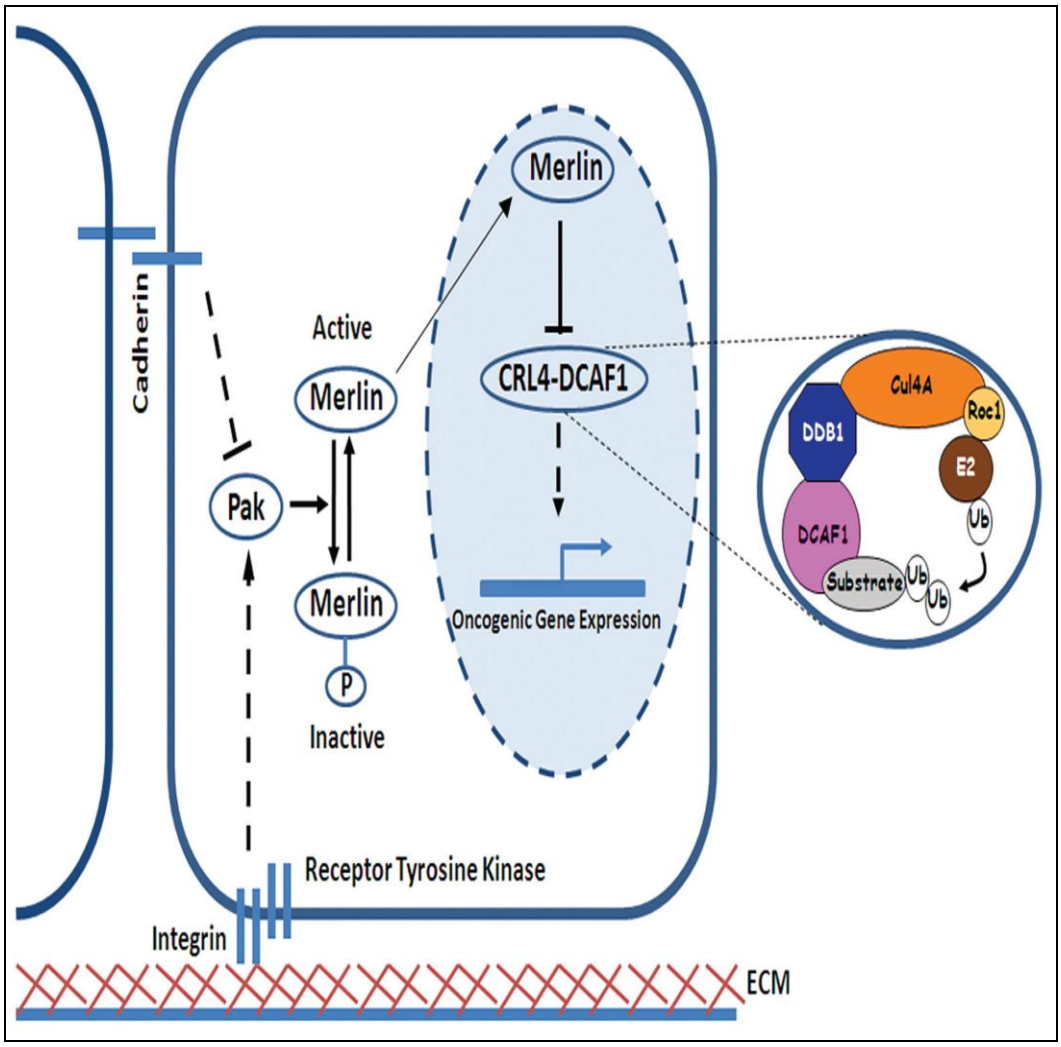


Figure 19. Model showing the Merlin tumor suppressor function in the nucleus. Upon growth signals, Pak phosphorylates the Merlin (in active) and cannot enter nucleus. The hypophosphorylated form of Merlin enters the nucleus and binds to the E3 ubiquitin ligase CRL4DCAF1, thereby inhibiting its activity. Inset showing the molecular organization of CRL4DCAF1 ligase. Through this model authors proposed that deregulated CRL4DCAF1 drives the oncogenicity of Merlin-deficient cells by upregulating the expression of multiple oncogenic genes (From Li and Giancotti, 2010).

4.6 Merlin in mTOR signaling pathway

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase shown to be involved in the development of endocrine tumors in diseases such as NF1 and tuberous sclerosis. There are two kinds of mTORs: mTOR complex 1 and 2 (mTORC1 and mTORC2, respectively). mTOR role has been well documented as a crucial regulator of cell proliferation

in tumor development and it is activated depending on the availability of growth factors at plasma membrane (Guertin and Sabatini, 2007; Lodish and Stratakis, 2010).

Concerning the role of mTOR in NF2 related tumors, originally it has been shown that integrin-mediated adhesion results in the inactivation of Merlin, and activation of mTOR1 signaling. This in turn results in elevated levels of cyclin D expression leading to cell cycle progression. The same study also showed the remarkable correlation between inactivation of Merlin and activation of mTORC1 in a group of malignant mesothelioma cell lines. These results indicate that inactivation of Merlin results in aberrant mTOR signaling which in turn promote malignant mesothelioma and other *NF2*-related malignancies growth (López-Lago et al., 2009). Another study nicely demonstrated that role of Merlin in mTORC1 signaling regulation. There, the authors have shown that loss of Merlin activity in meningioma cells and arachnoidal cells results in hyperactivation of mTORC1 signaling and increased growth. Also, they have shown that reexpression of active Merlin, but not a patient-derived L64P mutant, inhibited the mTORC1 signaling suggesting the role of Merlin in mTOR signaling in meningioma and schwannoma development. Finally these studies have shown that Merlin deficient cells are sensitive to growth inhibition by mTOR1 inhibitors such as rapamycin, suggesting that it might be a good candidate for NF2 therapeutics (James et al., 2009). Indeed several clinical trials are in progress with this drug for NF2 patients. On the other hand, recently it has been shown that Merlin promotes the kinase activity of mTORC2, the other mTOR complex. In contrast to mTORC1, mTORC2 is not sensitive to rapamycin and it is activated by Merlin upon growth factor (IGF1, EGF, or PDGF) stimulation in Schwann and arachnoid cells. This activated mTORC2 in turn leads to the activation of Akt1 by phosphorylation. Finally it has been shown that, by using a combination of rapamycin and Torin1 (mTOR kinase inhibitor), it is possible to block the mTOR-mediated signaling and proliferation in Merlin inactivated human meningioma cells suggesting the importance of these studies for the development of therapeutics for *NF2*-related tumors(James et al., 2012).

The summary of multiple function of Merlin in regulating cell proliferation, apoptosis, survival and growth summarized in **Fig 20.**

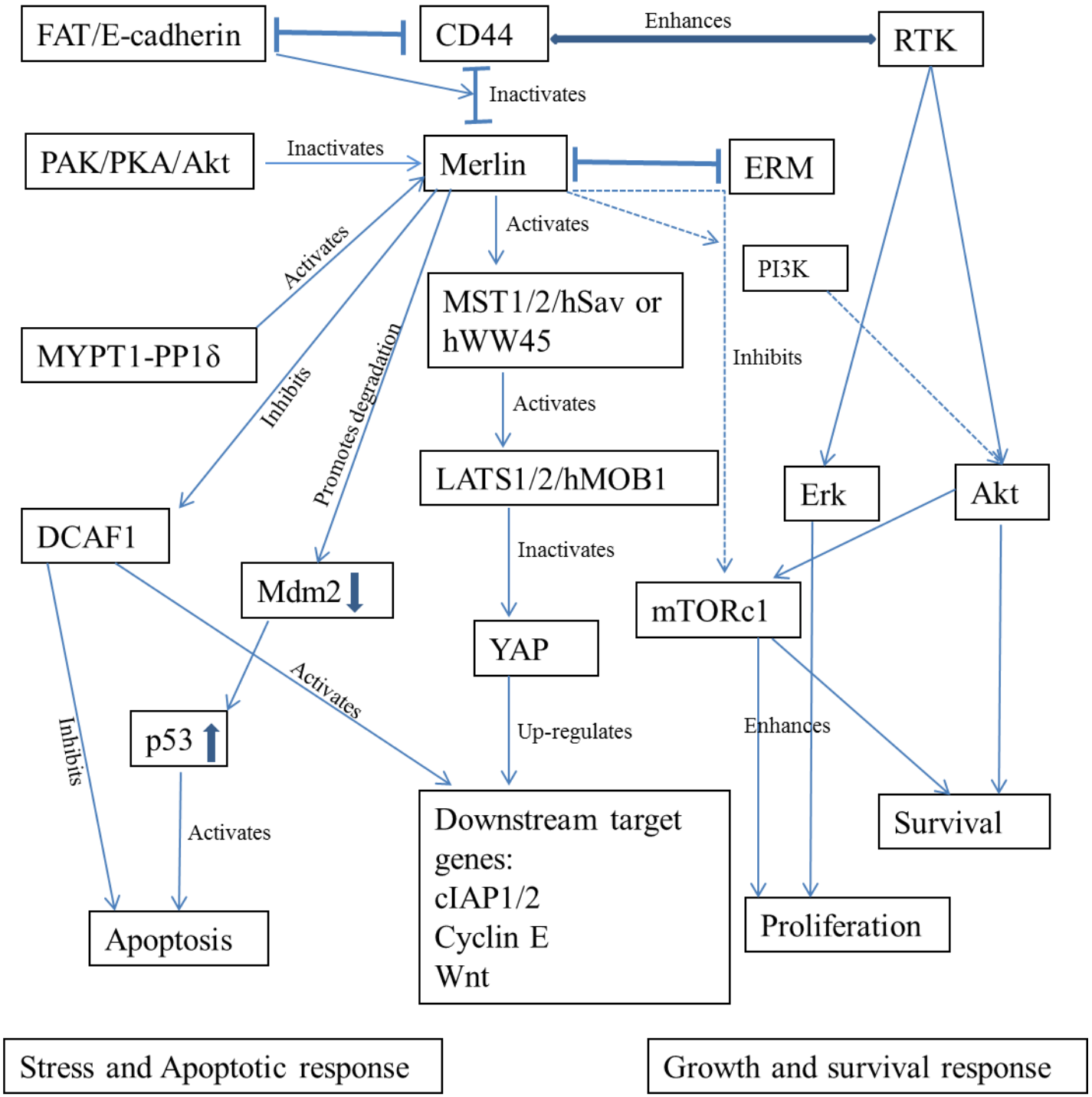


Figure 20. Scheme showing the summary of Merlin-mediated signaling events and their potential interplay to regulate the cell proliferation, survival, apoptosis and cell growth (Modified from Stamenkovic et al., 2010)

OBJECTIVES OF THE THESIS

When cells come in contact with one another, it leads to various significant changes in their behavior. This in turn leads to many stimulatory events, and contact inhibition of proliferation (CIP) is one of the major regulatory mechanisms to control cell proliferation when cell surfaces contact each other. The concept of inhibition of cell proliferation first coined in the 1960 to demonstrate the property of tissue culture cells to attenuate or decrease their proliferation rates when they reach confluency (density-dependent inhibition of proliferation). Many studies indicated that contact dependent inhibition of proliferation might be a key mechanism to determine tissue architecture and organ size control. It is believed that by inhibiting the over-proliferation, contact inhibition regulates the organization of cells within normal tissues. In contrast, loss of contact dependent inhibition is one of the major characteristic observed in tissue dysmorphogenesis displayed by cancers, and potentially promotes tumor progression. However, not all effects of increasing density need to be due to contact. So, it is suggested that three events play crucial roles in contact inhibition of proliferation. (1) Cell surface receptors that are engaged by the physical interaction between cell surfaces; (2) growth regulatory signaling pathways that are affected by those receptors in a contact-dependent fashion; and (3) molecular mechanisms that functionally and biochemically couple those surface receptors and intracellular signaling pathways together. Several studies demonstrated that Merlin is involved in organ size control through regulation of proliferation and apoptosis by acting on the three steps mentioned above.

Merlin, the product of the *Neurofibromatosis Type II* (*NF2*) gene, is unique in its capacity to inhibit the cell proliferation, and hence function as a tumor suppressor. This growth suppressive function of Merlin is explained by many mechanisms including its ability to negatively regulate Ras and Rac GTPase signaling, to inhibit the growth by activating canonical signaling cascades such as for Hippo pathway, and finally enter into nucleus to inhibit DCAF1 function.

Our laboratory has demonstrated that Merlin exerts its tumor suppressor function by regulating the transport of receptors at cell surface in primary Schwann cells (SCs). In this study we have shown that loss of Merlin expression results in accumulation of several growth receptors such as ErbB2, ErbB3, insulin-like growth factor 1 receptor (IGF1R), PDGFR-β at the plasma membrane and also we have shown that receptor accumulation is due to defects in

their delivery as Merlin over expression results in clearance of receptors from the cell surface. In our experiments we also found that the accumulation of receptors at plasma membrane, due to inactivation of Merlin, promotes the SCs proliferation and growth. In addition, we have shown overexpression of growth receptors in *Nf2*-mutant peripheral nerves of mice as compared with wild-type ones. Finally we have observed elevated expression levels of HER2, HER3, IGF1R, PDGFR-β and N-cadherins in human Schwannoma samples compared to normal nerves. Collectively this study highlights the role of Merlin in regulating the growth factor receptors at the plasma membrane of SCs and shown that alteration in this mechanism in absence of Merlin is an important step in the initiation of schwannoma development in mouse NF2 models as well as in NF2 patients. Based on these results, it has been proposed that when cells come in contact, Merlin down regulates the levels of various growth receptors then inhibiting the activation of downstream mitogenic signaling pathway which in turn results in inhibition of cell proliferation (Lallemand et al., 2009b). Our results are further supported by previous studies in *Drosophila* showing that inactivation of Merlin results in the accumulation of transmembrane receptors at cell surface (Maitra et al., 2006).

In the second step, in order to identify the specific domains which are important for the down-regulation of growth receptors to inhibit the SCs proliferation, we have tested the capacity of different domain specific mutants of Merlin to inhibit SCs proliferation. In this study interestingly we found that the F2 subdomains of the FERM domain along with a portion of the C-terminus domain corresponding amino acid 532-579 are crucial for the growth suppressive activity of Merlin. Finally this study for the first time has shown that the F1 sub-domain of Merlin is essential for actin remodeling. However, this actin binding is not necessary to inhibit the proliferation of SC, but it is necessary for the proper regulation of Merlin phosphorylation which contributes to the tuning of its tumor-suppressor functions. This study also documented that both Merlin isoform 1 and isoform 2 are able to rescue *Nf2* inactivation in SC explaining why no mutations are found in exon 16 and 17 (Lallemand et al., 2009a). It has been shown that mice expressing a NF2 protein with an interstitial deletion in the FERM domain (Merlin-Δ 39–121/ Δ exons 2-3 mutants /Mer Δ 2-3 mutant) showed high prevalence of Schwann cell-derived tumors and Schwann cell hyperplasia, whereas those expressing a carboxy-terminally truncated (Merlin Δ C-terminus 1-340) protein were normal. Importantly, deletions of exons in the FERM domain of *NF2* (Mer Δ 2-3) are found in human NF2 patients and have been associated with early tumor onset and poor prognosis

(Giovannini et al., 1999). Furthermore in *Drosophila* it has been shown that removal of a seven–amino acid (171YQMTPEM177-Blue Box ($Mer^{\Delta ABB}$) conserved sequence within FERM domain results in a dominant-negative form of Merlin that is stably associated with the plasma membrane and causes over-proliferation when expressed ectopically in the wing (LaJeunesse et al., 1998). Also, expression of a murine analog of this amino-terminal mutant of *Nf2* leads to complete transformation of NIH3T3 fibroblasts in culture. These studies demonstrated that Merlin Δ 2-3 mutants have dominant effect on SC cell proliferation in a WT background and promotes tumorogenesis (Johnson et al., 2002).

Based on all the above results, we hypothesized that Mer Δ 2-3 and $Mer^{\Delta ABB}$ mutants may act in a dominant negative manner by titrating components which are important for the growth suppressive function of Merlin. We believe that these interactions are mediated by carboxy-terminal domain of Merlin, as overexpression of Mer Δ C-terminus did not promotes tumorogenesis, whereas Mer Δ 2-3 trigger the tumorogenesis by over-proliferation in spite of similar levels of expression of both mutant proteins in the Schwann cells. Furthermore, we also hypothesized that new phosphorylation events affecting the function of the c-terminal domain or its interaction with important effectors, in addition to S518 phosphorylation, may play an important role in regulating Merlin tumor suppressive activity.

Therefore, the major aims of my PhD were defined as follows:

1. To evaluate already characterized C-terminus interacting partner of Merlin that could explain its function in receptor regulation. In this regard we focused our attention on HRS (Hepatocyte growth factor regulated kinase substrate) that is known to participate in the receptor trafficking.

2. To identify novel interacting partners specific of the C-terminal domain (532-579) of Merlin and characterize their role in the tumor suppressor function of Merlin.

3. To identify possible new phosphorylation sites in the same C-terminus domain of Merlin and evaluate their role in Merlin tumor suppressor function.

PART 1

INDENTIFICATION OF NOVEL INTERACTING PARTNERS OF THE C-TERMINUS MERLIN IMPORTANT FOR ITS TUMOR SUPPRESSOR FUNCTION

1. Introduction

1.1 Merlin and its interacting partners

There are more than 40 proteins interacting with Merlin and these interactions help for better understanding of its function. Most of these interacting partners bind to hypophosphorylated, closed form of Merlin and have been shown to be necessary for Merlin-mediated tumor suppressor function (Rong et al., 2004a; Surace et al., 2004). However, other studies have shown that the closed conformation masks the binding sites for several partners of Merlin (Gonzalez-Agosti et al., 1999; Gutmann et al., 2001; Neill and Crompton, 2001). The known binding partners of Merlin play important role in regulation of receptor transport and turn-over such as NHE-RF and HRS (Gutmann et al., 2001; Murthy et al., 1998), cell-cycle transit (HEI10) (Grönholm et al., 2006), cell-cell adhesion (CD44, beta1 integrin) (Gutmann et al., 1999; Lallemand et al., 2003; Morrison et al., 2001b; Obremski et al., 1998), cytoskeletal organization (actin) (Laulajainen et al., 2008; Manchanda et al., 2005), and signaling (Ras, Rac and PAK) (Kissil et al., 2003; Morrison et al., 2007; Okada et al., 2005). In this scenario, keeping the Merlin tumor suppressor function as our central focus, we can divide these binding partners into the following groups (Scoles, 2008)

1. Activators and suppressors of Merlin function (e.g. PAK and MYPT-1-PP1δ)
2. Structural proteins supporting Merlin function (e.g Actin and βII-spectrin)
3. Growth activators suppressed by Merlin (e.g ERMs and CD44 and RalGDS)
4. Growth inhibitors that function with Merlin (e.g HRS, Rho GDI)
5. Role in Merlin suppression of growth unclear (e.g CRM1/exportin and Riβ)

Since my aim is to understand the role of C-terminus interacting partners of Merlin in its tumor suppressive function, I focused on known interacting partners. Among all the binding partners, one molecule drew our attention, the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS or HGS). Firstly HRS binds to residues 453-557 of Merlin (Gutmann et al., 2001; Scoles et al., 2000a), which partially overlaps with C-terminus domain (532-579) which is essential for its growth suppressive function (Lallemand et al., 2009a). The Merlin

interacting region on HRS is a coiled-coil domain including residues 470–497 and HRS was shown to be essential for Merlin mediated inhibition of proliferation (Scoles et al., 2005; Sun et al., 2002b). In addition, HRS is able to regulate EGFR internalization and degradation, and blocks proliferation of schwannoma cells even in the absence of Merlin. Finally, HRS is a key regulator of receptor tyrosine kinase trafficking and their turn-over via lysosomal pathway (Raiborg et al., 2002; Shih et al., 2002). Interestingly HRS and Merlin co-localized to EEA1 positive early endosomes, suggesting that Merlin might have a role in HRS mediated receptor trafficking (Scoles et al., 2000b). Hence it is a possibility that down regulation of RTK by Merlin is mediated, at least in part, via its interaction with HRS.

1.2 Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS/HGS)

HRS was originally identified as a 115 kDa tyrosine phosphorylated protein in B16-F1 mouse melanoma cells treated with hepatocyte growth factor (HGF). HGF is one of the most potent mitogenic stimuli for Schwann cells and has been shown to promote cell motility in a variety of cell types. Human HRS contains 777 amino acids with several conserved protein–protein interaction domains, including a FYVE domain, a VHS zinc finger domain, a coiled-coil domain and two proline-rich regions (Komada and Kitamura, 1995; Krasnoselsky et al., 1994; Maulik et al., 2002). The FYVE and VHS domains have been implicated in the localization of HRS to the early endosome where HRS functions to modulate endocytosis and exocytosis. HRS is required for the trafficking of receptor tyrosine kinases (RTKs) from the early endosome to the lysosome where they are degraded. Also, HRS has been suggested to function in the TGF-β signaling pathway by binding to SARA, a Smad family adaptor protein (Komada and Soriano, 1999; Mao et al., 2000; Miura et al., 2000), as well as mediate cell growth regulation by binding to the STAT signal transducing adaptor molecule (STAM) and modulating STAT pathway signaling (Asao et al., 1997). The interaction between Merlin and HRS was first identified in yeast two-hybrid screening of an adult human brain cDNA library (Scoles et al., 2000b). Studies in rat SC demonstrated that HRS and/ or Merlin expression inhibited the EGF activation of Stat3 and its abundance (Scoles et al., 2002, 2005).

2. Results

Previous studies have demonstrated that HRS binds to the C-terminus domain of Merlin (Scoles et al., 2000a). Based on these observations, we asked whether Merlin may regulate, at least partly, receptor transport and turnover via interacting with this major regulator of endocytosis namely HRS.

2.1 Interaction of Merlin with HRS

Since Merlin and HRS are known to both localize at the plasma membrane, we first wondered if these proteins may co-localize in this compartment. To answer this question, we employed a technique called TIRF (Total internal Reflection Florescence) microscopy, a powerful microscopic technique allowing visualizing the proteins at or near the plasma membrane. In this experiments we transfected GFP-Merlin and HRS-DsRed and tested for the co-localization of these molecules at basal side of the plasma membrane. Using this technique, we could not observe co-localization of Merlin and HRS after over expression of the tagged versions of the both proteins in Hela cells (**Fig 1A**). However we observed that, indeed, both molecules did localize near the basal plasma membrane.

In the next step to test the interaction between Merlin and HRS, we performed two complementary sets of experiments. In the first step, we performed immunoprecipitation (IP) of endogenous Merlin from cellular extracts of Hela cells and checked for bound HRS by western blotting. In this experiment, endogenous HRS was not pulled down with endogenous Merlin (**Fig 1B**). In the second step, we analyzed the interaction between Merlin and HRS in HEK 293 cells after transient overexpression of GFP tagged full length Merlin (GFP-Merlin). We carried out IP of GFP-Merlin to test the co-immunoprecipitation of HRS. Again to our surprise, we did not found any interaction between HRS and Merlin even after IP of ectopic overexpressed HRS (data not shown). Collectively, these results suggest that the previously described Merlin binding to HRS was very likely an artifact due to the sticky nature of the proteins. Similarly, S. Urbe group also observed the same sticky nature of HRS and Merlin in their biochemical experiments (personal communication). To support our conclusions, a two-hybrid screen performed at Curie Institute using Merlin as bait identified HRS as an interactor. However, the interaction was reported as nonspecific and due to the "sticky" nature of HRS. Hence, it appears that HRS is unlikely to be an essential effector of Merlin function.

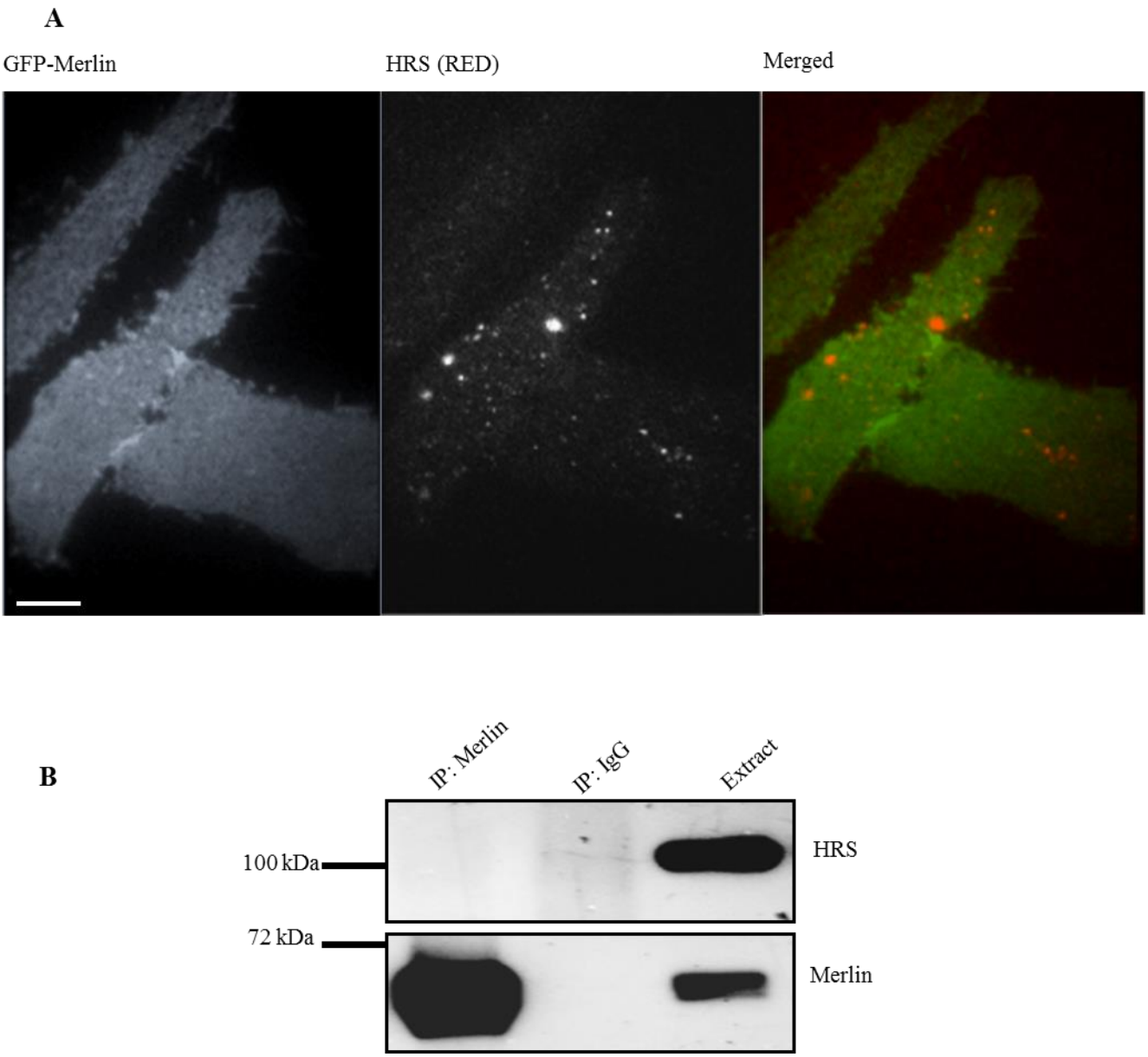


Figure 1. Interaction and co-localization between Merlin and HRS. A. IF analysis of Hela cells transfected with GFP-Merlin (Full length) and HRS-Red. TIRF images were taken at 100X magnification (White scale bar =10um). B. Immunoprecipitation (IP) reactions were performed using control immunoglobulin G (IgG) or anti-Merlin antibody.Western blotting was conducted with indicated antibodies.

2.2 Experimental strategy to fish for novel interacting partners of C-terminus Merlin

Since we did not succeed in establishing the role of a known interacting partner of Merlin, HRS, we decided to search for novel interacting partners of C-terminus Merlin. To achieve this goal, we used immunoprecipitation of Merlin from human embryonic kidney 293T cells (HEK 293) coupled to mass spectrometry **(Fig 2A)**. In this experimental strategy we expressed various constructs of Merlin fused to GFP. We used GFP-Merlin 1-595 (full length), GFP-Merlin 1-480, GFP-Merlin 330-595, and GFP alone as a nonspecific control **(Fig 2 B)**. To identify Merlin-associated proteins, we co-immunoprecipitated (co-IP) Merlin-containing complex from HEK293T cells after transient transfection of the GFP-tagged Merlin constructs using anti GFP high affinity antibody covalently coupled to beads, a technique called GFP Trap. These purified Merlin containing complexes were then separated on SDS-PAGE and chopped gel slices were analyzed by mass spectrometry following trypsin digest. MALDI-TOF-MS/MS followed by peptide mass fingerprinting and mass spectrometric sequencing was performed. Hits were considered positive if detected for GFP-Merlin (full length), and C-ter Merlin (330-595) but absent in Merlin (1-490) and GFP control. In the end three proteins only were identified that bind specifically to the region between amino acids 480 to 595: Angiomotin (Amot), Angiomotin-like 1 (Amotl1) and Angiomotin- like 2 (AmotL2) - (Angiomotin family proteins). We focused on the characterization of Merlin-Angiomotin family protein interactions as two Angiomotin family proteins (Amot, AmotL1) exhibited the highest peptide match numbers in our purified Merlin-containing complexes. Before getting to the investigation we performed of the function of Merlin/AMOTs interactions, let me give you a structural and function insights of AMOT family proteins from the literature.

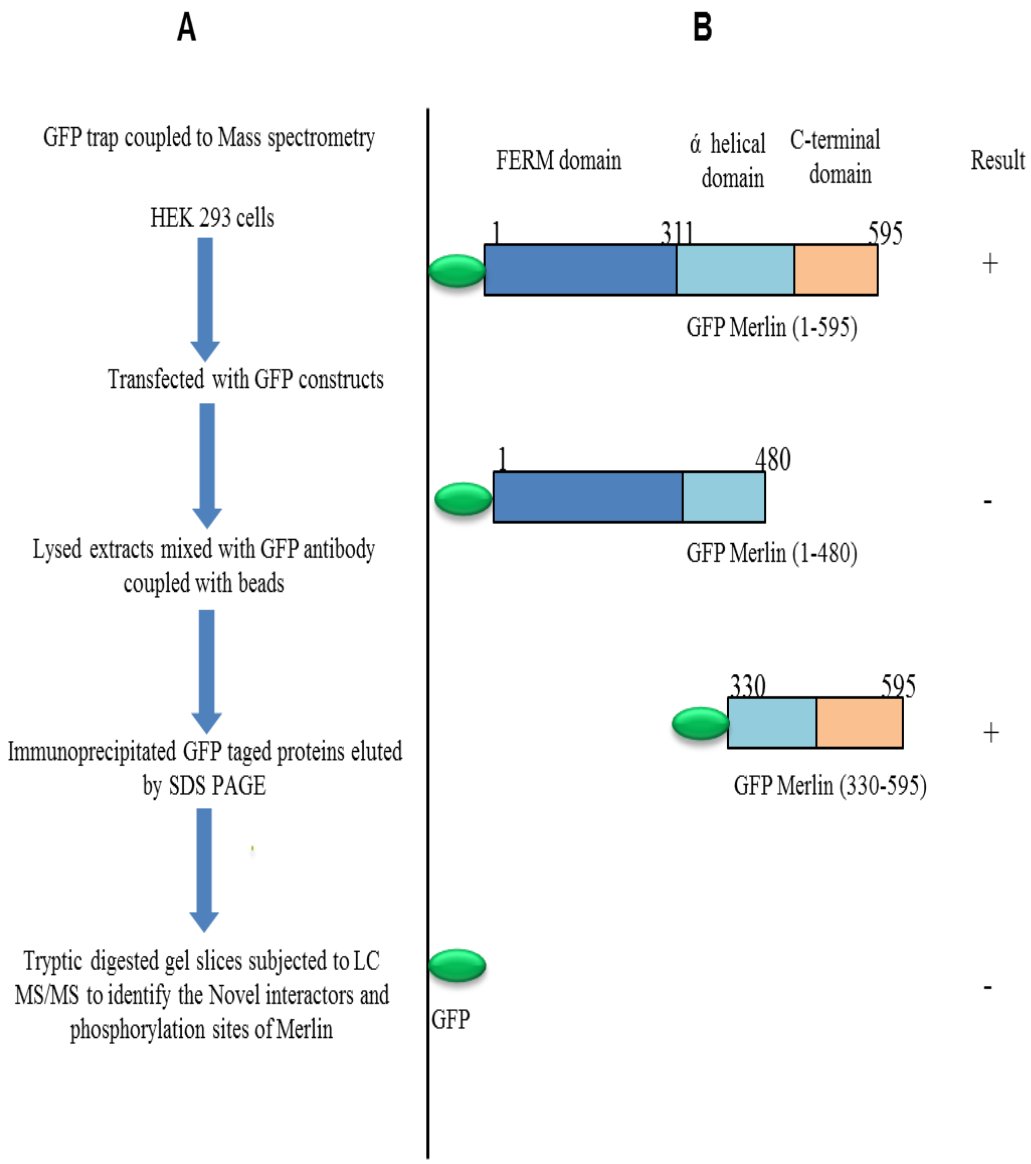


Figure 2. A. Experimental strategy to fish for novel interacting partners of C-terminus Merlin. B. Various GFP tagged constructs used in our study to search for new interacting partners of C-terminal Merlin. Result indicates the criteria for the new interactor of Merlin in which we are interested (+).

2.3 AMOT family proteins

In vertebrates, sprouting is involved in the formation of new blood vessels from the existing ones and this process is called angiogenesis. This phenomenon involves several steps:

proliferation of endothelial cells, modification of the extracellular matrix, cell migration and morphogenesis. Angiogenesis is a vital function for the growth of normal tissues during embryogenesis as well as for the pathological growth of tumors (Folkman, 1995; Hanahan and Folkman, 1996). So, tight balance between specific growth factors and inhibitors is necessary during this process. One inhibitor that was shown to suppress angiogenesis in mice is angiostatin (O'Reilly et al., 1994, 1996). It is the proteolytically cleaved product of the first four Kringle domains of plasminogen. Angiostatin plays an important role in the inhibition of endothelial cell proliferation, migration (Claesson-Welsh et al., 1998; Ji et al., 1998), and also apoptosis. Angiostatin has been shown to inhibit endothelial cell invasion and tube formation in 3D angiogenesis assays (Barendsz-Janson et al., 1998; Griscelli et al., 1998). However the mechanism through which angiostatin inhibits the angiogenesis is not fully understood.

In 2001, it has been shown that Angiomotin (AMOT= motility in Latin) mediates the inhibitory effect of angiostatin on endothelial cell migration and tube formation in vitro. Amot was first identified as angiostatin-binding peptides in a two-hybrid screen from placental cDNA library (Troyanovsky et al., 2001). Hence, Amot is a receptor. At the tissue level, it is expressed in brain, skeletal muscle and placenta and at the cellular levels it is expressed in endothelial cells of the larger vessels and in the capillaries of the placenta. In the absence of angiostatin, or when it is overexpressed, Amot promotes cell migration by localizing at the leading edge of endothelial cells *in vivo* and *in vitro* which is consistent with its role in angiogenesis (Levchenko et al., 2003; Troyanovsky et al., 2001).

2.3.1 Structure of Amot family proteins

Human Amot is composed of 675 residues. The AMOT family has three paralogs in humans and mice: Amot, AmotL1, and AmotL2. All these share similar domain organization, including two PPXY motifs in the N-terminal half, a coiled-coil domain, and a C-terminal PDZ-binding motif (Bratt et al., 2002; Ernkvist et al., 2006) (**Fig 3A**). An actin binding domain is also present between the second PPXY motif and the coiled-coiled domain. PPXY domains are important to target the proteins to specialized locations by interacting with WW domain containing proteins such as MAGI (membrane- associated guanylate kinase with inverted domain), a component of endothelial tight junctions (Patrie, 2005). AMOT family proteins are localized to tight junction through interaction with Patj-Pals and MUPP1 (multi-

PDZ domain protein 1) proteins and this interaction is mediated by their PDZ domain. In addition, Amot was shown to bind to Rich1, a Cdc42 RhoGAP and involved in maintaining cell polarity. In contrast, AmotL1 and AmotL2 do not bind to Rich1 (Bratt et al., 2005; Sugihara-Mizuno et al., 2007; Wells et al., 2006). The major difference among AMOT family proteins is that Amot contains angiostatin binding domain and a transmembrane binding domain, whereas Amotl1 and Amotl2 do not. Therefore, only Amot can mediate the angiostatin action although all the AMOTs are involved in endothelial cell biology, which in turn suggests that AMOT family proteins may harbor non redundant functions although probably nobody focused on this aspect (Bratt et al., 2002).

Due to alternative splicing there are many isoforms within each group of AMOT family members. Amot has two isoforms, a short P80 form (P80) and long P130 form (P130). P80 do not have PPXY motif at its N-terminus whereas P180 have a long N-terminus region with two PPXY motifs (Ernkvist et al., 2006). Similarly AmotL1 exists in two isoforms; short p90 from lacking PPXY motif and long p100 from (Moreau et al., 2005; Zheng et al., 2009). In addition, the short forms lack the actin binding domain and cannot directly bind to actin filaments. AMOT family proteins are well conserved in evolution and they are present in Xenopus laevis, the puffer fish Fugu rubripes and the nematode Caenorhabditis elegans. Further phylogenetic analysis indicates that AmotL2 is an evolutionary out groups in relation to Angiomotin and AmotL1.

2.3.2 AMOT family protein functions

The tight control of endothelial cell (EC) migration, proliferation, and polarization play crucial roles in establishing a functional blood vessel network. Angiostatin is important for the regulation of these processes by inhibiting the Angiomotin (AMOT) mediated cell migration. As we discussed, Amot is exist in p80 and p130 forms. With respect to their functions, p80 enhances cell migration and stabilizes tubes in vitro, whereas p130-Amot associates with actin cytoskeleton and affects cell shape (Ernkvist et al., 2006; Levchenko et al., 2004). Furthermore, one study showed that the ratio of p80 to p130 expression in endothelial cells regulates a switch from migratory to more stable non-motile cells (Ernkvist et al., 2008). However the mechanism by which Angiomotin proteins regulate cell migration is largely unknown. Several studies have shown that Amot is important for the epithelial cell

polarity and tight-junction integrity by acting as a scaffold protein at tight junctions to mediate the interaction between Rich1, Pals1, Patj, and Par-3 and by recruiting ZO-1 and MAG-1b to tight junctions (Bratt et al., 2005; Wells et al., 2006). In this context recently it has been shown that polarity complex, Patj: Pals1 connects Amot to the RhoGEF Syx and this ternary complex regulates RhoA activity at the leading edge of migrating cells. Interestingly, in this study authors have shown that knockdown of Amot or Syx results in inhibition of migration of intersegmental vessels during zebra fish angiogenesis suggesting a strong role of Amot in cell migration in vitro and in vivo. In addition, recently it has been shown that Amot localizes to endocytic recycling compartment via its coiled-coiled lipid binding domain allowing it to redistribute the protein complexes such as Pat J and Mupp1 to endosome and which in turn is important for the maintenance of cell polarity (Ernkvist et al., 2009). Genetic studies in mice and zebra fish demonstrated that Amot is essential for embryogenesis and development as Amot knockout mice die between embryonic days 7 (E7) and E11 of a general defect in vessels formation. From the pathological point of view, elevated levels of Amot were observed in many diseases including Kaposi's sarcoma (Troyanovsky et al., 2001) and breast tumors tissues (Jiang et al., 2006) and is linked to angiogenesis. Finally, DNA vaccines and antibodies that neutralize the pro-migratory activity of Amot were shown to inhibit angiogenesis in xenografts tumors and appear to constitute potential therapeutic approaches for the inhibition of tumor growth in vivo (Ernkvist et al., 2006; Levchenko et al., 2008).

Although AmotL1 and AmotL2 are structurally similar to Amot, their functions are less well understood. AmotL1 and Amot L2 also localize to tight-junctions of epithelial cells and interact with several tight-junction associated proteins such as MAGI-1 and MUPP1 via their LPXY motif and PDZ-binding domain, respectively. Like Amot, endogenous Amotl-1 and Amotl-2 localize to punctate structures in the cytoplasm of cultured epithelial cells (Sugihara-Mizuno et al., 2007). Amotl-2 plays a role in cell migration by promoting the membrane translocation of activated c-Src (Huang et al., 2007). However, the precise role of AmotL1 in cell migration remains to be determined. In this context, one study demonstrated that AmotL1 rescues the phenotype caused by the inactivation of Amot on intersegmental vessels development in zebra fish, suggesting the functional redundancy among AMOT family proteins (Aase et al., 2007). *In vivo,* Amot appears to control the polarity of vascular tip cells whereas AmotL1 mainly affects the stability of cell– cell junctions of the stalk cells (**Fig 3B**)

(Zheng et al., 2009). Also, AmotL1 forms complexes with Amot80 through which AmotL1 not only co-localizes with filamentous actin but also significantly modifies the architecture of the actin cytoskeleton. In relation to migration, Angiomotin-like 1 is known to increases the velocity of migration and decreases the persistence of migration directionality (Gagné et al., 2009). However, the underlying mechanisms of the role of AmotL1 in cell migration remain elusive.

Having identified interactions between Merlin and AMOT family of proteins in our mass spectrometry experiments, we focused on the importance of this interaction for the tumor suppressor function of the former and more generally for cancer development.

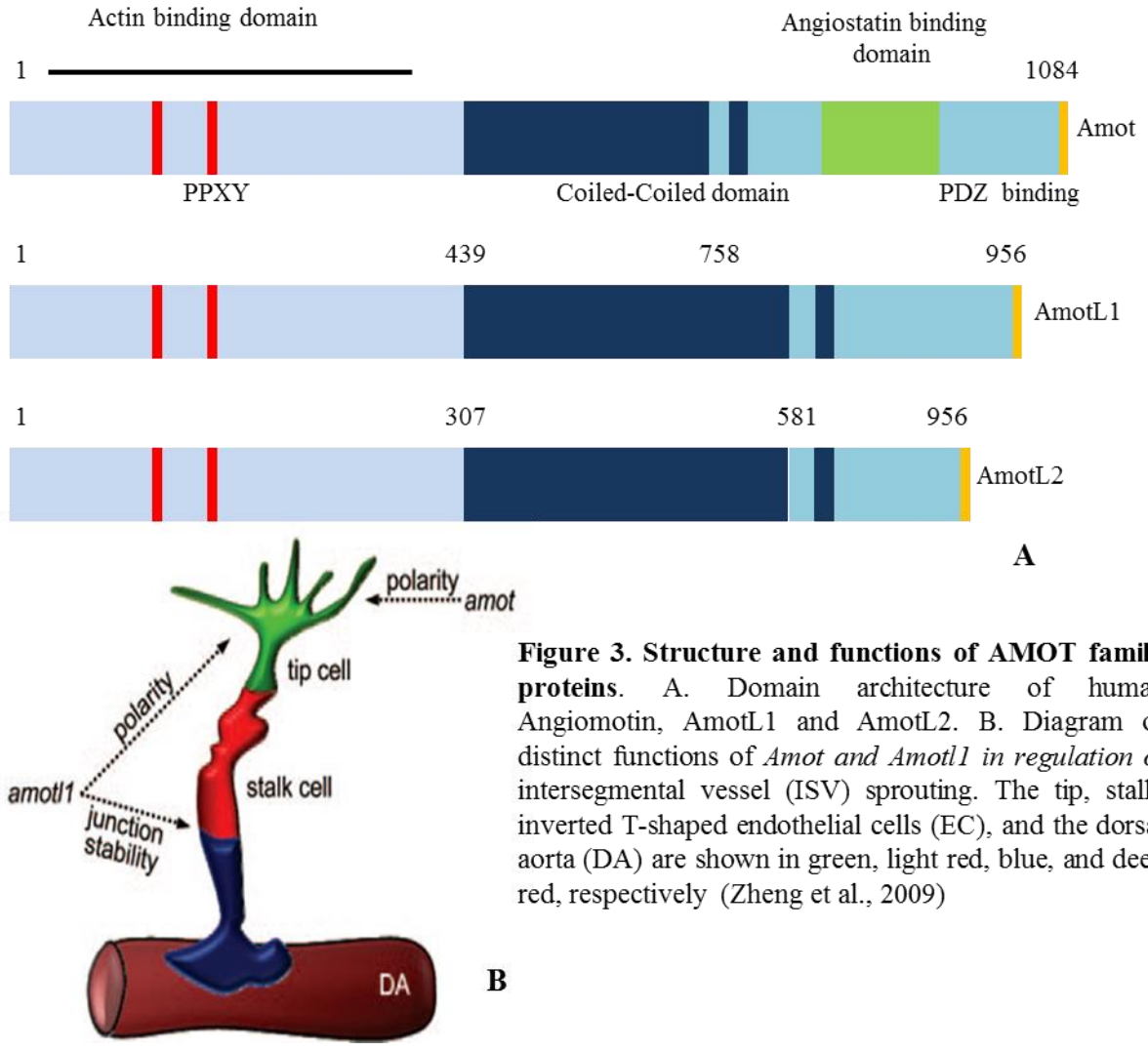


Figure 3. Structure and functions of AMOT family proteins. A. Domain architecture of human Angiomotin, AmotL1 and AmotL2. B. Diagram of distinct functions of *Amot and Amotl1 in regulation of* intersegmental vessel (ISV) sprouting. The tip, stalk, inverted T-shaped endothelial cells (EC), and the dorsal aorta (DA) are shown in green, light red, blue, and deep red, respectively (Zheng et al., 2009)

2.4 Merlin interacts with AMOT family proteins through its coil-coiled domain.

In our initial western blot experiments on AMOT family proteins, we found that HEK293 cells express significant amounts of Amot, AmotL1 at endogenous level. We did not pursue much on AmotL2 due to lack of good antibody against this protein at the time. To confirm the interaction between Merlin and Amot, we transfected GFP tagged Merlin constructs such as GFP-Merlin full length, GFP-Merlin 1-480, GFP-C-ter Merlin 330-595 in HEK293 cells, then performed IP using anti GFP antibody and looked for co-Immunoprecipitated Amot or AmotL1 by western blot. As we have shown previously through mass spectrometry, Merlin C-terminus 330-595 as well as full length protein binds to Amot, but not other shorter constructs (**Fig 4A**). In addition, we confirmed that Merlin specifically interacts with Amot under physiological condition hence endogenous Merlin immunoprecipitation using a specific antibody, but not a control IgG, pulled down endogenous Amot from HEK293 cells. Altogether indicates that Amot selectively binds to Merlin *in vivo* (**Fig 4B**).

To further map the Amot binding domain within Merlin precisely, we used GFP tagged C-terminus deletion constructs of Merlin harboring progressive sequential deletion of twenty amino acids from the C-terminus (**Fig 5A**). We transiently transfected the HEK 293 cell with the different GFP tagged C-terminus deletion fragments of Merlin and immunoprecipitated them with GFP-trap in order to pull down the Amot family proteins. These experiments showed that GFP-Merlin 330–555 and GFP-Merlin 330–575 interact with the Amot proteins, Amot and AmotL1 (**Fig 5B**). In contrast, GFP-Merlin 330-495 and GFP-Merlin 330-515 and GFP-Merlin 330-535 failed to bind to Amot and AmotL1 (**Fig. 5B**). Collectively, these results suggest that Amot and Amot L1 binding domain on Merlin lies between amino acid 535-575 of the C-terminus domain of Merlin. Very interestingly, AMOT proteins binding domain on Merlin exactly matches with the C-terminus domain of Merlin which is required for its growth suppressive function in SC (Lallemand et al., 2009a). This observation supports the idea that Merlin may exert its tumor suppressor function via its interaction with the AMOT proteins.

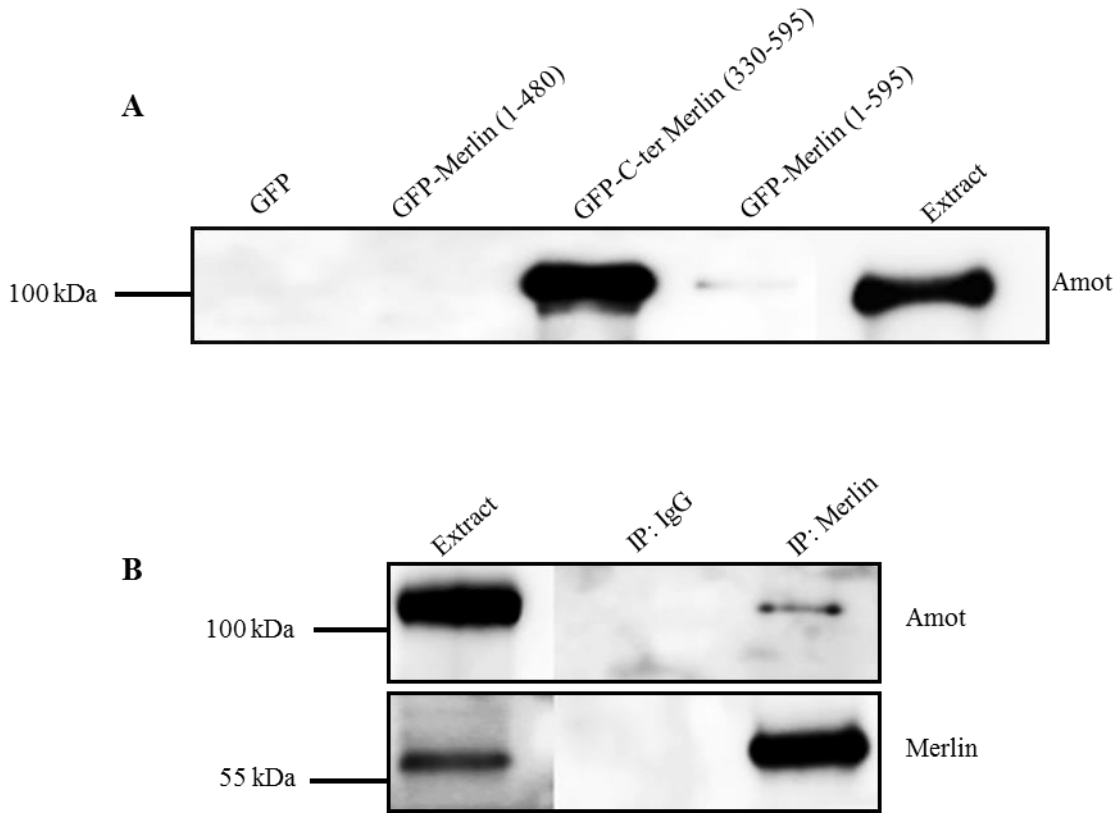


Figure 4. Merlin interacts with Amot via its C-terminus. A. HEK293T cells were transfected with the indicated constructs (GFP, GFP-Merlin (1-480), GFP-CTM (330-595) and GFP-Merlin (full length)) and lysates were immunoprecipitated with GFP antibody. Co-IP of Amot is analyzed by immunoblot using the specific antibody against Amot. B. Immunoprecipitation (IP) was performed using IgG control or Merlin antibody and western blotted for endogenous Amot and Merlin with specific antibodies.

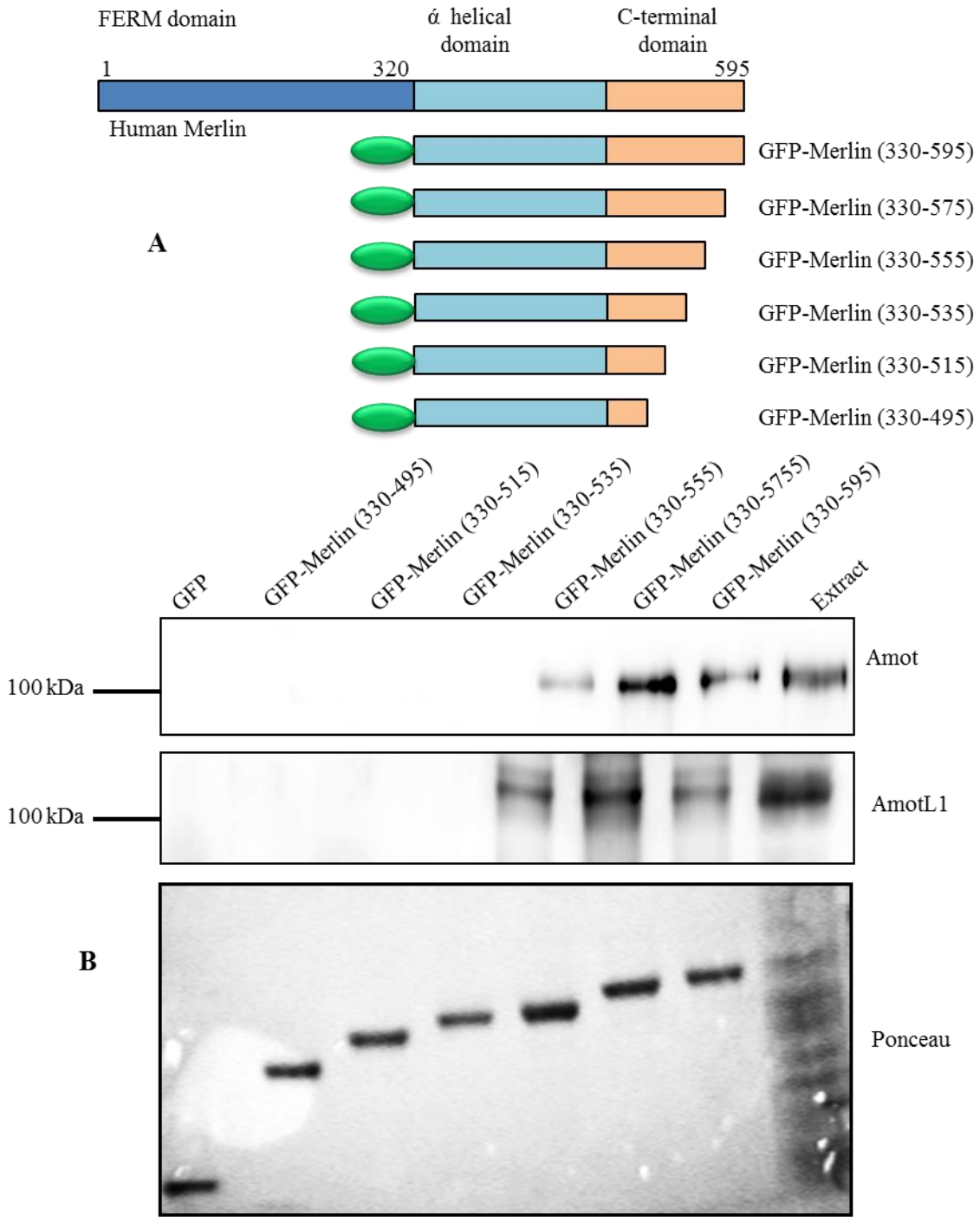


Figure 5. Mapping of Amot and AmotL1 binding sites on Merlin. A. Schematic representation of C-ter Merlin constructs used in the study. B. HEK 293 cells were transfected with indicated GFP-tagged Merlin constructs. Immunoprecipitation (IP) of GFP was performed from lysates using anti GFP antibody. Analyzed for Amot (Upper panel) and AmotL1 (middle panel) by western blot using specific antibodies. Lower panel; Ponceau of corresponding western blot showing the loadings of the different Merlin constructs.

2.5 Merlin phosphorylation (S518) does not affect its interaction with Amot and AmotL1

Many studies have demonstrated that phosphorylation of Merlin at serine 518 abolishes its growth suppressive activity (Kissil et al., 2002; Okada et al., 2005). Furthermore, hyperphosphorylated form of Merlin on S518 fails to bind interacting partners such as CD44 and HRS (Morrison et al., 2001b; Rong et al., 2004b). When we compared the binding capacity of S518D and S518A mutants of full length as well as C-terminus Merlin to Amot and AmotL1 by co-IP, we found that both mutants retained the capacity to bind to Amot proteins equivalently. However in this experiment, we observed that Merlin C-ter domain fused to GFP binds better to Amot and AmotL1 than the GFP fusion to full length Merlin. We believe that this might be due to the more open conformation of C-terminus Merlin, not masked by the FERM domain (**Fig 6A and B**).

It has been shown that Merlin exists as two major isoforms in the cell that bind with distinct affinities to molecular partners (Ramesh, 2004). In this context, we tested whether the two isoforms display differential binding capacities to Amot and AmotL1. To answer this question, we immunoprecipitated GFP-tagged full length Merlin isoform 1 (M17) and Merlin isoform 2 (M16), and looked for bound Amot and AmotL1 by western blot. Again, we found that both isoforms of Merlin bind equally to Amot or AmotL1 (**Fig 6A and B**). Collectively our results indicate that inhibition of Merlin tumor suppressor function by phosphorylation is not mediated by loss of contact with Amot or AmotL1.

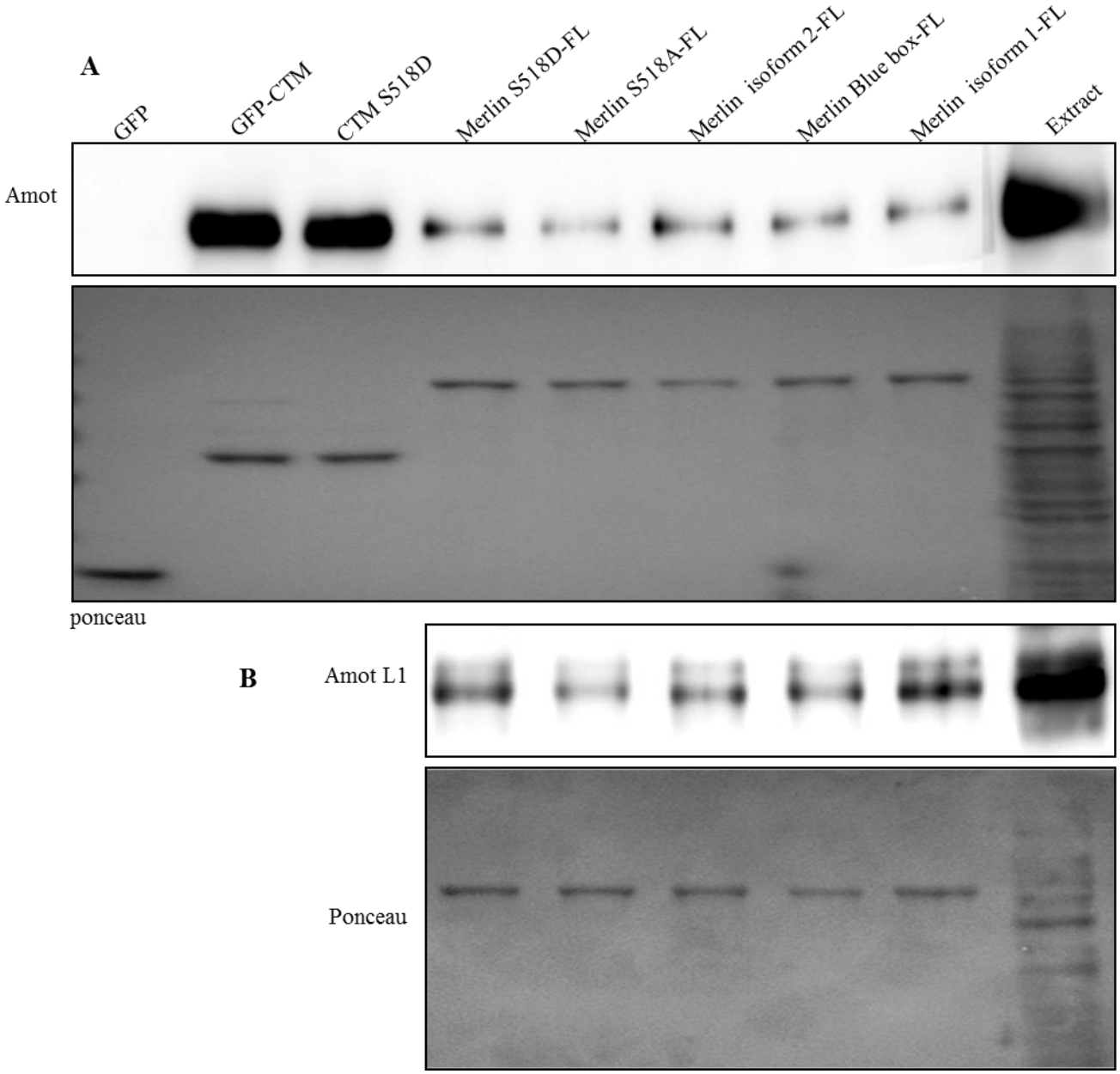


Figure 6. Phosphorylation of Merlin at S518 does not alter binding of Merlin to Amot and AmotL1. A. HEK293 cells were cotransfected with indicated GFP-tagged Merlin constructs and IP was performed with GFP specific antibody. Bound proteins were then analyzed by western blot using an anti-Amot antibody. Lower panel showing the expression of GFP-tagged Merlin constructs by Ponceau. B. Experiment carried out same as in A, but bound protein AmotL1 was detected by western blot using anti-AmotL1 antibody. Lower panel showing the expression of GFP-tagged merlin constructs by Ponceau. CTM=GFP-C-ter Merlin; FL=Full length.

2.6 Merlin binds to the coiled-coiled domain of AmotL1

We have shown that Amot and AmotL1 bind to coiled-coiled domain (535-575) of Merlin. We then set to map the interaction domain of Merlin on AmotL1. We generated constructs of GFP-AmotL1 with progressive C-terminal deletions (**Fig 7A**). We observed that AmotL1 constructs were mostly insoluble as we barely saw them by ponceau after immunoprecipitation, except for the shortest one, GFP-AmotL1 (1-429) construct and despite high levels of expression as assessed by fluorescence microscopy. This may indicates that the coiled-coiled domain of AmotL1 might target the protein to specific cellular domains that renders it less extractible. We immunoprecipitated AmotL1 constructs and looked for co-immunoprecipitation (co-IP) of Merlin by western blot. By doing so, we observed that Merlin could interact with GFP-AmotL1 1-565, GFP-AmotL1 1-720 and GFP-AmotL1 1-765 but not to GFP-AmotL1 1-429 (**Fig 7B**). This result indicates that Merlin binds to the coil-coiled domain of AmotL1. Collectively our results demonstrated that Merlin and AmotL1 bind to each other through their coil-coiled domain.

While we are mapping the interacting domain of Merlin on AmotL1, we also observed that AmotL1 and Amot are able to form heterodimers. In this experiments we transfected the HEK 293 cells with various deletion constructs of AmotL1 and checked for the co-IP of Amot by western blot after performing the IP of GFP tagged AmotL1. Interestingly, we found that Amot binds to the same domain on AmotL1 where Merlin binds (**Fig 7B and C**). It would be very interesting to check whether Merlin inhibits the heterodimerization of AMOT family proteins in the cell and whether this leads to modification of AMOT family proteins functions or distribution in the cell.

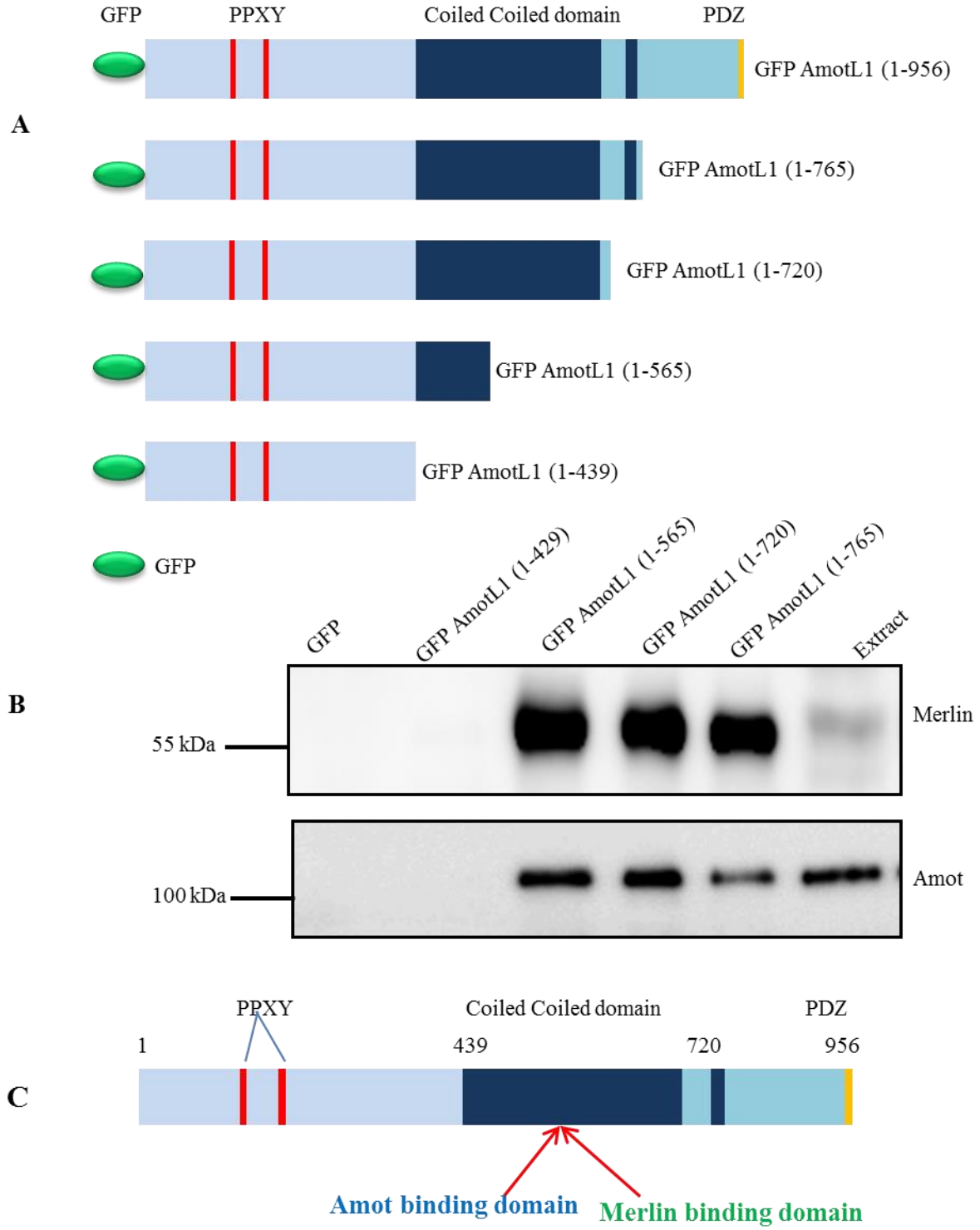


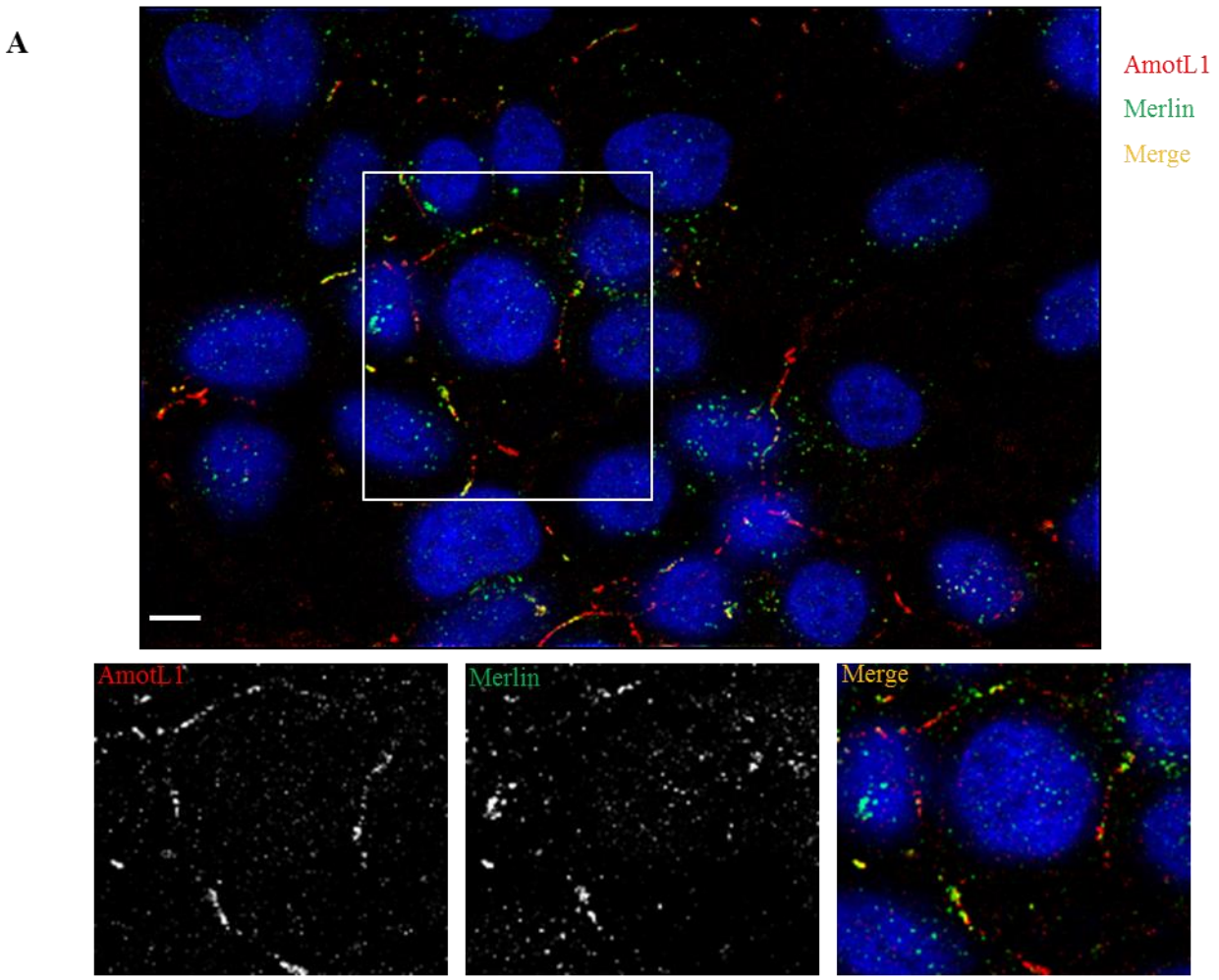
Figure 7. Merlin and Amot bind to coiled-coiled domain of AmotL1. A. Schematic representation of the human AmotL1 deletions constructs used in this study. B. Western blot analysis with Merlin (upper panel) and Amot (lower panel) antibodies after performing the immunoprecipitation of GFP tagged AmotL1 C-terminus deletion constructs from HEK cells transfected with indicated constructs. C. Scheme showing Merlin and Amot binding sites on AmotL1 C-terminus domain.

2.7 Merlin and AmotL1 co-localize at plasma membrane as well as in the cytosol

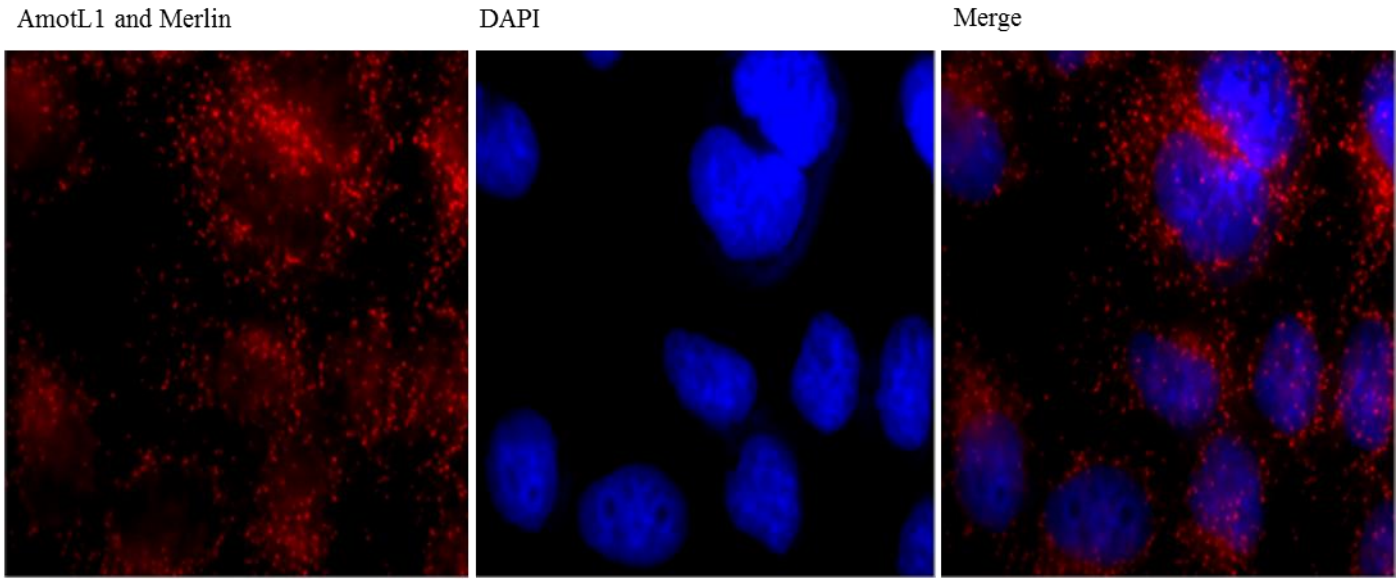
Many studies described that Merlin (Lallemand et al., 2003) and AmotL1 (Zheng et al., 2009) 2009) localize to cell-cell contacts. To test where in cells Merlin and AmotL1 might co-localize, we used two experimental strategies: 1. by immunofluorescence, we investigated the cellular distribution of endogenous Merlin and AmotL1 in HEK 293 cells that express high levels of AmotL1. 3D microscopy was used to visualize the proteins and we found that Merlin and AmotL1 indeed co-localize at the cell-cell contacts suggesting a possible role for the two proteins in the regulation of cell junctions (**Fig 8A**). In addition, we ectopically overexpressed Merlin and AmotL1 in Hela cells to evaluate their co-localization. We again employed 3D microscopy and confirmed the co-localization of the two proteins. By doing so, we observed that these overexpressed proteins were localizing frequently in punctuate structures in cytosol (**Fig 8B**). Taken together these results suggest that AmotL1 and Merlin co-localize both at plasma membrane and cytosol. However, co-localization in the cells does not mean necessarily that the two partners interact at this location. To tackle this question, we used a very sensitive imaging technique called Duolink. This is a PCR based imaging technique allowing the visualization of two proteins when they are closer than 30nm distance. Hence, the two proteins either interact or are in the same complex. In this experiment, we used Hela cells expressing endogenous AmotL1 and Merlin. In this cell line we carried out the Duolink experiment using Merlin and AmotL1 antibodies from different species. We found that these two proteins are co-localized in the cells at endogenous level (**Fig 8C**). Co-localization was seen in the cytoplasm mainly but not clearly at the membrane. This might be due differences in the dynamic or the nature of junctions between Hela and HEK 293 cells. Taken together our results show that AmotL1 and Merlin colocalize in the cells in various subcellular compartments.

Figure 8. Merlin and AmotL1 co-localize at plasma membrane and as well as in the cytosol. A. Immunofluorescence (IF) analysis of endogenous Merlin (green) and AmotL1 (red) in HEK 293 cells. 3D microscopy images were taken at 100X magnification. The lower panels represent an enlargement of the region delimited by a square in the upper image. B. Hela cells were transfected with Merlin and GFP-AmotL1. IF staining of Merlin (red) and AmotL1 (green) carried out and 3D microscopy pictures were taken at 100X. C. IF analysis of endogenous Merlin and AmotL1 colocalization by Duolink. Images were acquired by 2D epi-Florescence microscopy. Scale bar =10 um. Red = Duolink signal and blue = DAPI

Figure 8. Merlin and AmotL1 co-localize at plasma membrane and as well as in the cytosol

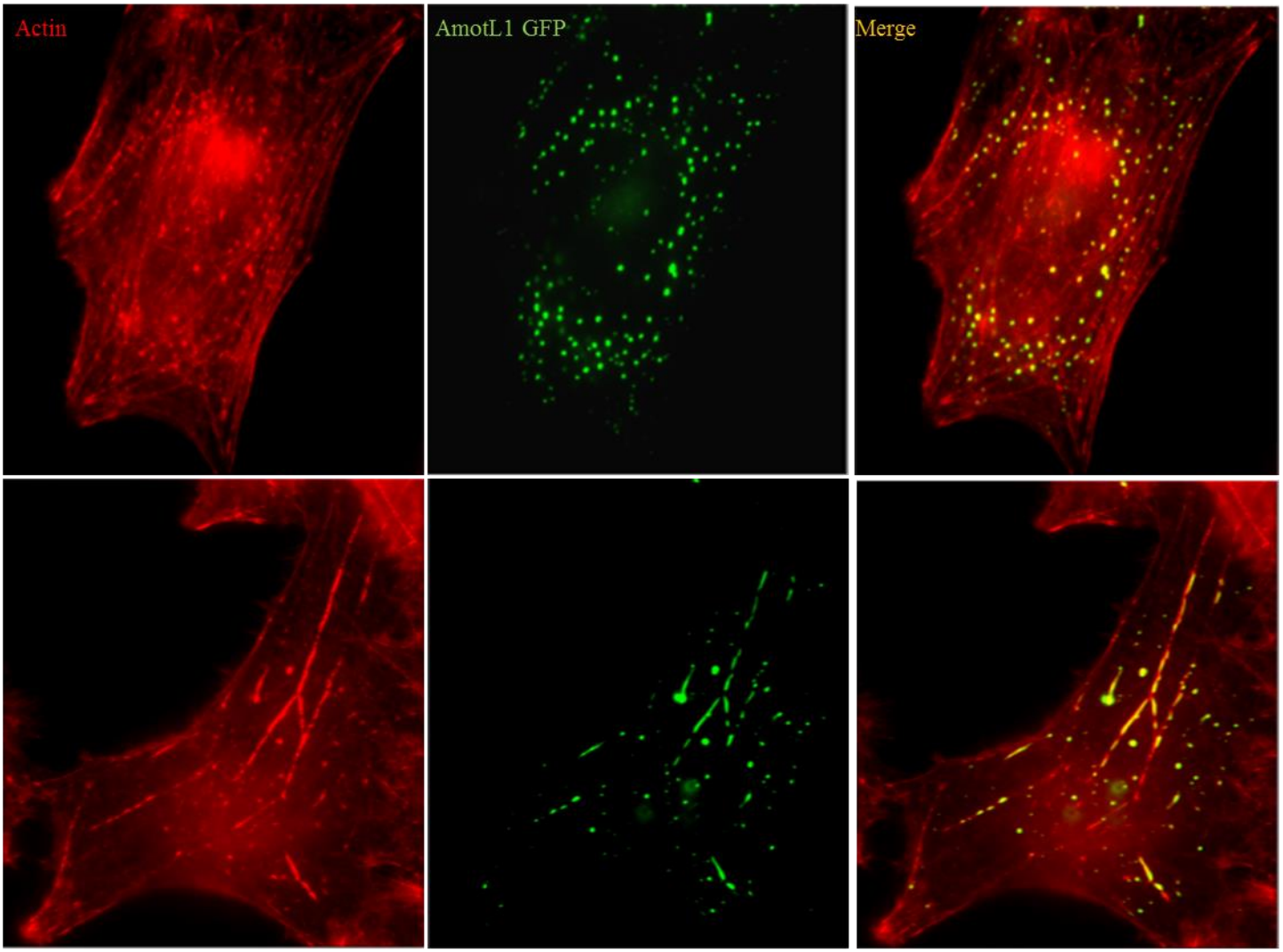


C



When we over expressed GFP-AmotL1 in Hela cells, interestingly we observed the formation of long filaments and these filaments evoked actin cables. Since AmotL1 is known to bind actin, we further investigated the potential link between AmotL1 and filaments. By overexpressing AmotL1 in Hela cells and staining with fluorescent phalloidin, we confirmed that AmotL1 is decorating actin filaments (**Fig 9A lower).** Interestingly, the overexpression of AmotL1 leads to the formation of very thick actin cables. In addition, AmotL1 not only co-localizes with actin filaments but also is present in actin rich punctate-like structures (**Fig 9A upper**). Collectively, our results further support the idea that AMOT family proteins not only localize to actin filaments but also modify the architecture of cytoskeleton and trigger the formation of thick actin cables in the cells (Gagné et al., 2009). To exclude the nonspecific effect of the GFP fused protein, we have generated a stable cell line expressing untagged AmotL1 under the control of the tet inducible promoter that can be induced by addition of doxycycline in the culture media. Using this cell line, we overexpressed wild-type AmotL1 and found that it still generate the formation of thick actin cables (data not shown). When we overexpressed Merlin by transient transfection in Hela cells we found that it also co-localized with AmotL1 on actin filaments (**Fig 9B**). Hence AmotL1 likely remodels the actin cytoskeleton and recruits Merlin to actin cables where it is not naturally visible. In the laboratory, we are currently investigating the role of the interaction between Merlin and AmotL1 in the formation of actin cables and the remodeling of the cytoskeleton.

A



B

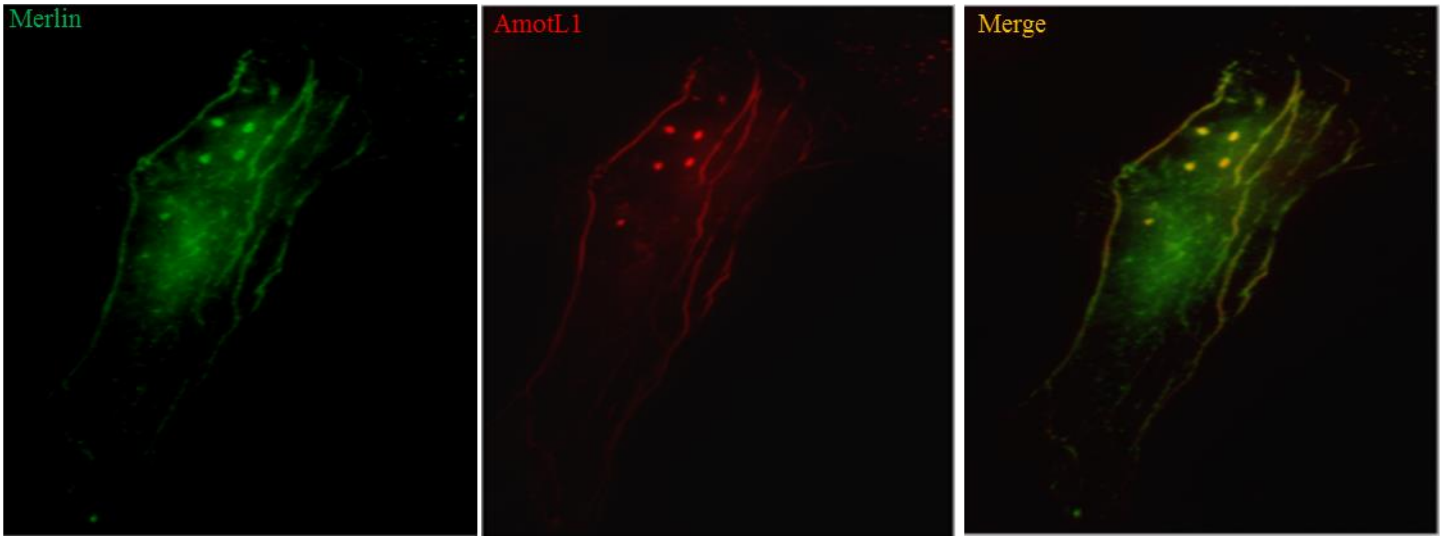


Figure 9. Amotl-1 co-localizes with Merlin on actin filaments. A. Hela cells were transfected with GFP-AmotL1 cells and then immunofluorescence (IF) was performed for AmotL1 (green) and actin (red). Images were taken using 3D microscopy at 100X. The upper panel shows the localization AmotL1 on punctuate structure of actin. The lower panel shows the localization of AmotL1 on actin cables. B. Hela tet-on cells overexpressing AmotL1 were transfected with Merlin expression vector and IF was performed for AmotL1 (red) and Merlin (green) showing co-localization.

Many studies described the role of Amot in cell migration by regulating the cytoskeleton organization and cell shape (Bratt et al., 2005; Gagné et al., 2009; Levchenko et al., 2004; Wells et al., 2006). In addition, as we discussed before, it has been shown that Amot levels are up-regulated in some cancer syndromes that include Kaposi's sarcoma and breast tumors. (Jiang et al., 2006; Troyanovsky et al., 2001). In contrast, AmotL1 was shown to localize at leading edge of cell and may play important role in cell migration. However, in comparison to Amot, the mechanisms involved in the cell motility/migration regulation by AmotL1 remain to be elucidated. In addition, whether AmotL1 plays a role in cancer development is not known. In this context, we decide to study the role of AmotL1 in tumorogenesis in relation with Merlin as they are strong binding partners in our study.

2.8 Merlin affects AmotL1 functions at different levels

Concerning the role of AmotL1 in cancer, Christophe Couderc, postdoc in the lab, observed by western-blot that in 14 out of 16 breast cancer cell lines, there is an opposite pattern of expression levels between Merlin and AmotL1 protein **(Fig 10A).** Hence cells expressing a low level of Merlin (including MDA-MB-436, MCF-10A and MCF-12A) displayed a higher level of AmotL1, whereas cells with a high level of Merlin expressed a lower level of AmotL1 (including HCC1143, MDA-MB-468 and BC52). These results suggest that there might be a cross-regulation between Merlin and AmotL1. We then investigated the interplay between Merlin and AmotL1. To do so, first we inhibited the expression of Merlin by two different shRNA in MDA-MB-468 and BC52 breast cancer cell lines and analyzed the expression levels of AmotL1 by western blot. We found that Merlin knockdown lead to increased expression levels of AmotL1 (**Fig 10B**). In contrast, overexpression of AmotL1 did not affect Merlin levels (data not shown). Collectively our results indicate that Merlin down-regulate the expression levels of AmotL1.

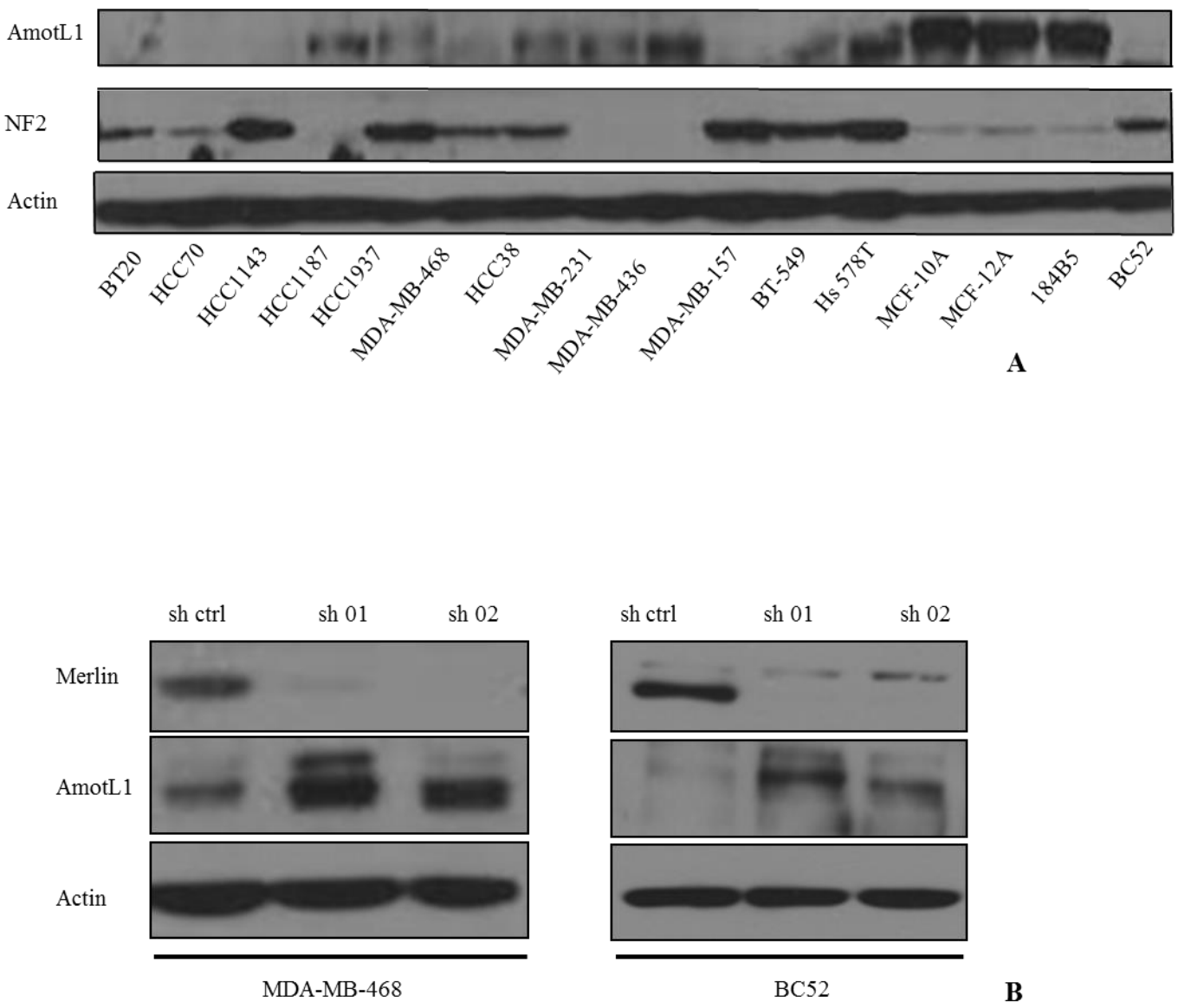


Figure 10. AMOTL1 regulation by Merlin. A. Expression analysis of AmotL1 and Merlin expression levels in a serie of 16 breast cancer cell lines was performed by western blot. The different cell lines are mainly derived from the basal cluster tumors. B. Immunoblot analysis of AmotL1 and Merlin levels in BC52 and MDA-MB-468 cells stably expressing a shRNA control (sh ctrl) or two different shRNAs against Merlin (sh 01 and sh 02). Actin was used as internal control loading.

2.8.1 Merlin regulates AmotL1 expression at transcriptional level

In the next step, we have investigated how Merlin may regulate AmotL1 expression levels, whether it is at the transcriptional level or at the protein level. In order to determine if Merlin regulates AmotLl transcriptional expression, we used RT-qPCR to measure AmotL1 mRNA levels after inhibiting Merlin expression using two different shRNAs in BC52 breast cancer

cell lines. Interestingly, we found that AmotL1 mRNA levels increased by 2 fold upon Merlin inhibition when compared to control scrambled shRNA **(Fig 11A)**. This confirmed that Merlin regulates the expression levels of AmotL1 at the transcriptional level. Then we further investigated the underlying mechanism involved in the regulation of AmotL1 transcript levels by Merlin. It has been shown that Merlin regulates the activity of the co-transcription factors YAP (Yes Associated Protein)/TAZ (transcriptional co-activator with PDZ-binding motif) through the Hippo signaling pathway (Baia et al., 2012; Zhang et al., 2010a). In addition, we observed that AmotL1 promoter regulatory sequences contains a TEAD responsive element (data not shown), suggesting that AmotL1 is a target gene of YAP/TAZ. We then made use of a TEAD luciferase reporter (8xGTIIC-lux) assay to measure the transcriptional of activity of YAP/TAZ and found that inhibition of Merlin by shRNA leads to a 1.8 fold increase of YAP/TAZ transcriptional activity (**Fig 11B)**. Finally, when we inhibited the expression of YAP by using siRNA in BC52 cells depleted for Merlin expression, it resulted in down regulation of AmotL1 transcript levels **(Fig 11C)** as well as decreased AmotL1 protein levels (**Fig 11D)**. Then to evaluate the importance of the link between YAP and AmotL1 in breast cancer, the proteomic and transcriptomic profiles of 250 breast tumors were obtained at Curie Institute. When we analyzed the data, we found a significant positive correlation ($p=0.002237$) (**Fig 12A**) between YAP protein levels and AmotL1 transcript levels suggesting that AmotL1 expression depends on the YAP protein levels in the tumors. Interestingly, this correlation was significant ($p=0.008988$) in tumors expressing low Merlin protein levels (defined as tumors displaying an expression level of Merlin protein below the median of total Merlin levels) whereas it was not significant anymore in tumors with high levels of Merlin (defined as tumors with Merlin level above the median of total Merlin levels) (**Fig 12B and 12C**). These results suggest that Merlin regulates AmotL1 transcriptionnal expression levels by inhibiting Yap activity. Hence, when Merlin levels are high, AmotL1 expression is independent of YAP, which is then inhibited by Merlin. Taken together, these observations demonstrate the strong crosstalk between Merlin, YAP and AmotL1 in breast cancer cell lines, where the loss of Merlin triggers YAP activity which in turn induces Amotl1 expression.

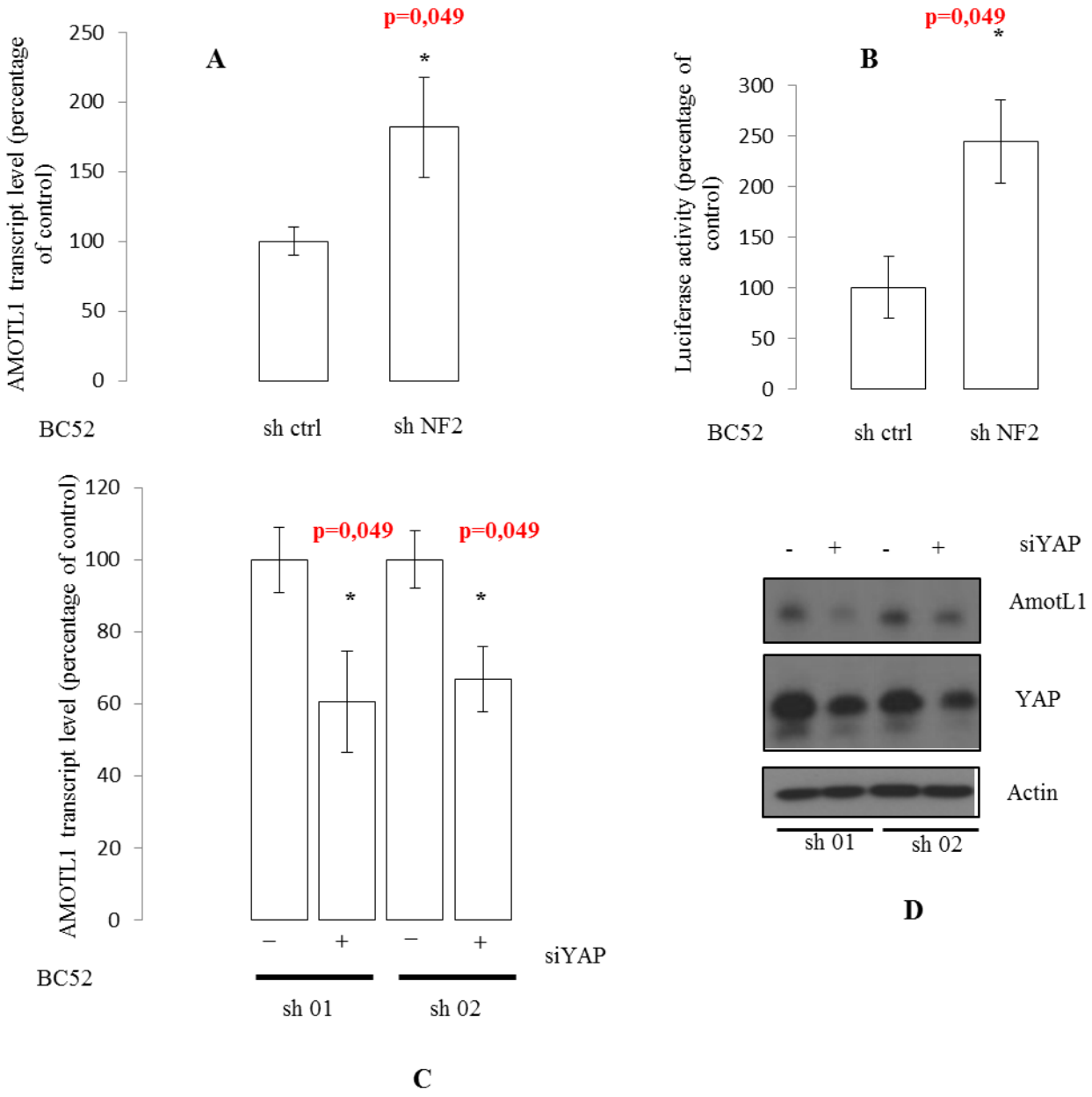


Figure 11. Merlin regulates AmotL1 expression by inhibiting YAP. A. Evaluation of Merlin inhibitory effect on the mRNA level of AmotL1 by real time PCR, using BC52 cells stably expressing shRNA (sh ctrl) control or shRNA against Merlin (sh NF2). B. Evaluation of YAP/TAZ transcriptional activity in BC52 cells expressing shRNA control or shRNA against NF2, using the established YAP/TAZ-responsive reporter 8xGTIIC-lux vector. C. Real time PCR analysis of the AmotL1 transcript level upon siRNA mediated YAP inhibition in BC52 cells stably expressing shRNA control (sh ctrl) of NF2 (sh NF2). D. Immunoblot analysis of AmotL1 and YAP proteins level upon siRNA mediated YAP inhibition in BC52 cells stably expressing shRNA control (sh ctrl) of NF2 (sh NF2).

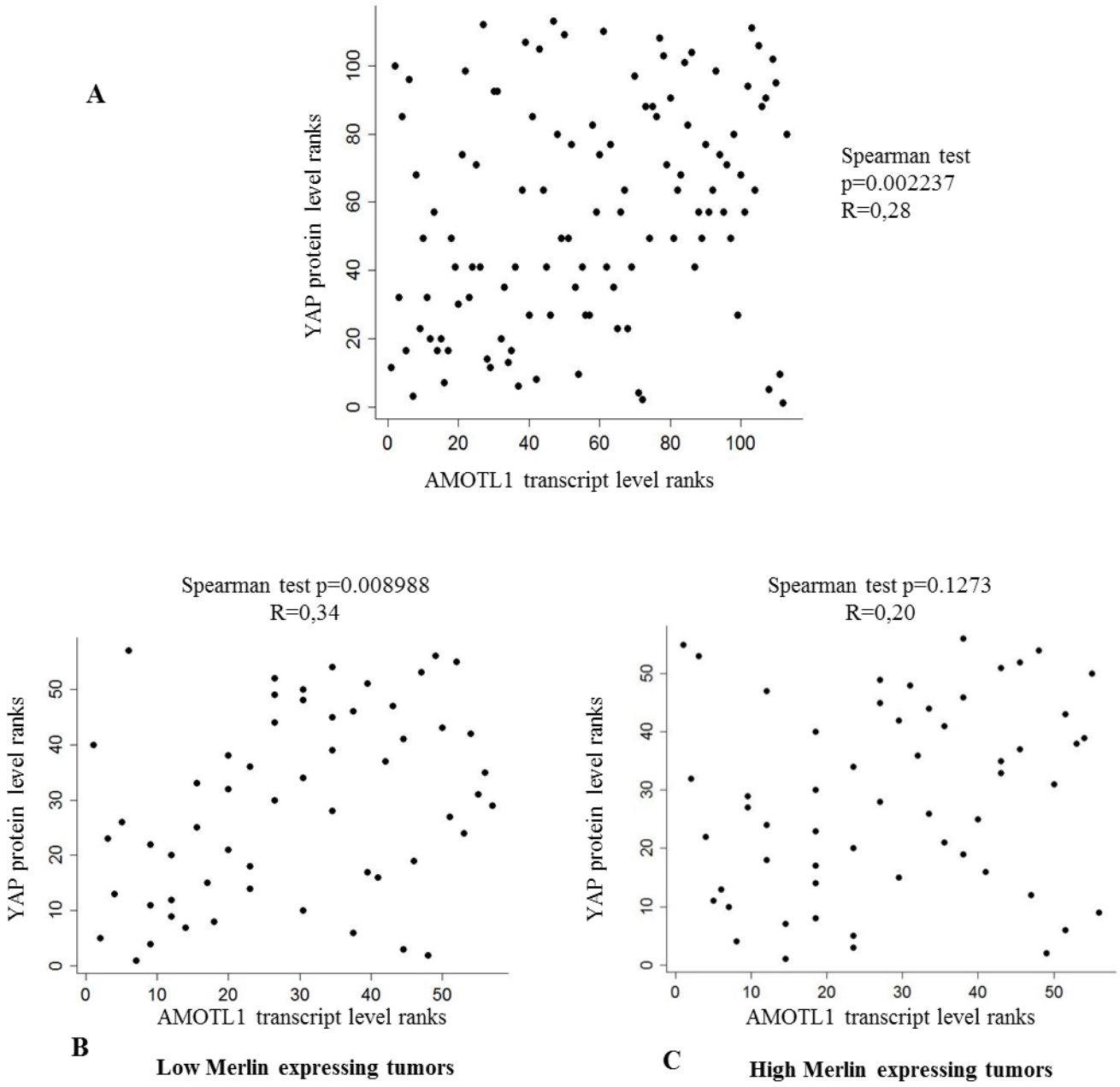


Figure 12. Transcriptional regulation of AmotL1 by YAP. A. Correlation between AmotL1 transcript levels and YAP protein levels given by RPPA (Reverse phase protein array), in 150 tumors. B. Correlation between AmotL1 transcript levels and YAP protein levels given by RPPA, in low Merlin expressing tumors. C. Correlation between AmotL1 transcript levels and YAP protein levels given by RPPA, in low Merlin expressing tumors. Spearman test has been used for each correlation evaluation.

2.8.2 Merlin regulates AmotL1 at protein level

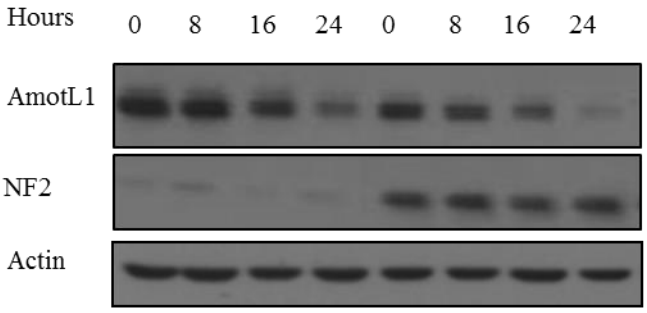
Simultaneously, in an attempt to determine if Merlin also affects AmotL1 protein stability, we checked AmotL1 half-life in presence and absence of overexpressed Merlin. To do so, we transfected BC52 cells with a tet inducible Amotl1 expression vector together with a vector constitutively expressing Merlin or with a GFP expression vector as a control. AmotL1 expression was induced with a short pulse of doxycycline and its decay was followed by western-blot. In this experiment we found that AmotL1 levels decreased more rapidly in the presence of Merlin, with a half-life 40% shorter compared to control (from 1246 +/- 46 minutes to 861 +/-206 minutes) (**Fig 13A)**. To determine the pathway that mediates the degradation of AmotL1 by Merlin, we treated the BC52 cells with proteasome inhibitor MG132 and measured the expression levels of endogenous AmotL1 in presence or absence of Merlin (inhibited by shRNA). After treating the cells with MG132 for 24 hours, we found an increase in AmotL1 expression levels in control cells but not in Merlin expression inhibited BC52 cells (**Fig 13B and 14 A)**. All together our results indicate that AmotL1 degradation is mediated by Merlin through the proteasomal pathway. Having mapped the precise interacting domain of AmotL1 on Merlin, we asked whether the interaction is important for the proteasome mediated degradation of AmotL1 by Merlin. To test this hypothesis, we transfected the BC52 cells overexpressing the AmotL1 with a vector expressing GFP-Merlin or a deleted construct of Merlin (GFP Merlin1-532) which is unable to bind AmotL1. After transfection, we evaluated the proportion of cells with high, medium and low AmotL1 expression as defined in **Fig 14B.** Strikingly, the proportion of high, medium and low AmotL1 expressing cells were very similar in control (GFP alone) and GFP-Merlin 1-532 transfected cells. In contrast, GFP-Merlin transfected cells displayed a much higher proportion of low AmotL1 expressors and a lower percentage of high expressors of AmotL1 (**Fig 13C**). Taken together, our results suggested that that the binding of Merlin to AmotL1 is mandatory for the proteasomal mediated degradation of the later.

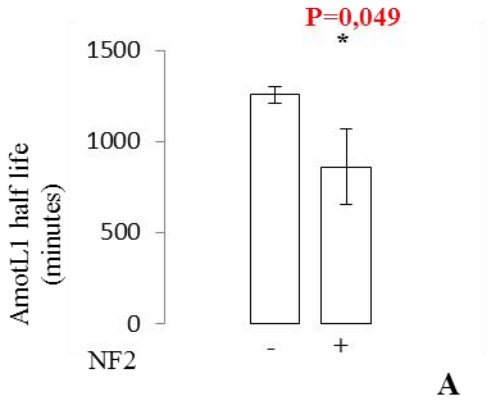
Finally we checked the interplay between Merlin and AmotL1 at the leading edge of the BC52 cells, because it has been shown that AmotL1 localizes at the leading edge of endothelial cells (Zheng et al., 2009). To test this, we transfected the BC52 cells expressing AmotL1 with Merlin GFP vector and we found that over expression of GFP-Merlin led to the

delocalization of AmotL1 away from the leading edge (**Fig 13D**). In a second step we investigated the role of Merlin and AmotL1 interaction in delocalizing the AmotL1 from the migrating front of cells. Thus, we used the truncated form of GFP-Merlin (1-532), unable to bind AmotL1, to check whether the interaction between Merlin and AmotL1 is involved in this delocalization. In cells transfected with GFP alone, the percentage of cells with a strong AmotL1 expression at the leading edge was 69%, whereas in Merlin full length-GFP transfected cells, this percentage dropped to 24% (**Fig 13E**). In contrast, expression of GFP-Merlin 1-532 showed similar percentage for Amotl1 expression at the leading edge (68%) than that of GFP alone (**Fig 13E**). These results indicate that interaction with Merlin is necessary for AmotL1 delocalization. Finally, treatment with MG132 had no impact on AmotL1 delocalization process indicating that local degradation was not the reason for AmotL1 removal from the leading edged by Merlin (**Fig 14C**). In conclusion, AmotL1 function in the cell is tightly controlled by Merlin through three distinct mechanisms, namely the regulation of its expression, protein stability and subcellular localization. For all these functions, the interaction between Merlin and AmotL1 is compulsory, and underlines the importance of the new interactor AmotL1 in Merlin biology.

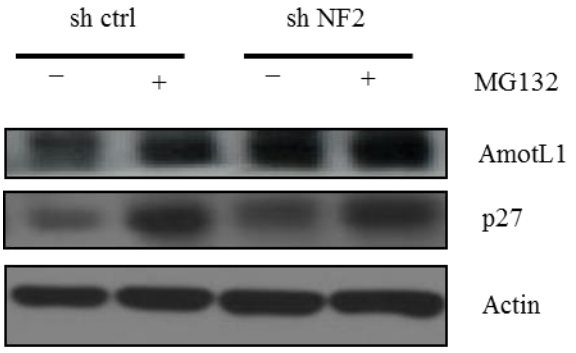
Figure 13. Merlin regulates AmotL1 stability and localization in the cell.

A. BC52 tet on AL1 cells were transfected with Merlin expression vector or control vector and treated during 24h by doxycycline. Then, doxycycline was washed out and AmotL1 levels have been evaluated by immunoblot over time (left panel), at the different time points mentioned, and quantified. The AmotL1 half-life, determined as the time needed to get 50% decrease of the initial amount of AmotL1 (at time=0h), has been calculated based on three independent experiments and the result is represented in a graph (right panel). B. BC52 cells stably expressing either shRNA control or shRNA against AmotL1 were treated overnight by MG132. Effects of MG132 on AmotL1 levels have been evaluated by western blot. p27 protein is used as a positive control of the MG132 efficiency. C. Doxycycline treated BC52 tet on AL1 cells were transfected by GFP-Merlin full-length, GFP-Merlin (1-532) and GFP alone. Intensity of AmotL1 expression (defined as high, medium and low) in transfected cells was evaluated by immunofluorescence. The proportions of cells expressing a high, medium or low level of AmotL1 in the different conditions were represented in a graph. D. Localization of AmotL1 (green) in BC52 tet on AL1 treated with doxycycline and transfected with Merlin full length (red). *: designates the AmotL1 localization at the leading edge in absence of Merlin; §: designates the presence of Merlin at the leading edge and the absence of AmotL1. E. Evaluation of the percentage of doxycycline treated BC52 tet on AmotL1 cells displaying an AmotL1 localization at the leading edge, evaluated by immunofluorescence, when transfected with various constructs: GFP alone, GFP-Merlin full length or GFP-Merlin 532 (n=3).

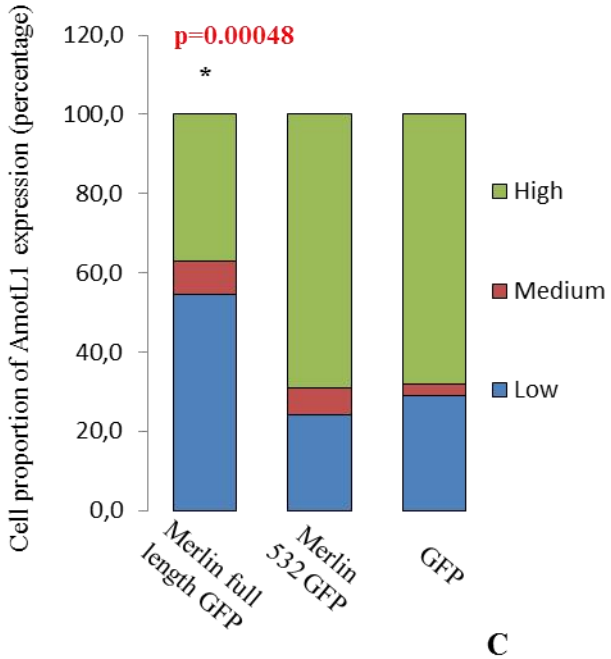

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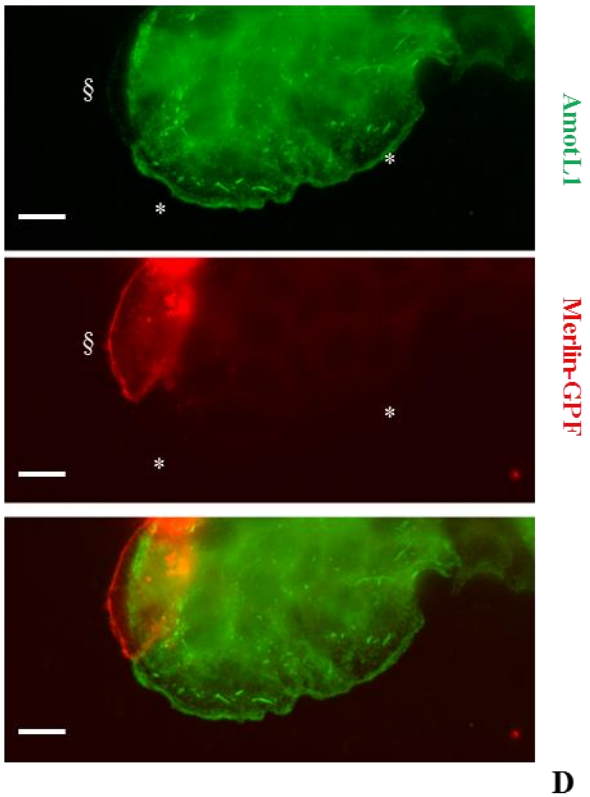
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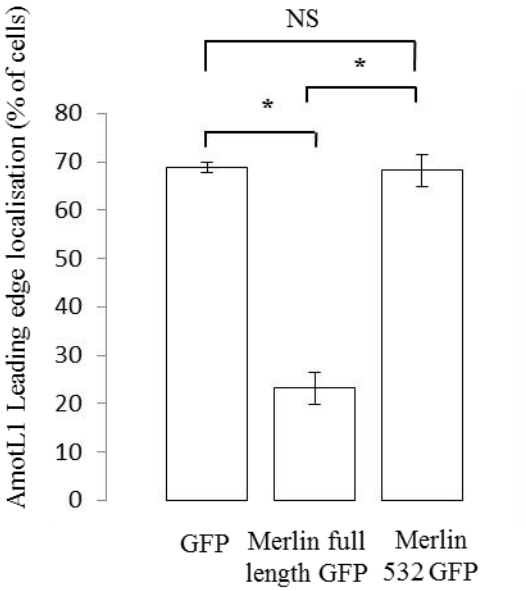
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Cell proportion of AmotL1 expression (percentage)
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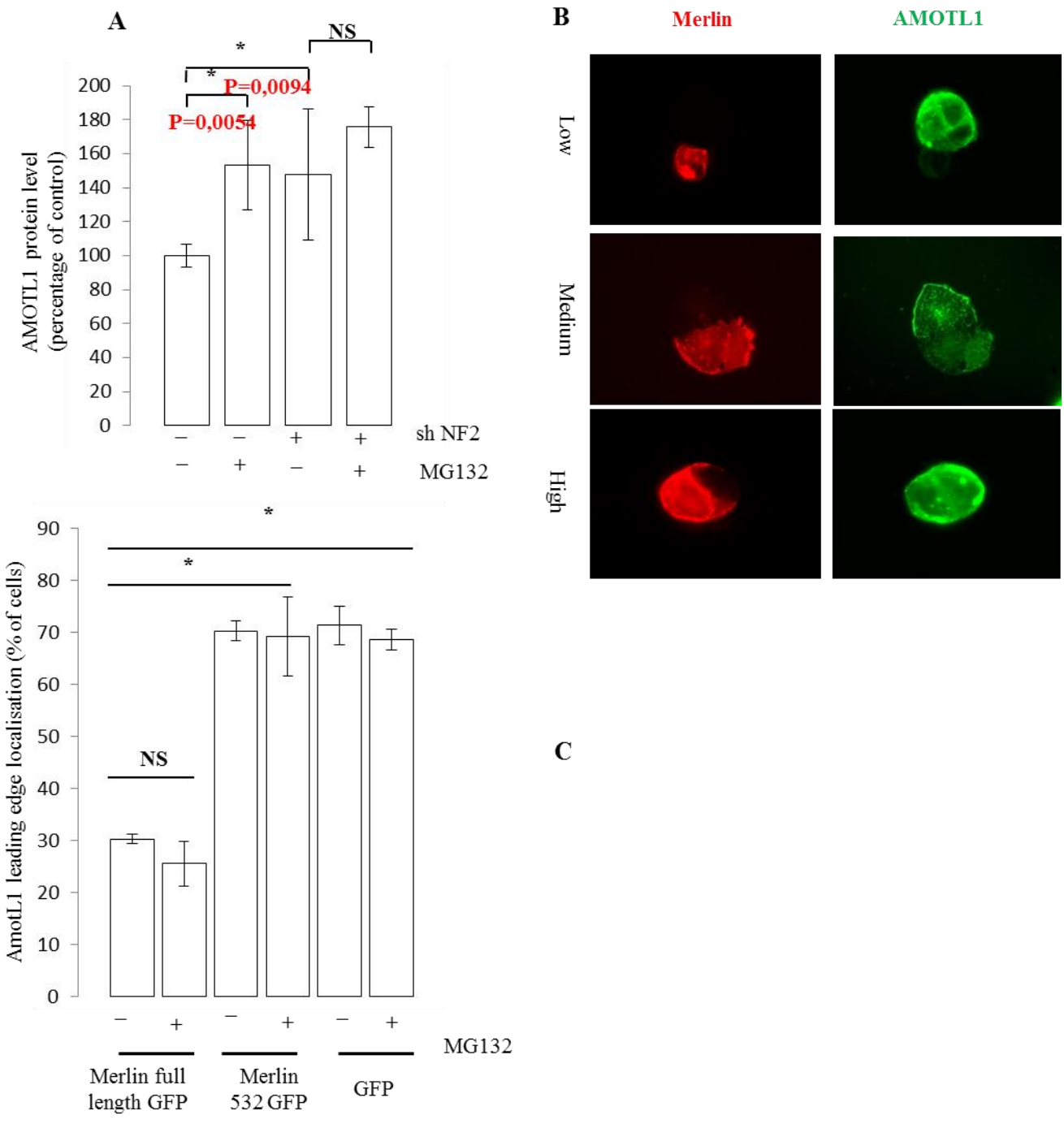


Figure 14. AmotL1 regulation by Merlin. A. Quantification of immunoblot analysis related to Figure 7B (n=3): measurement of the consequences of MG132 addition on AmotL1 levels in BC52 cells depleted or not for NF2. B. Illustration of the different levels of AmotL1 expression in Merlin transfected cells: high, medium and low. C. Quantification of AmotL1 localization at the leading edge evaluated by immunofluorescence in doxycycline treated BC52 tet on AmotL1 cells in presence or absence of MG132 and transfected with various constructs: GFP alone, GFP-Merlin full length or GFP-Merlin 532 (n=3).

2.9 Merlin regulates proliferation and migration of breast cancer cells through AmotL1

Interestingly, when we knockdown the expression of Merlin in breast cancer cell lines, we observed the increase of cell growth and migration. Then we hypothesized that these pro-proliferation and pro-migration effects upon Merlin depletion are mediated by AmotL1. To check this, we knock-down AmotL1 expression using shRNA and looked for cell growth of breast cancer cell lines in 3D in presence or in absence of Merlin. When we knock-down the expression of AmotL1 in Merlin depleted BC52 cells, we observed that number of cell aggregates back to the level obtained with shAmotL1 alone. Similarly, we used siRNA to knock-down the expression of AmotL1 in MDA-MB-468 cells depleted or not in Merlin and we looked for the effect of AmotL1 expression on cell migration. Interestingly, we observed that down regulation of AmotL1 expression bought the number of migrating cells back to the level obtained with siAmotL1 alone, demonstrating that the increase of migration mediated by the loss of Merlin is dependent of AmotL1 expression. Altogether, our results demonstrate that AmotL1 is a major mediator of proliferation and migration effects upon Merlin loss in breast cancer cells such as BC52 and MDA-MB-468.

3. Interactions between Hippo core components

We have shown that Merlin, a member of the Hippo pathway, interacts with a new partner, AmotL1 and regulates it. This observation strongly suggests that AmotL1 is a new component of Hippo signaling, adding to the complexity of this pathway. Hence, in the subsequent part of my work, I've tried to evaluate the interactions between various members of the Hippo pathway in order to get a better understanding of their hierarchy and of the functional importance of Merlin in this context.

3.1 Interaction between YAP and AMOT family proteins

In the course of studying the role of Merlin and AmotL1 interaction in cell migration, we found an unpublished report from NCBI data bank indicating a possible interaction between AmotL1 and YAP (Yes-Associated Proteins).

To confirm the interaction between YAP and AMOT family members in mammals, we have transfected HEK 293T cells with GFP-AmotL1. Cell lysates were immunoprecipitated with by

GFP-Trap. Then we tested the co-IP of endogenous YAP by western blot. Indeed, we pulled down YAP with AmotL1, but not in GFP control (data not shown). We further confirmed the interaction between Amot and YAP by IP of endogenous Amot and looking for endogenous YAP and vice versa. This indicates that AMOT family proteins are specific interacting partners of YAP (**Fig 15A and B**). Then to precisely map the interacting domain of YAP on AmotL1, we employed the immunoprecipitation of GFP-tagged AmotL1 C-ter deletion constructs (**Fig 7A**) in HEK 293T cells and analyzed for the endogenous YAP by western blot. In doing so, we found that YAP binds to the N-terminus half of AmotL1 (**Fig 15C**) and it is different from Merlin binding domain on AmotL1. More recently, other groups have shown that AMOT family proteins (Amot, AmotL1 and AmotL2) interacts with the YAP/TAZ and that this interaction is mediated by two PPXY motifs of AMOTs and two WW domains of YAP/TAZ (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011b). These studies also showed that AMOT family proteins recruit YAP/TAZ to tight junctions (TJ) and to the actin cytoskeletons, which results in decrease YAP/TAZ nuclear localization (**Fig 16**). This function is not dependent on phosphorylation of YAP/TAZ. However, later, it has been shown that AMOT proteins trigger YAP/TAZ phosphorylation at LATS target sites and this might be due to a scaffolding function of AMOT on Hippo pathway components such as MST2, LATS2, and YAP (Paramasivam et al., 2011), suggesting that AMOT family proteins suppress the YAP/TAZ activity both by phosphorylation-dependent and phosphorylation-independent mechanisms.

Collectively these results and other studies lead us to think that the presence of a large number of PPXY motif containing members (AMOTs, MST 1/2, LATS 1/2) might regulate the WW domain (YAP/TAZ) containing molecules in the Hippo pathway (Genevet and Tapon, 2011; Sudol, 2010). In addition in *Drosophila*, it has been shown that many of the Hippo core components interact with each other for the tight regulation of YAP to control the growth. However the interaction between mammalian Hippo pathway core components is less described. So we decided to investigate how members of the hippo pathway interact with each other with a specific emphasis on the AmotL1 and Merlin interactions.

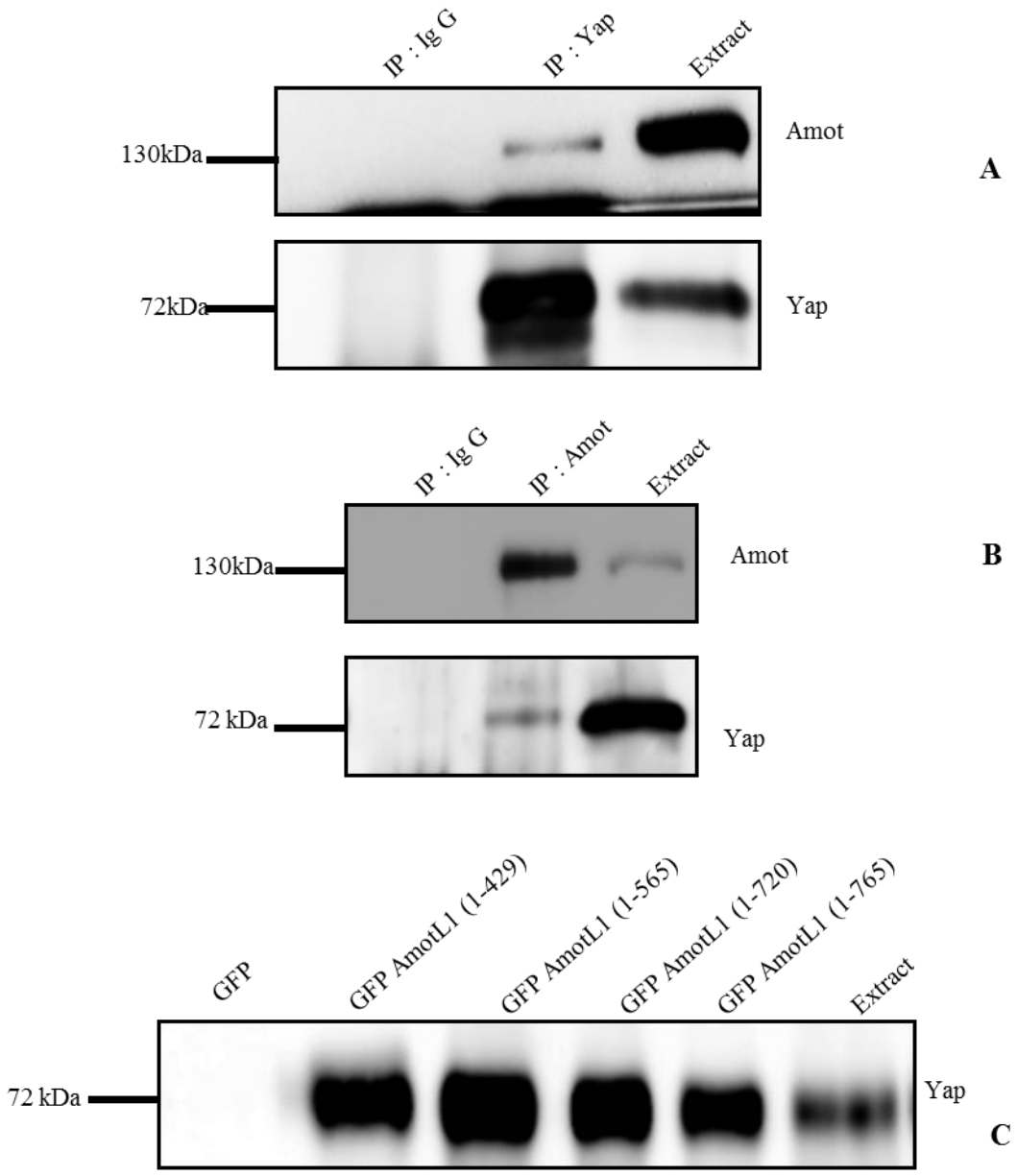


Figure 15. Interaction between YAP and AMOT family proteins. A. Immunoprecipitation (IP) with control IgG or anti-YAP was performed from the extracts of HEK 293T cells and analyzed for the indicated proteins by western blot. B. IP of Amot performed and western blotted to look for co-IP of indicated proteins. C. HEK 293 cells were transfected with GFP-tagged AmotL1 C-terminus deletion constructs and IP of GFP performed using anti GFP antibody coupled to beads (GFP-trap) and analyzed by western blot for YAP.

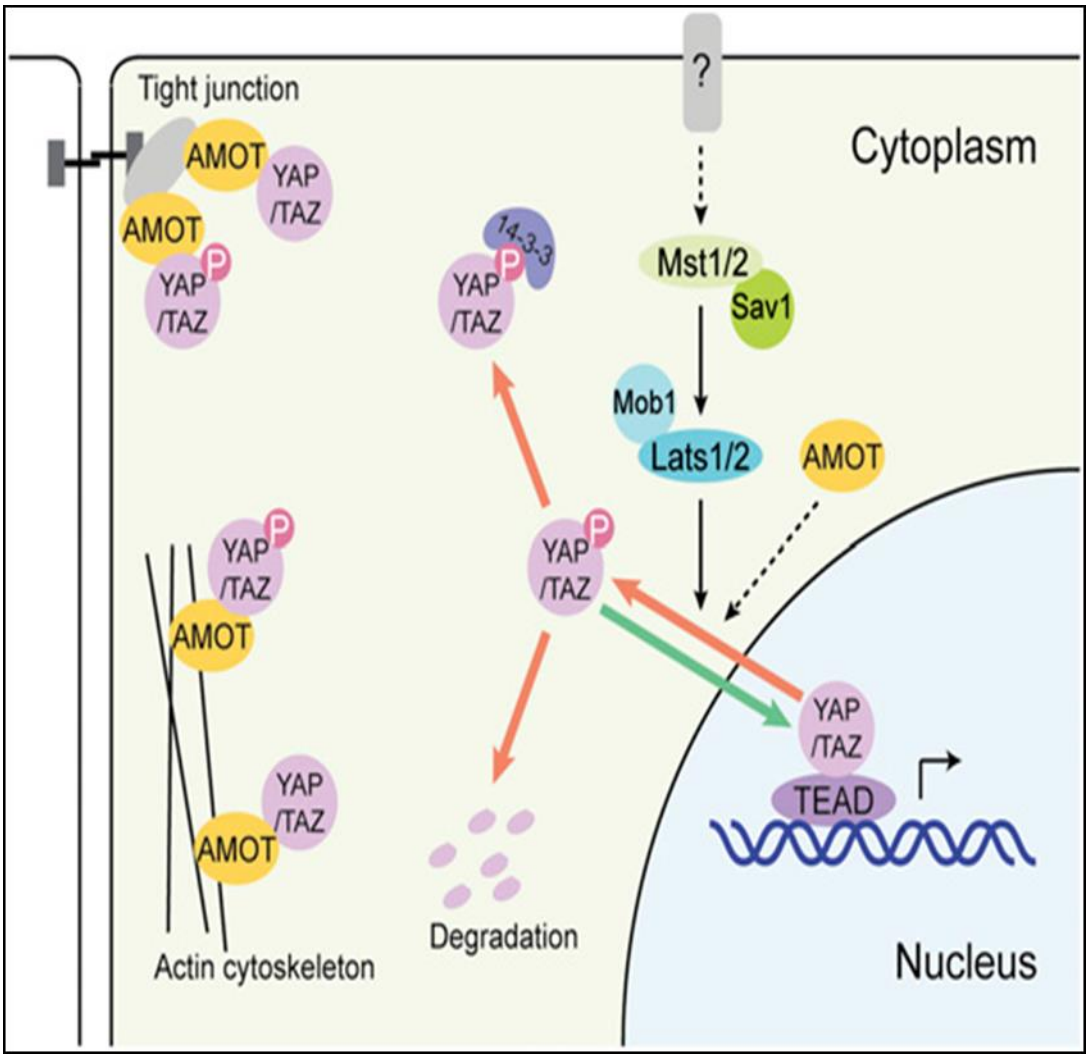


Figure 16. A model of AMOT in YAP/TAZ regulation. In this model, AMOT inhibits YAP/TAZ by regulating YAP/TAZ localization via physical interaction and promoting YAP/TAZ phosphorylation by the Hippo pathway (From Zhao et al., 2011b).

3.2 Interaction between Merlin, AmotL1 and KIBRA

In *Drosophila* and mammals, it has been well documented that the interplay between Merlin and KIBRA is important for the activation of Hippo pathway (Yu et al., 2010; Zhang et al., 2010a). Since we identified the interaction between Merlin and AmotL1, we thought that KIBRA might form complex with Merlin and AmotL1. We first confirmed the interaction

between Merlin and KIBRA in mammalian cells. For this we transfected GFP-tagged KIBRA in HEK 293 cells and carried out the GFP trap to look for partners. In this experiment, we found that Merlin is pulled down efficiently with KIBRA but not with GFP alone (**Fig 17A**). In addition, we also observed the co-localization between Merlin and KIBRA at cell-cell contacts by immunofluorescence (IF), (data not shown**)** suggesting that Merlin and KIBRA interaction may be involved in cell to cell communication.

Next step we tested whether AmotL1 and KIBRA may co-IP. To do so, we conducted immunoprecipitation of GFP-KIBRA in HEK 293T cells and looked for the endogenous AmotL1 by western blot. Indeed we could co-IP AmotL1 with KIBRA (**Fig 17B**). In the same experiment, we also looked for YAP. Strikingly we found that YAP is pulled down with KIBRA. Of course it is possible that this may be an indirect interaction, because YAP binds to AmotL1 which in turn binds to KIBRA. We then mapped the interaction domain of KIBRA on AMOTL1 by immunoprecipitating GFP-AmotL1 deletion constructs in HEK 293 cells and looked for the KIBRA by western blot. Interestingly we found that KIBRA binds to N-terminus domain of AmotL1 (likely to PPXY motifs due to presence of WW motif in KIBRA) which is different from Merlin binding domain on AmotL1 (**Fig 17C**). Collectively our results revealed new associations between Hippo core components which were unknown in the field: 1. Merlin, Kibra and AmotL1 appear to form complex together. 2. Merlin and KIBRA bind to different domain on AmotL1. 3. YAP is pulled down with AmotL1 and KIBRA. 4. In contrast, KIBRA and YAP (WW domain containing proteins) may compete for the PPXY motif of the AmotL1. In this context it is interesting to know where KIBRA binds on Merlin (on going work). The putative binding sites of these molecules on each other are represented in **Fig 18**.

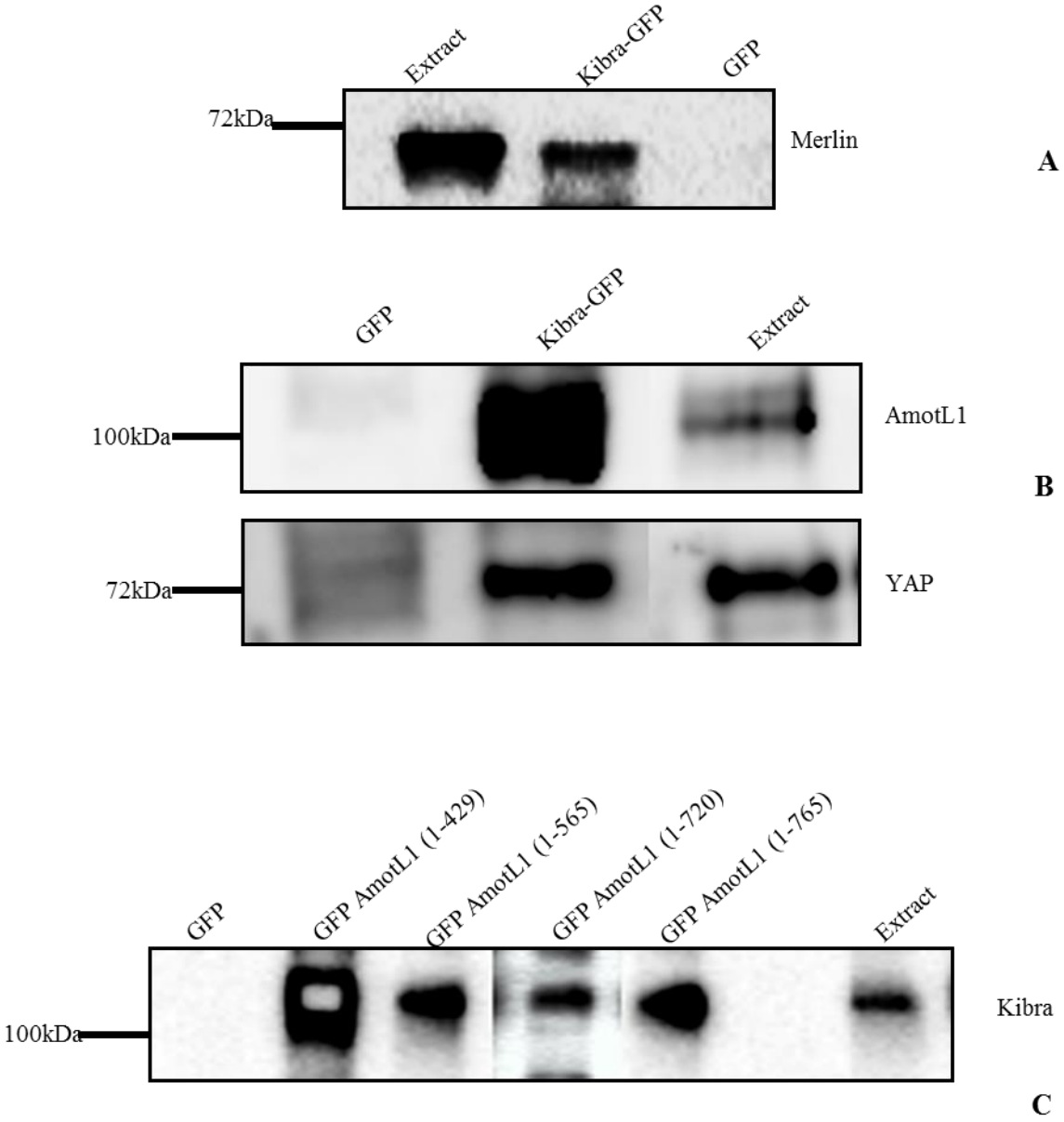


Figure 17. Interaction between Merlin, AmotL1 and KIBRA. A. HEK 293 cells were transfected with KIBRA-GFP and Immunoprecipitation (IP) of GFP was carried out. Immunoprecipitates were analyzed for Merlin by western blot. B. IP of GFP performed after transfecting the HEK 293 cells with KIBRA-GFP and analyzed for indicted proteins by western blot. C. HEK 293 cells were transfected with various GFP-AmotL1 C-terminus deletion constructs and IP of GFP was performed and analyzed for co-IP of KIBRA by western blot.

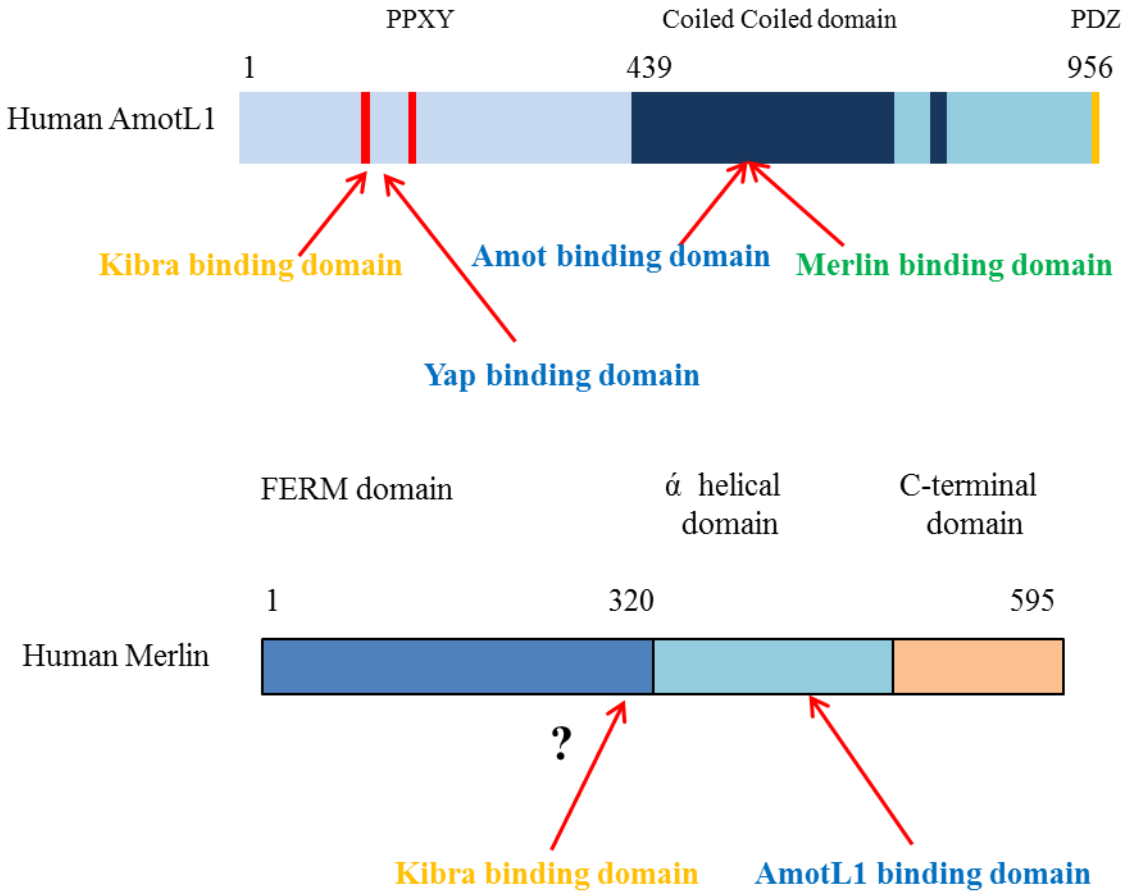


Figure 18. Summary interactions between Merlin, AmotL1 and Kibra

3.3 Interaction of Merlin with LATS and YAP

Having uncovered new interaction between Merlin and other Hippo members, we tested if Merlin may interact with downstream effectors such as YAP and Lats. To test this, we transfected HEK 293T cells with GFP-FERM (Merlin), GFP-C-terminus Merlin (CTM), and used GFP as negative control. When we carried out the immunoprecipitation of GFP constructs of Merlin, we were able to pull down LATS with FERM domain of Merlin but not with the GFP C-terminal half nor GFP alone (**Fig 19A**). We also transfected HEK 293T cells with mutant version of FERM domain of Merlin (FERM Blue Box- that is known to acts as a dominant negative over WT Merlin), wild type FERM domain and GFP as control. Interestingly, we found that LATS does not come down with the mutant FERM domain of

Merlin (**Fig 19B**). Collectively these experiments identify the specific interaction between Merlin and LATS via the FERM domain of Merlin and suggest that the loss of interaction with LATS could play a role in the dominant negative function of the Merlin Blue Box mutant.

It is well documented that LATS binds to YAP and phosphorylates YAP on serine on five consensus HXRXXS motifs (Hao et al., 2008; Zhao et al., 2010b). In the next step, we analyzed whether Merlin and YAP could co-IP. We hypothesize that Merlin could act as adaptor molecule for the LATS and YAP. To test this, we transfected HEK 293T cells with GFP-FERM (Merlin) and GFP-C-terminus Merlin and GFP as control. Strikingly we found that endogenous YAP co-IP with the C-terminus Merlin but not with the FERM domain of Merlin (**Fig 19C**). To further map the interaction domain of YAP on Merlin, we carried out immunoprecipitation of GFP-tagged deletion constructs of C-terminus Merlin and found that YAP binds to ~555-575 region of C-terminus Merlin (**Fig 20**). In conclusion, Merlin co-IP with Lats via its FERM domain and also co-IP with YAP through its C-terminus coiled-coiled domain. Hence, Merlin does not bring down YAP through interaction with Lats. Work is in progress to test if YAP binding to Merlin is direct or not. Altogether our results suggest that Merlin might act as adaptor molecule bringing LATS and YAP to facilitate the phosphorylation of YAP. It is also possible that Merlin directly regulate YAP activity by binding to it and modifying its localization in the cells (like AMOT family proteins) (Zhao et al., 2011b). This question is being investigated in the context of other projects in the lab.

Finally, using these results we biochemically characterized the Hippo core components interactions with special focus on Merlin and AmotL1. Based on our results we prepared an upgraded Hippo interactome shown in **Fig 21 (please see at the end of this part)** which may prove helpful for better understanding of the functional interplay between Hippo components. In conclusion, this part of my project shows that the network of interaction in the Hippo pathway is more complex than previously proposed. Several of the interactions that I discovered are now being explored and gave rise to new projects in the lab to which I am participating.

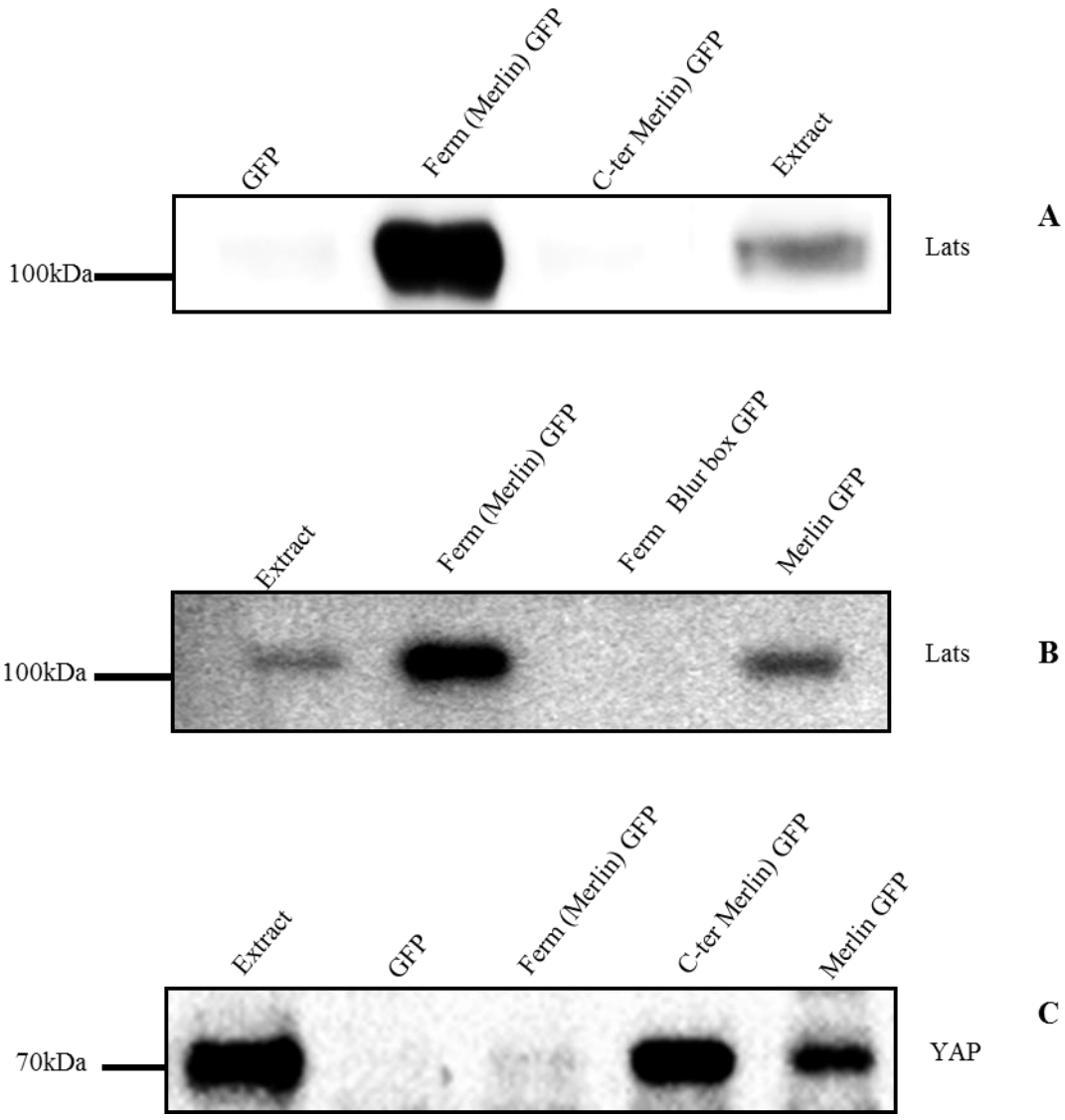


Figure 19. Interaction of Merlin with LATS and YAP. A. Western blot analysis with LATS antibody of immunoprecipitation of FERM and C-terminal Merlin GFP fusions transfected in HEK 293 cells. B. Immunoprecipitation (IP) of GFP tagged Merlin, FERM Blue Box and WT FERM and analyzed for Lats binding by western blot. C. HEK 293 cells were transfected with indicated Merlin constructs and IP of GFP was performed. YAP interaction was analyzed by western blot.

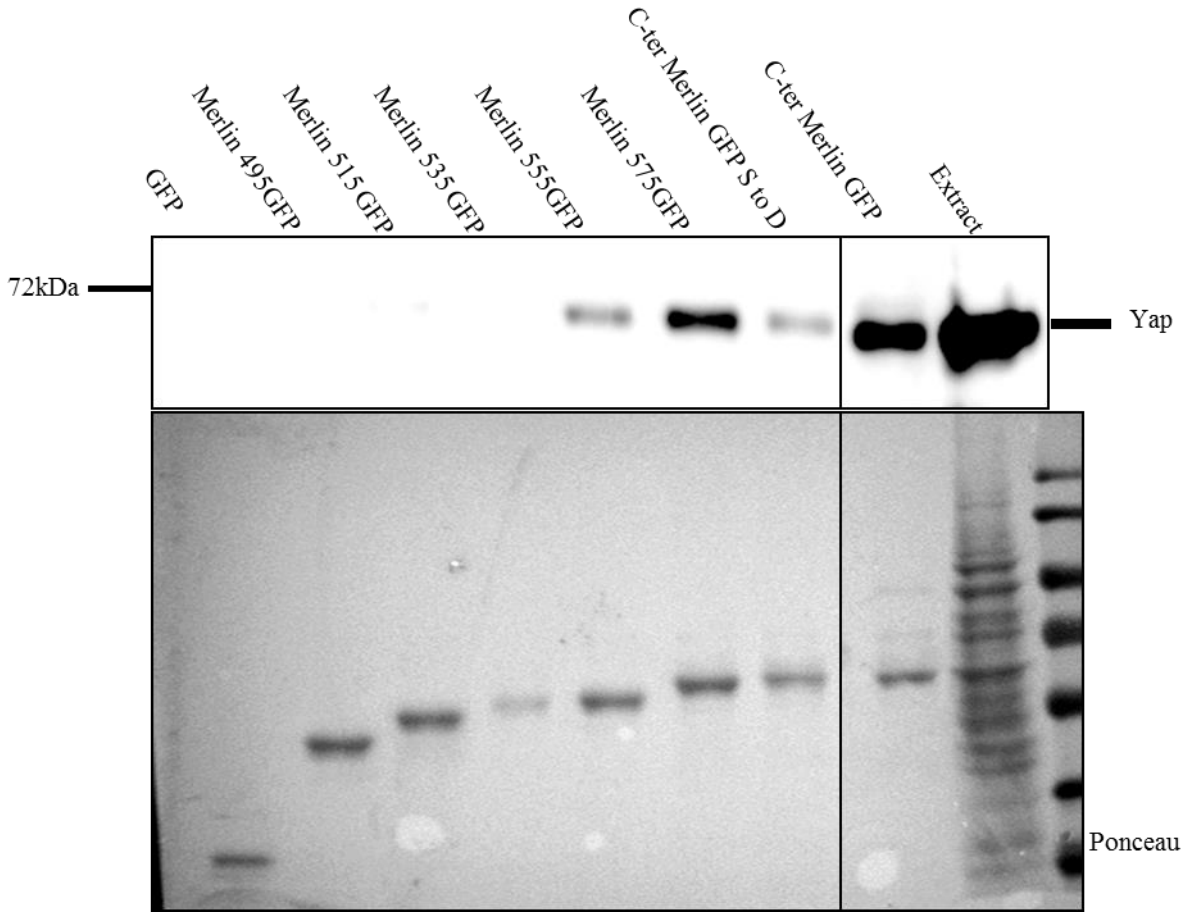


Figure 20. Mapping of Yap binding site on Merlin. HEK 293T cells were transfected with indicated GFP Merlin constructs and GFP trap was performed. Analyzed for YAP binding by western blot.

3.4 Merlin, a magnet of Hippo pathway

By observing several interactions between Hippo core components, we hypothesized that being localized at the plasma membrane, Merlin might acts as scaffold protein and recruit Hippo pathway core components to this location. The sequential extension of these protein–protein interactions suggests the existence of large multi-molecular complex that control the Hippo pathway activity. We hypothesize that Merlin acts as a "magnet" for Hippo components and enable the formation of functional molecular complexes. Therefore, it is possible that loss of *NF2* renders the complex formation impossible. To test this hypothesis,

we employed size exclusion chromatography (Gel-filtration). In this experiment, we have grown the *NF2*+/+ Schwann cells (SC) and *NF2*-/- Schwann cells at two different densities: 1. low density (where Merlin is inactive) and 2. high density (where Merlin is active). High and low density cell cultures were harvested and lysed in mild extraction buffer to preserve the complex. After clearing at 50,000 rpm for 30 minutes, supernatants were loaded onto Sephadex column 200 and 20 fractions are collected with constant volumes. Then the fractions are analyzed by western blot to look for the nature of the complexes in presence or absence or Merlin. By comparing the fractions from *NF2*+/+ Schwann cells (SC) and *NF2*-/- Schwann cells, we did not find any significant changes in the pattern of elution of Hippo members. This means that Hippo core components elute in the same fractions regardless of presence or absence of Merlin, and elution profiles were also independent of cell density (**Fig 22 and 23**). These results collectively suggest that Merlin, at least in our experimental set up, does not seem to effect the formation of complexes. The reasons are multiple. It is possible that our experimental set up may not be the best to study Hippo multi protein complexes or these protein complexes may be very dynamic and might not be separable using size exclusion chromatography. Further, the association of Hippo signaling components with cytoskeleton elements may render them difficult to extract and analyze. Finally, it is also possible that this kind of complexes may not exist in the cells, and instead may exist only as small size associations (combination of three to four components) as we have seen previously in co-IP experiments and in some of the fractions from chromatography.

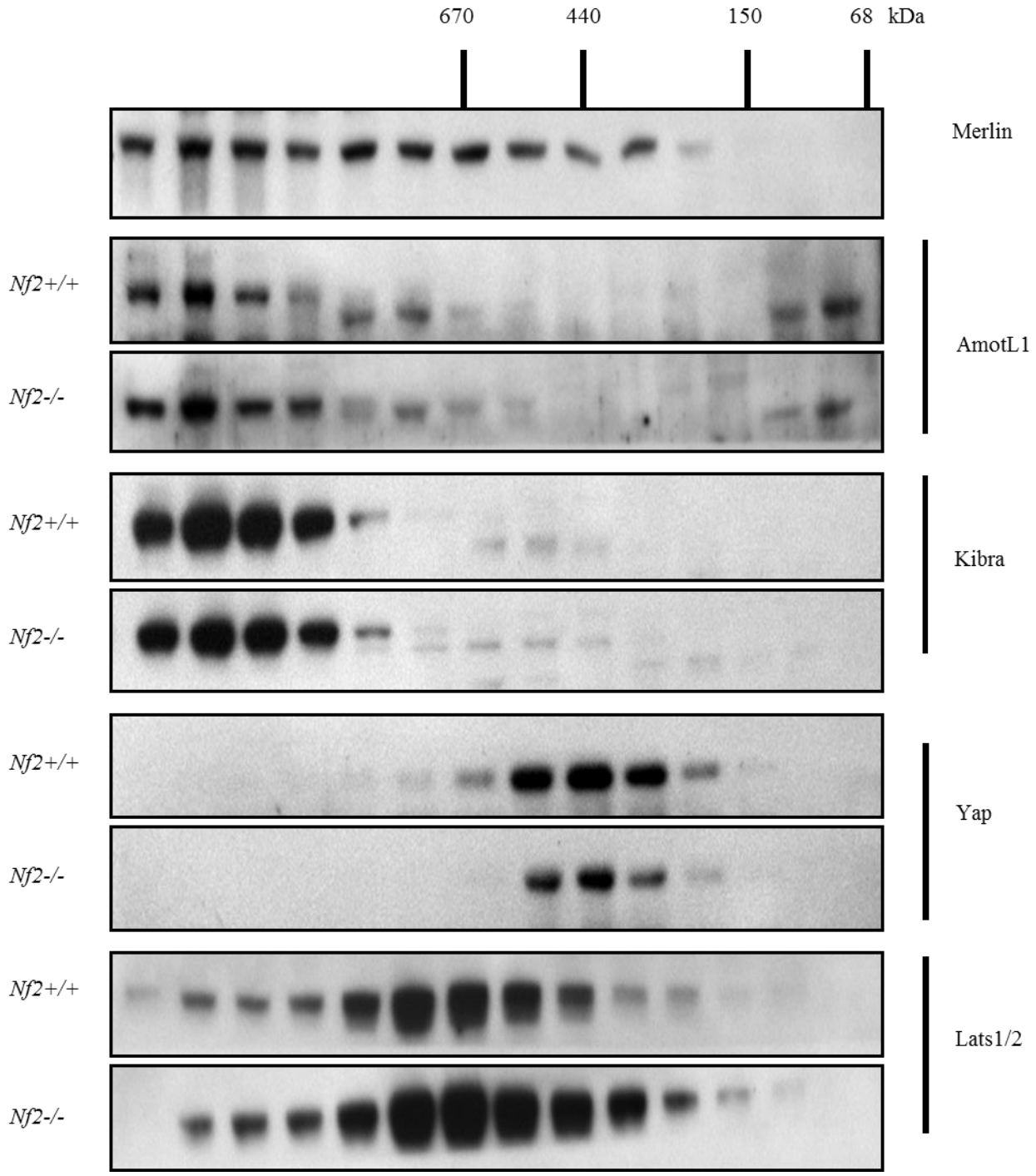


Figure 22. Effect of Merlin inactivation on the formation of molecular complexes containing Hippo components in confluent cells. Total extracts from *NF2+/+* and *NF2-/-* cells were subjected to gel filtration. Proteins from the different fractions were analyzed by immunoblotting with specific antibodies to the indicated proteins.

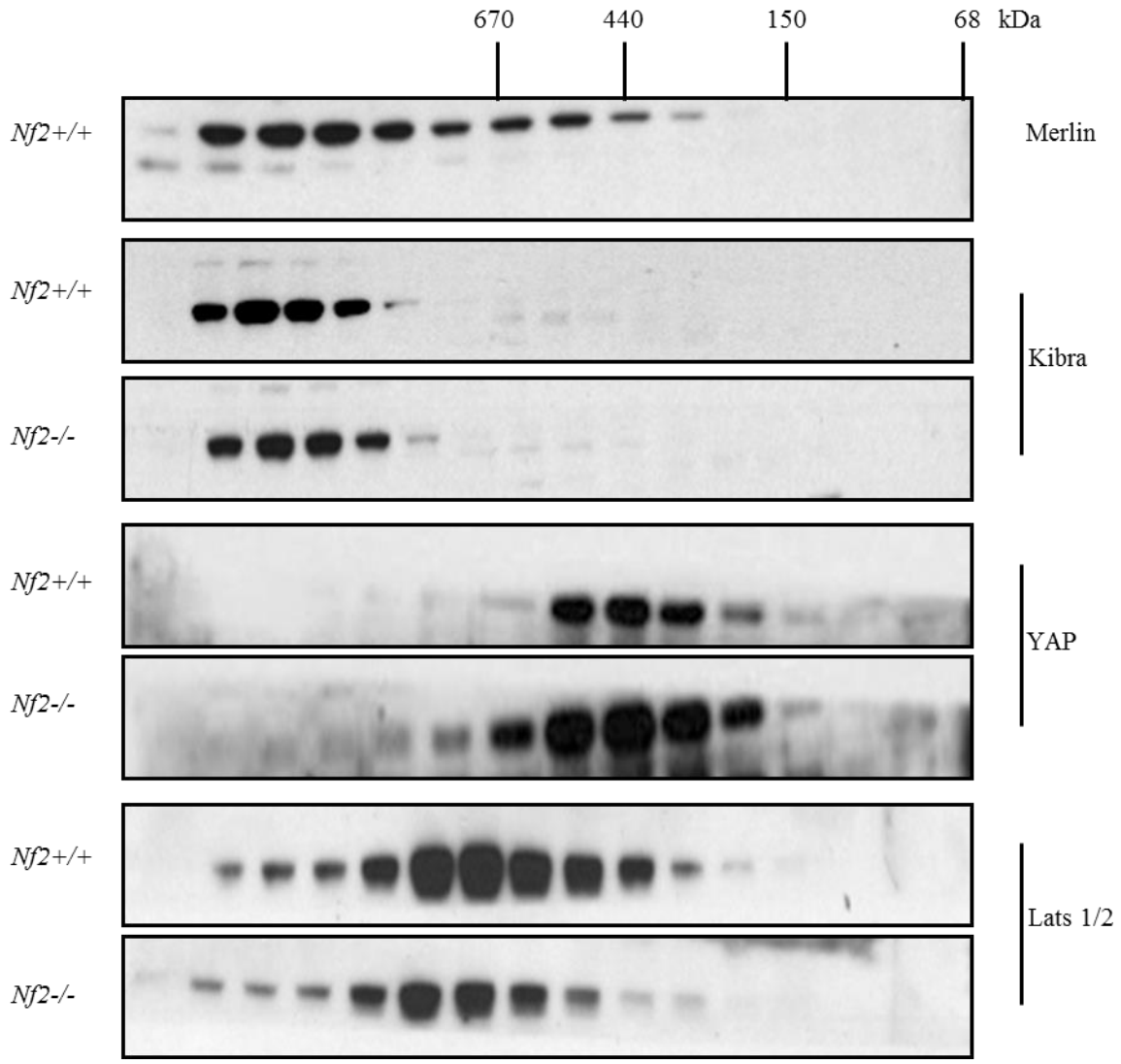


Figure 23. Effect of Merlin inactivation on the formation of molecular complexes by Hippo components in sub-confluent cells. Total extracts from *NF2+/+* and *NF2-/-* cells were subjected to gel filtration. Proteins from the different fractions analyzed by immunoblotting with antibodies to the indicated proteins.

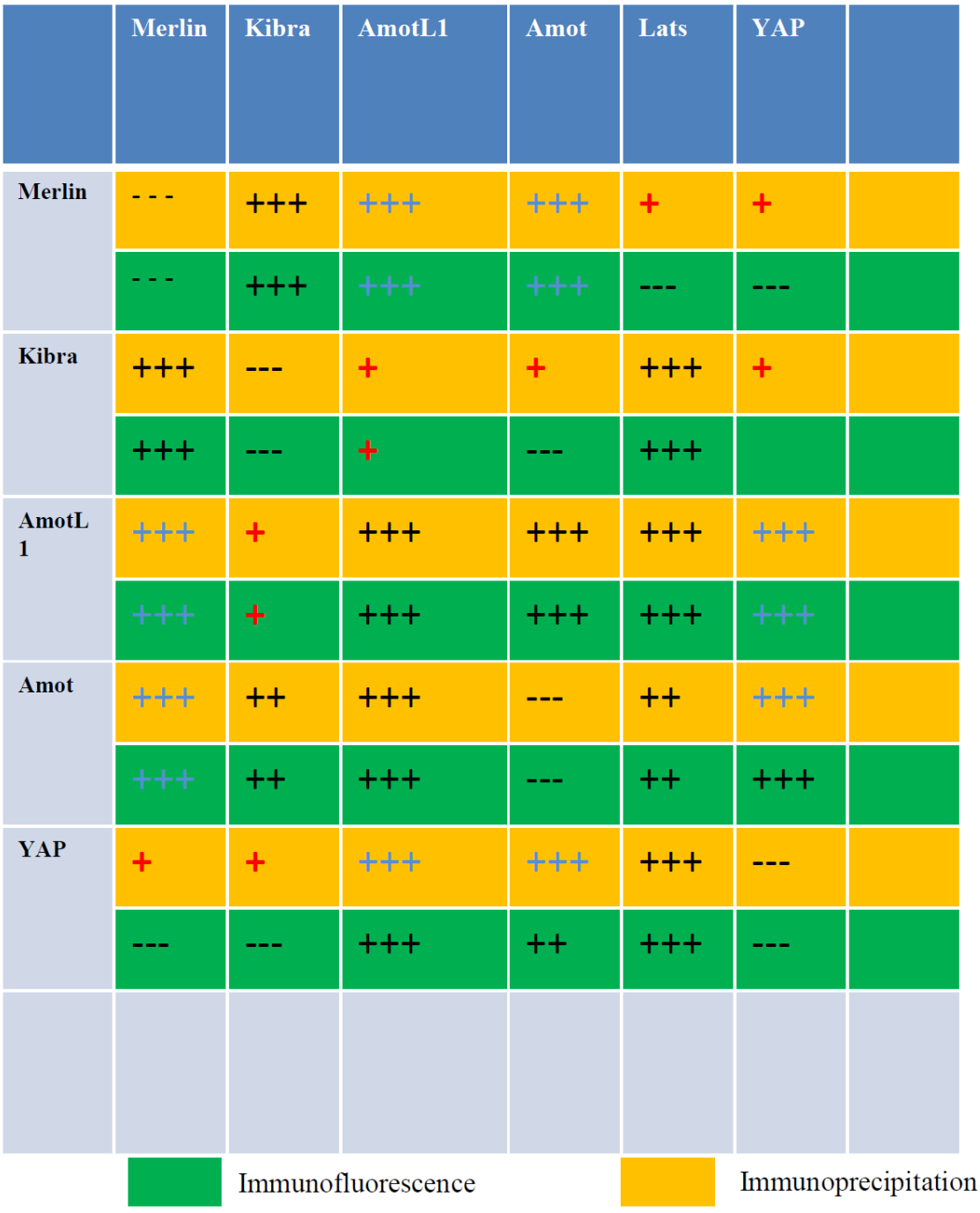


Figure 21.The summary of protein–protein interactions between components of the Hippo pathway

+ : Represents new interactions we identified in our laboratory
++: Indicates interactions observed by others
+++: Represents the interaction observed by us and others as well.
- - - : Represents negative result
+++ : Interaction at endogenous level

PART 2

INDENTIFICATION OF NOVEL PHOSPHORYLATION SITES ON C-TERMIMUS OF MERLIN

1. Introduction

1.1 Generalized view of cell cycle and its role in cancer development

The cell cycle is an ordered set of events, combination of cell growth and division into two daughter cells. In prokaryotic cells (without a nucleus), the cell cycle proceeds through a process termed binary fission. In eukaryotes cells (with a nucleus), the cell cycle can be divided in two periods: interphase and mitosis. During interphase cell grows, accumulating nutrients needed for mitosis and duplicating its DNA. In mitotic (M) phase, cell splits itself into two distinct cells, often called "daughter cells" and finally cytokinesis results in completion of cell division (**Fig 24**). Through this vital process, a single-cell fertilized egg develops into a mature organism. Based on morphology, the cell cycle is divided into Interphase and Mitosis (M) phase.

Under the microscope, interphase cells simply grow in size, but different techniques revealed that the interphase includes G1, S and G2 phases (Norbury and Nurse, 1992). The first phase is called G1 (gap). During this phase (G1) the cell is preparing for DNA synthesis. The second phase of Interphase is called S Phase during which cells are synthetizing DNA. DNA content doubles as it is replicated into two copies. The final phase of interphase is G2, second gap phase and during which cell prepares for the mitosis. Cells in G0 are not actively in cycle but have the potential to divide. The term "post-mitotic" is sometimes used to refer to both quiescent and senescent cells in G0 phase.

Mitosis is a combination of nuclear division and cytokinesis, and produces two identical daughter cells. The process of mitosis is complex and highly regulated. Mitosis is subdivided into a series of events that include prophase, prometaphase, metaphase, anaphase, and telophase. These sequential phases of mitosis are highly complex and tightly regulated.

Prophase: During this phase, the nuclear membrane starts to breakdown and the nucleolus disappears. Subcellular organelles like Golgi and Endoplasmic reticulum fragment and localize beneath the plasma membrane. Sister chromatids in the nucleus begin to condense and become visible under the light microscope as chromosomes. Centrioles start to move to opposite ends of the cell and microtubule fibers extend from the centromeres.

Prometaphase: In this phase, nuclear envelope completely vanishes and the mitotic spindle continues to mature as more microtubules assemble. Chromosomes become more condensed and proteins bind to centromeres forming the kinetochores. Mature microtubules attach to kinetochores to move the chromosomes.

Metaphase: In this phase, microtubules align the chromosomes between the two poles of the cell on a line referred as metaphase plate (equatorial plane). This metaphase plate helps in equal separation of chromosomes into the daughter cells.

Anaphase: During this phase, the paired chromosomes at metaphase plate separate by kinetochores attachment to spindles and helps in movement to the opposite poles of the cell. The separation of chromosomes results from combination of kinetochore movement along with spindle and polar microtubules.

Telophase: Nuclear membrane start to form around chromosomes and the chromosomes arrive at opposite poles and begin to uncoil. The microtubules depolymerize into monomers that helps in providing the cytoskeleton components for newly formed daughter cells. Finally partitioning of the cell will start in this phase.

Cytokinesis: In animal cells, cytokinesis begins following the onset of anaphase. A contractile ring made up of actin and non-muscle myosin II forms just inside the plasma membrane at the equatorial plane. Then, myosin II moves along the actin filaments to pull the equator of the cell inward to form a cleavage furrow. Further movement of myosin II along with actin filaments pull cleavage furrow inwards and finally the plasma membrane pinching results in the mother cell giving two identical daughter cells.

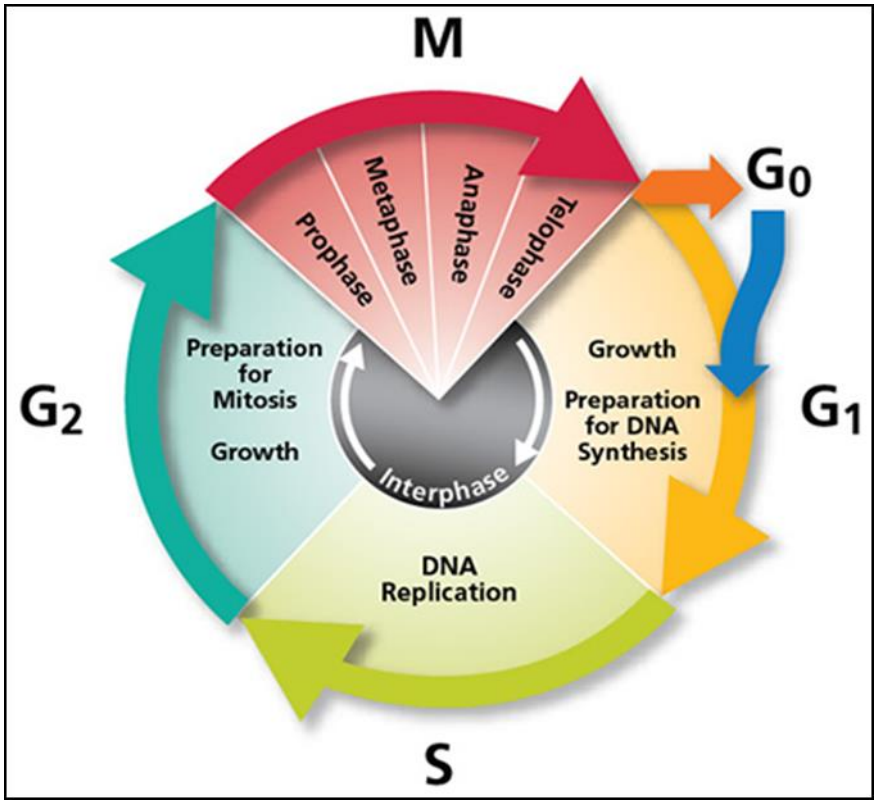


Figure 24. A schematic overview of the different phases of the cell cycle.

1.2 Control of the cell cycle

The transition from one cell cycle phase to another occurs in a sequential fashion and is regulated by different cellular protein kinases. Among them, cyclin-dependent kinases (CDKs), Polo-like kinases (PLKs) and Aurora kinases play crucial role in controlling the mitotic entry, and make sure that an accurate coordination of chromosomal and cytoskeletal events leads to the correct partition of the genetic material into two daughter cells. It would go beyond the scope of this manuscript to give a detailed account of the molecular mechanisms involved in the mitotic progression. I will focus specifically on Aurora kinases because of their importance for the following parts of our work.

1.3 Mitotic kinases in cell cycle regulation and cancer

During the mitosis, cells undergo numerous changes that include nuclear envelope breakdown, chromosome condensation, centrosome maturation, bipolar-spindle assembly, centrosome separation, chromosome segregation and cytokinesis to separate the replicated genetic materials into two daughter cells. Various studies in lower organisms have demonstrated that these events are tightly regulated by phosphorylation events mediated by several serine/threonine kinases called mitotic kinases. One major group of mitotic kinases which are involved in the regulation of mitosis is Aurora Kinases.

1.3.1 Aurora kinases

The Aurora kinases belong to Ser/Thr kinases family and emerged as important regulators of cell cycle processes covering from mitotic entry to cytokinesis. Homologues of Aurora related kinases are conserved in various organisms including yeast, nematodes, fruit flies and vertebrates. Depending on the species, the number of family members of Aurora kinases varies: lower organisms like fungi have only one Aurora gene whereas, in contrast, in higher eukaryotes the family has branched. Human have three Aurora kinases including Auroras A, Aurora B and Aurora C. Aurora A and B are closely related in their sequence and structure, their catalytic domains shares 70% identity (**Fig 25 and 26**). In contrast, their distribution and functions are quite different in mitosis progression (Barr and Gergely, 2007; Ruchaud et al., 2007; Vader and Lens, 2008). Both kinases are expressed in most normal cells and involved in the control of cell cycle during G2 through to cytokinesis in a coordinated manner. Aurora C is expressed in testis and is known to be crucial for spermatogenesis (Hu et al., 2000; Li et al., 2004).

The gene encoding the Aurora-A protein kinase is located in the 20q13 in humans and its amplification is observed in various epithelial malignant tumors, including breast, colon, and bladder, ovarian and pancreatic cancers. Human Aurora B and C genes are located on chromosomes 17p13.1 and 19q13.43 respectively and are not amplified in human cancers. However, the consequences of overexpression of Aurora A in cancers are far from being fully appreciated (Hu et al., 2000).

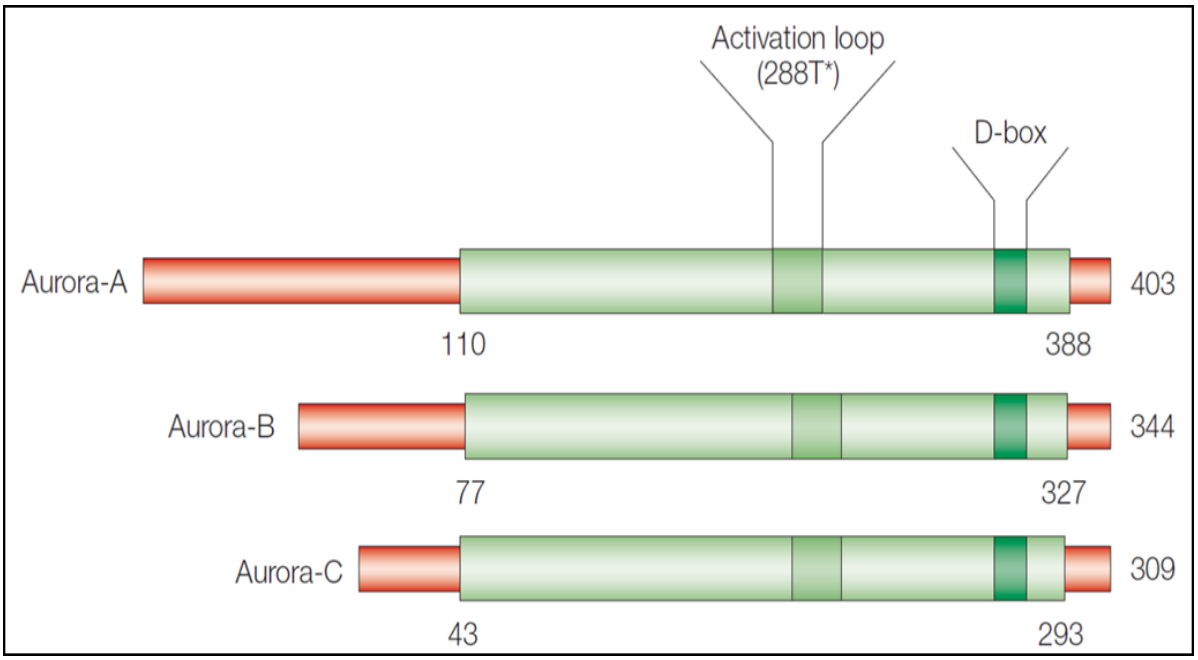


Figure 25. Schematic representation of human Aurora kinases structure. Aurora kinase A, B and C has highly conserved catalytic domain (green region). T288 in the activation loop of Aurora Kinase-A is required for the activation of its kinase activity. All the Aurora kinases (A, B and C) has a short amino-acid peptide motif called the 'destruction box' (D-box) in the carboxyl-terminal region which is recognized by adaptors of the anaphase-promoting complex/cyclosome and thereby targets these proteins for degradation through the ubiquitin/proteasome-dependent pathway (From Marumoto et al., 2005).

1.3.2 Aurora A functions and its role in cancer development

Aurora A was identified in a screen for Drosophila mutations affecting the poles of the mitotic spindle (Glover et al., 1995). In human cells, it has been shown that Aurora A undergoes dynamic changes in subcellular localization during the mitosis suggesting a key role in each step of mitosis (**Fig 26**). Full activation of Aurora A requires binding to specific protein cofactors which in turn leads to change in the conformation of kinase domain, results in auto-phosphorylation of T288. Activated Aurora A mediates its functions by interacting with many proteins. It has been shown that in Hela cells, active form of Aurora is crucial for mitotic entry. The activation of Aurora A is mediated by LIM protein called Ajuba. In this mechanism, first, Ajuba get phosphorylated and binds to Aurora A. The binding of Ajuba to Aurora A results in activation of Aurora A at late G2 centrosome, which is an important step in the commitment of cells to mitosis (Hirota et al., 2003). In addition, in various organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* it was shown that activation of

Aurora A is important for the centrosome maturation and separation (Berdnik and Knoblich, 2002; Giet et al., 2002; Hannak et al., 2001). Similarly, depletion of Aurora A in human cells results in inhibition of centrosome maturation (Hirota et al., 2003). Concerning the role of Aurora A in spindle formation, many studies observed that interaction between TPX2 (targeting protein XKLP2) and Aurora A is crucial for the mitotic spindle formation (Eyers et al., 2003; Kufer et al., 2002). Moreover, it also has been demonstrated that activation of Aurora A is important for the correct attachment of microtubules to the kinetochore during prometaphase which is mediated by phosphorylation of CENP, Kinetochore-specific histone H3 variant, through Aurora A (Kunitoku et al., 2003; Marumoto et al., 2003). Finally, the inactivation of Aurora A by anaphase promoting complex (APC) triggers cytokinesis (Castro et al., 2002; Honda et al., 2000). Altogether, Aurora A acts at many steps to tightly regulate the mitosis. Alterations in its expression therefore will likely results in mitotic defects such as centrosome maturation and separation, delay in cytokinesis.

Overexpression of Aurora A results in colony formation in soft agar and tumor formation in mice, indicating that Aurora A can act as an oncogene (Bischoff et al., 1998). Amplification of *Aurora A* gene is observed in many malignant tumors including breast cancer. In addition, elevated levels of Aurora A at mRNA and protein levels are frequently observed in various tumors such as HCC (hepatocellular carcinomas) and colon cancers (Gritsko et al., 2003; Jeng et al., 2004; Li et al., 2003; Takahashi et al., 2000; Tanaka et al., 1999). However they are not correlated with the gene amplification which was observed in other cancers such as breast cancer (Zhou et al., 1998), gastric cancer (Sakakura et al., 2001). Overexpression of Aurora-A triggers abnormalities in mitotic checkpoints and cytokinesis failure which in turn results in chromosome instability (Anand et al., 2003; Meraldi et al., 2002). Interestingly, it was observed that inhibition of Aurora A activity leads to suppression of cell growth and induces apoptosis in cancer cells, suggesting that Aurora A can be good drug target for the treatment of many human malignancies. All these studies collectively indicate that proper timing of activation and expression of Aurora A is important for the mitotic progression and disturbances in these processes result in abnormal cell division which in turn can lead to tumorigenesis. Given the importance of Aurora kinases in mitosis and their oncogenic potential, new chemotherapy agents targeting Aurora-A and B have been developed, and some of them are in phase II trials for various cancers (Kollareddy et al., 2012; Nigg, 2001).

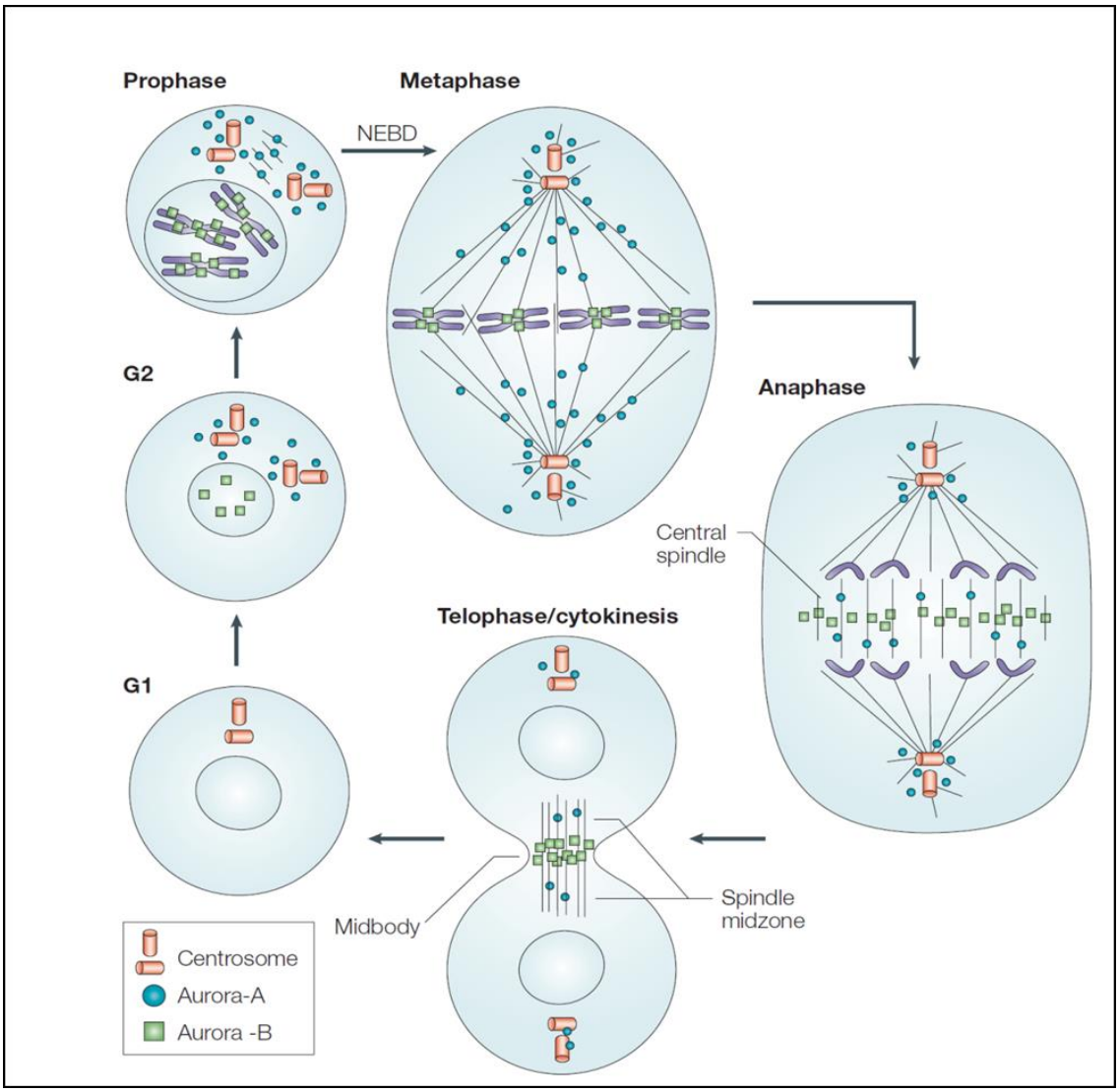


Figure 26. Model showing the localization of Aurora-A and -B kinases during the cell cycle. A small amount of Aurora kinase A (blue circles) is activated at centrosomes was first evident of mitosis at late G2 phase. During the prophase both the amount and activity of Aurora-A rapidly increase in the centrosome, and a fraction of active Aurora-A subsequently translocates into nucleus in parallel with chromatin condensation. Then nuclear-envelope breakdown (NEBD) proceeds and activated Aurora-A is observed at the spindle poles and bipolar spindles during prometaphase as well as metaphase. Only a small fraction of Aurora-A remains on the centrosomes and the spindles at the onset of anaphase and localizes on the spindle midzone and centrosomes during late anaphase and telophase. To proceed to cytokinesis, Aurora-A is degraded by the CDH1/Fizzy-related form of the anaphase-promoting complex/cyclosome (APC/C). The amount of Aurora-A starts to decrease at the metaphase–anaphase transition and most of the Aurora-A protein becomes undetectable at the cytokinesis. On the other hand, Aurora-B (green squares) is localizing on chromosome arms during prophase and the interface between sister centromeres (inner centromere region) during prometaphase and metaphase. During the later phases of mitosis, it relocalizes to the central spindle and the cell cortex, at the site of cleavage-furrow. Finally Aurora-B localizes at midbody during cytokinesis (From Marumoto et al., 2005).

2. ERM proteins and Merlin in cell cycle progression

2.1 Phosphorylation mediated activation of ERMs is important for the mitotic progression

Mitosis is a unique feature of animal cells that involves many complex sequences of morphological changes, from cortical retraction and cell rounding at the entry into mitosis to spindle organization and elongation, and finally cytokinesis at the exit of mitosis. In the Interphase, cells are either flat or often irregular in their shapes and the actin can be found in stress fibers. In contrast, upon onset of mitosis cells become round and almost spherical by retracting the cell margin and increasing cortical stiffness. This is achieved by rearrangements of actin cytoskeleton at the cell cortex to form a continuous layer of actin filaments positioned under the plasma membrane whereas stress fibers are absent**.** Concerning the cell rounding during mitosis, it has been shown that actin cytoskeleton plays a major role in this process in a Rho A dependent manner, suggesting a role for myosin II (Cramer and Mitchison, 1997; Maddox and Burridge, 2003). However the molecular mechanism underlying this phenomenon is poorly described. In this scenario, several groups have made significant contributions in understanding the role of ERM in mitosis. Moesin, the only ERM in *Drosophila*, is phosphorylated by Slik (a member of the Sterile-20 family of serine/threonine kinases) at T559 residue during mitosis (Hipfner et al., 2004; Hughes and Fehon, 2006b). This Slik mediated phosphorylation of Moesin is important for increasing the cortical stiffness to trigger the cell rounding during the mitosis (Carreno et al., 2008; Kunda et al., 2008). Indeed, phosphorylation of Moesin by Slik "opens" the protein and unmasks FERM and actin binding domain, leading to crosslinking of actin cytoskeleton to the plasma membrane. In this context, it has been shown that inhibition of Moesin phosphorylation either by knockdown of Moesin or Slik resulted in many mitotic defects including disruption in cell rounding process and caused cells to bleb continuously. In addition, Moesin knockdown resulted in defects in mitotic spindle shape and orientation which in turn lead to slow mitotic progression. In contrast, reducing the Myosin II activity did not affect either retraction or cell rounding, though these cells show abnormal cortical morphologies. Based on these results it has been proposed that Myosin II and Moesin are necessary for cortical regulation during mitosis as it was shown that cell size increases during anaphase, phospho-Moesin disappears from the poles and concentrates along with myosin II, at the future furrow just before cytokinesis. Concerning the regulation of Moesin during the mitosis, it has been shown recently that Pp1-

87B phosphatase restricts high Moesin activity to early mitosis and reduces the levels of Moesin at the polar cortex, after anaphase onset. In addition, two mitotic kinases, Pten and Slik, play important roles in the spatiotemporal regulation of activated Moesin by producing PIP2 throughout the mitosis (Roubinet et al., 2011) (**Fig 27**). Collectively, these results support a model in which *D. melanogaster* Moesin, along with myosin II, promotes cortical rigidity by binding to cortical actin filaments so that they lie parallel to the plasma membrane.

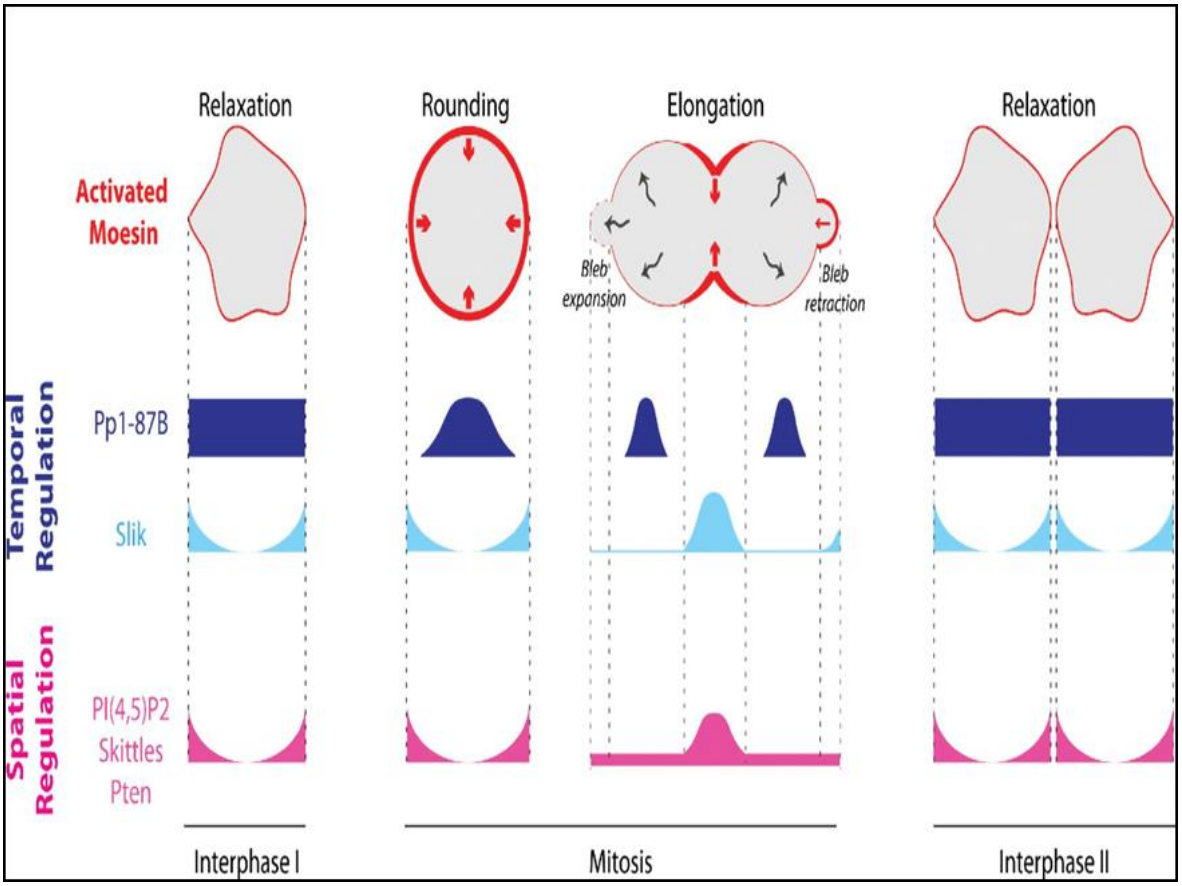


Figure 27. Proposed model of the spatiotemporal regulation of Moesin activity the cell cycle progression. The Pp1-87B phosphatase redistribution at mitosis entry is proposed to promote an increase of Slik- dependent phosphorylation of Moesin at early mitotic stages and then ensures that Moesin levels goes down at the end of division and in interphase. The PI (4, 5) P2 levels at the anaphase equator are increased by localized enrichment of Skittles and Pten. This in turn promotes Moesin recruitment at the equator and release at the polar cortex to allow cell elongation. The same proteins are important for the transient recruitment and activation of Moesin at the cortex of polar blebs, which is important for their efficient retraction (Modified from Roubinet et al., 2011).

2.2 Role of Merlin in cell cycle regulation

A rather large proportion of the tumor suppressors exert their functions by nuclear localization in a cell cycle-specific manner (Fabbro and Henderson, 2003), in contrast, Merlin is close to ERM family of proteins which are associated with the actin cytoskeleton and cell membrane components. Merlin partially co-localizes with ERM in actin-rich surface protrusions of the plasma membrane such as membrane ruffles, microspikes and in the cleavage furrow (Bretscher et al., 2002; Gautreau et al., 2002b). Due to its predominant localization to the plasma membrane, Merlin has been regarded as a unique type of tumor suppressor whose growth inhibitory mechanism would be linked to cell membrane. However few studies have shown that Merlin is present in the nucleus where it could also impact the cell cycle (Kissil et al., 2002; Kressel and Schmucker, 2002; Shaw et al., 1998b). For example, increased expression of Merlin suppresses the cell proliferation and promotes G0/G1 arrest in primary schwannoma and mesothelioma cells partly by inhibiting the cyclin D1 expression (Schulze et al., 2002; Xiao et al., 2005). Concerning the function of Merlin in nucleus, one group has demonstrated that Merlin exits the nucleus in the late G1 phase and that it is majorly localized to cortical membrane during the S and G2 phases. In the G2/M phase transition, Merlin is mostly perinuclear and associated with mitotic spindles during the mitosis. In addition, cell cycle-dependent nucleo-cytoplasmic shuttling of Merlin was shown to not depend on phosphorylation at S518 by PAK and PKA (Muranen et al., 2005; Shaw et al., 1998b). Furthermore, it was shown that Merlin can inhibit proliferation by promoting the degradation of cell cycle regulator HE I10, a substrate of cyclin B/cdc2 (Grönholm et al., 2006). Recently it has been proposed that hypophosphorylated form of Merlin translocates into nucleus and exerts its growth suppressive function by interacting with an E3 ubiquitin ligase DCAF1(Li et al., 2010). Inspiet of these results, the role of Merlin in cell cycle regulation is poorly investigated

3. Results

3.1 Role of novel phosphorylation sites of C-terminus Merlin in its tumor suppressor activity.

The general notion is that phosphorylation of Merlin results in the inactivation of its growth suppressive function. During growth arrest by serum deprivation, loss of adhesion, and increased cell density, Merlin is mostly in hypophosphorylated form. Whereas higher levels of hyperphosphorylated form of Merlin is present in growth permissive conditions. Merlin is known to get phosphorylated on both N- and C-terminal halves which in turn results in modification of its cellular functions (Shaw et al., 2001). In addition, phosphorylation of Merlin targets it for degradation (Laulajainen et al., 2011).

As we discussed previously, the phosphorylation of Merlin at S518 affects its localization and tumor suppressor functions (Kissil et al., 2002; Lallemand et al., 2003; Rong et al., 2004a), conformation and binding capacity to other proteins. In spite of all these studies, the exact mechanism by which S518 phosphorylation regulates Merlin's tumor-suppressive activity is still poorly understood. In this context our laboratory has shown that C-terminus domain of Merlin (~532-579) is important for the growth suppressive function of Merlin. In the first part of my thesis, we have focused on the interacting partners of C-terminus of Merlin, which are seem to be important for the growth suppressive function of Merlin. In this part, we have hypothesized that new phosphorylation at the C-terminus of Merlin alone or in association with S518 might be important for the tumor suppressor function of Merlin.

3.2 Identification of a novel phosphorylation at C-terminus of Merlin using mass spectrometry.

To identify new phosphorylation sites in Merlin, we transfected HEK 293 cells with GFP tagged Merlin and immunoprecipitated Merlin using the GFP-Trap. Then immunoprecipitated

protein were separated by SDS-PAGE and visualized with commassie staining, after which the protein bands were cut out from the gel, in-gel digested with trypsin into peptides, and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an Ultimate 3000 nano-LC (Dionex, Sunnyvale, CA) and a QSTAR Elite hybrid quadrupole TOF-MS (Thermo scientific/MDS Sciex, Foster City, CA) with nano–electrospray ionization. The fragmentation spectrum is derived from a tryptic (1 missed cleavage) Merlin peptide. The peptide sequence and the observed ions of the phopho-peptide are shown with the spectrum. MS/MS spectrum is labeled to show singly charged b and y ions, as well as ion corresponding to neutral losses of water (o), NH3 (*) and H3PO4 group (98Da). LC-ESI MS/MS spectrum for peptide with the position of the phosphate group KLpT (581) LQSAK (484.762+ m/z) (**Fig 28**). This phosphorylation peak corresponds to Threonine 581 (582 in mouse) residue in the human Merlin isoform 1. In addition to this new phosphorylation site, we also detected previously well documented sites including T230, S10 in N-terminus of protein and S518, T576 in the c-terminus of Merlin. Hence, we have identified a new phosphorylation site for Merlin. This site is specific of isoform I since it is localized beyond the last common amino acid with isoform II in position 579. In support of our study, a systematic screen for phosphorylated proteins in mouse tissues performed at Harvard Medical School also reported the detection of phosphorylated Merlin at T581 residue *in vivo* (https://gygi.med.harvard.edu/phosphomouse/).

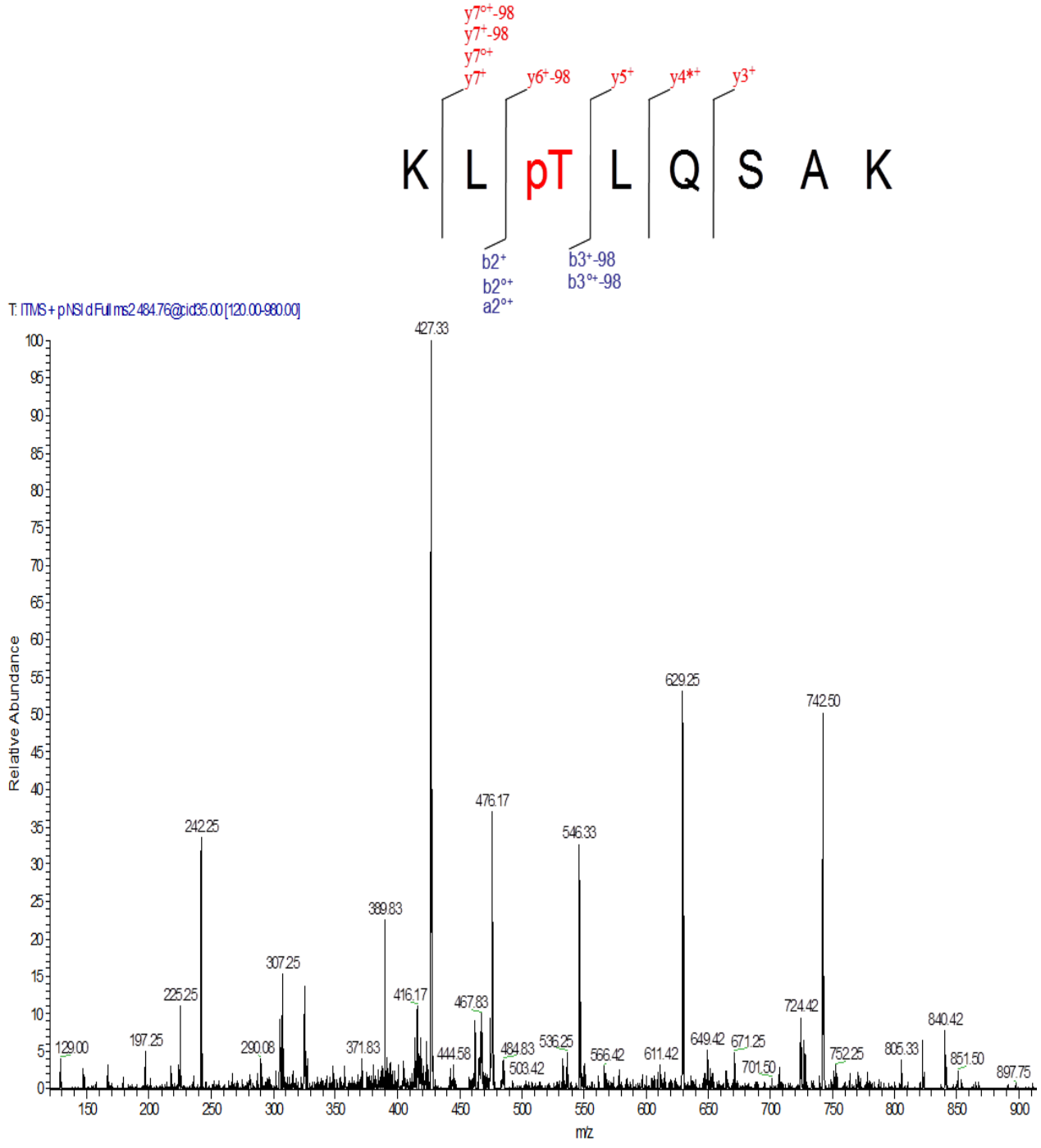


Figure 30. Identification of a new phosphorylation (T581) site on C-terminus of Merlin. Mass spectrometric peak (highest one) corresponding to T581 in human Merlin. The peptide sequence where T518 is indicated in red is presented above the graph (see the text for more details).

3.3 Generation and characterization of polyclonal antibody that specifically recognizes phosphorylated Merlin at T581 position

To study the role of T581 in Merlin function, first we generated a phospho-specific polyclonal antibody against this site. A chemically phosphorylated peptide was used to immunize the rabbits. A standard protocol was used to immunize the rabbits and was performed in collaboration with the Covalab company. After giving enough booster doses to rabbit, antibodies were purified by passing through an affinity column of phospho-antigen. In this step the antibodies present in the serum may recognize the phosphorylated and un-phosphorylated epitopes. In the following step, the eluted antibodies are incubated with a second column of the same peptide sequence but un-phosphorylated. Hence the antibodies that would recognize the non-phospho epitopes are captured. The eluent, which at this step should contains only the antibodies to the phospho-sequence, was collected and used for further characterization.

To further characterize the phospho specific antibody to T581, we developed various Hela Tet-on Merlin stable cell lines expressing the wild type and as well as nonphosphorylatable mutants of Merlin at T581 (T518A). Then we used these cell lines to verify the specificity of the phospho-specific antibody against T581 by western blot. We then tested the antibody on the lysates from wild-type or mutant Merlin expressing cells. We found that the phospho-specific antibody gave a positive signal only in wild type Merlin cells but not in mutants. In this experiment, we used S518 phospho-specific antibody as positive control and pre-immune sera as negative control (**Fig 29**). Inhibitor of phosphatase Na2Vo3 was also added to cell culture in order to artificially increase phospho-signal. However, interestingly, it had no effect on the intensity of the band although it increased total phospho-tyrosine signal. Collectively these results suggest that the antibody which we raised against pT581 is specific to phosphorylated Merlin at T581 and has only minimal cross-reactivity with non-phosphorylated epitope. We could not detect endogenous phosphorylation of Merlin in Hela cells and only a very feeble signal in 293T cells that were used for mass spectrometry. When Merlin was overexpressed in Hela and whereas the affinity of the antibody measured by Elisa was very high (not shown), the signals were very low by western-blot indicating that either the antibody efficiency is low in this technique or that the levels of phosphorylation in growing Hela cells are themselves low.

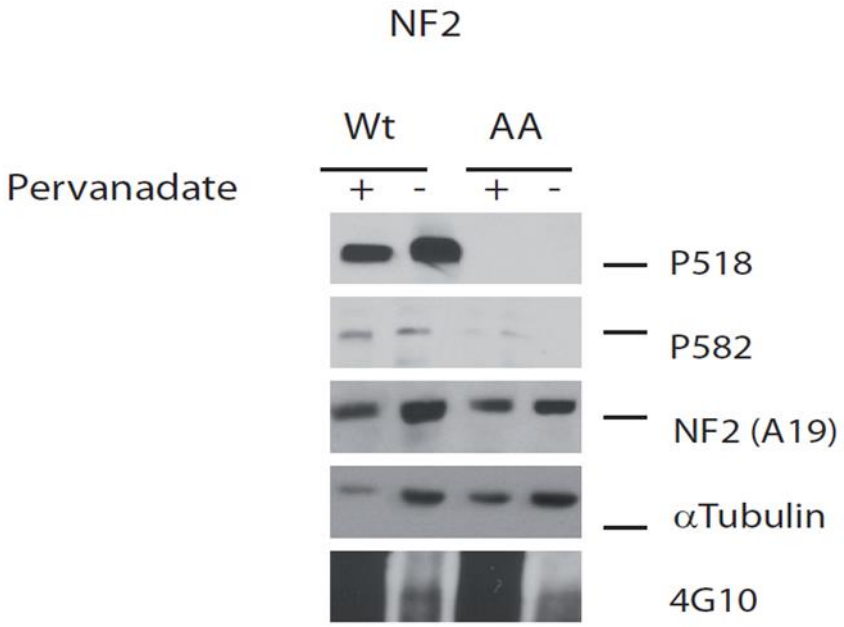


Figure 29. Characterization of a polyclonal antibody that specifically recognizes the phosphorylated Merlin at T581 position. A. Western blot analysis of Hela cells expressing either WT Merlin or mutant Merlin (AA indicates 518A581A) with and without phosphatase inhibitor. Extracts were analyzed for indicated phosphorylation sites of Merlin using phosphor-specific antibody**.** 4G10 is pre-immune serum. α-Tubulin used as a loading control.

3.4 Phosphorylation of Merlin at T581 does not affect its subcellular localization in Hela cells.

Previously it has been shown that phosphorylation of Merlin on S518 effects its localization (Kissil et al., 2002) in LLCPK cells. Instead, we used Hela Tet-on cells expressing wild type Merlin as well as various phosphomutants of Merlin T581 (T581A and T581D) alone or in combination with S518 (518A581A and 518D581D) to visualize subcellular localization of the different forms of Merlin. Interestingly upon over-expression, we did not find any significant differences in the localization between phospho-mutants and compared to wild type Merlin (data not shown) by immunofluorescence and using 3D microscopy. This results suggest that phosphorylation of Merlin at T581 do not drastically affects its subcellular

localization. Finally, we could not use anti pT581 antibody by IF to visualize the subcellular localization of phosphorylated Merlin because of the lack of specificity in the technique.

3.5 Merlin is hyper-phosphorylated during mitosis at T581 and S518

It is well demonstrated that Merlin is phosphorylated in response to various growth conditions such as serum stimulation (Laulajainen et al., 2008; Okada et al., 2009). In relation to this, we checked for the phosphorylation of T581 under various growth conditions including serum stimulation, serum starvation and in confluent cells. By western blot, we did not see phosphorylation of overexpressed Merlin at T581 residue in these conditions suggesting that phosphorylation of T581 is not regulated in these conditions (**data not shown)**. These results lead us think that the weak signal seen by western blot in the 293T cells might come from a specific sub-population of the cells and we thought that it could come from mitotic cells. To test the phosphorylation of T581 in mitosis, we synchronized Hela cells overexpressing Merlin in mitosis using nocodazole (1µM), a drug that interferes with microtubule polymerization and causes mitotic spindle arrest, thereby arresting cells in M phase. In this experiment we seeded the cells and then induce the Merlin overexpression by adding the doxycycline to the media (1µM). After 24 hours, we gave nocodazole treatment for 16 hours and mitotic cells were collected by mitotic shake off. Using this strategy we were able to collect cells which are exclusively in mitosis and then we prepared whole cell extracts to investigate the phosphorylation of T581 by western blot. Strikingly, we found a 5 fold increase in the phosphorylation of Merlin at T581 position in mitosis compared to interphase (**Fig 30 A and B**). In addition, we also checked the status of S518 phosphorylation in mitosis. Remarkably, S518 was also phosphorylated 2.5 fold more when compared to interphase (**Fig 30 C and D**). In contrast to T581, we were able to find phosphorylation of Merlin at S518 in interphase at detectable level, suggesting that Merlin might be phosphorylated during interphase and mitosis at S518 by different kinases (**Fig 30 A-D**). In addition, our results also suggest that S518 might be important for the control of Merlin activity in interphase as well as in mitosis, whereas T581 might be involved in the regulation of Merlin function specifically in mitosis. Collectively these results demonstrate that Merlin is hyper-phosphorylated at S518 and T581 during mitosis.

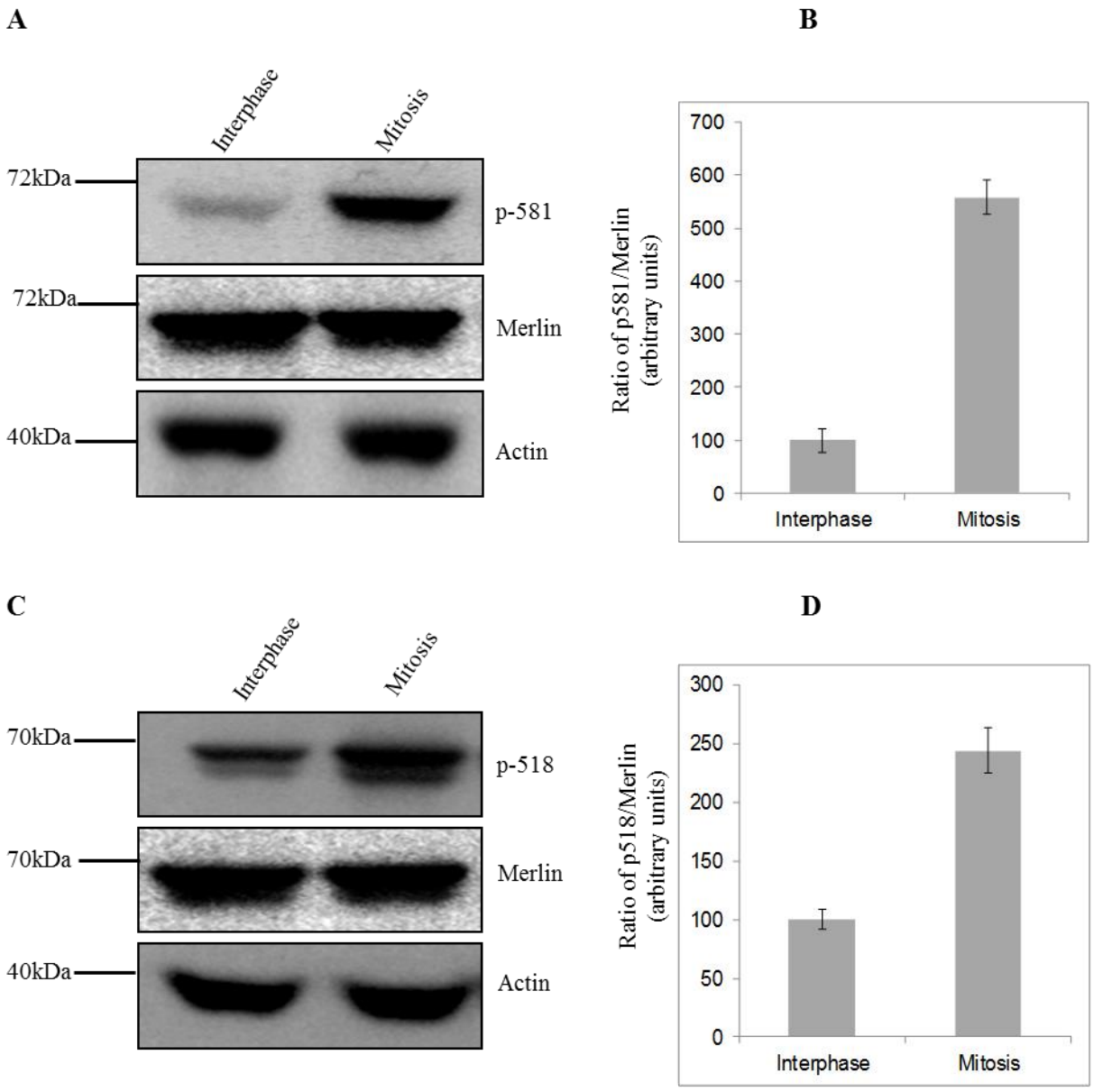


Figure 30. Merlin is hyper-phosphorylated at S518 and T581 during mitosis. A. Hela cells overexpressing Merlin were synchronized in mitosis using nocodazole (1uM). Interphase cells (without nocodazole) were also collected. Western blot was performed to analyze for p581 and total Merlin proteins. Actin is used as loading control for this experiment. B. Quantification of three independent experiments corresponding to experiment A with standard deviations. C. Experiments carried out as in A but analyzed for p518 and Merlin by western blot. D. Quantification of three independent experiments corresponding to experiment C with standard deviations.

3.6 Aurora Kinase A phosphorylates Merlin at S518 but not T581

In order to determine the mitotic kinases which phosphorylate the Merlin at S518 and T581 sites, we tested Merlin sequence for the various consensus sites for mitotic kinase. Interestingly we found that Merlin S518 is an Aurora kinase A consensus site with a basic amino acid (R) followed by hydrophobic amino acid and then S518 (**Fig 31A**). Interestingly we also found that this site is conserved in human and mouse Merlin but not in *Drosophila* as well as in ERMs indicating a specific function of this site in mitosis in mammals (**Fig 31A**).

To test whether Aurora Kinase A phosphorylates Merlin during mitosis, we inhibited Aurora A activity using the specific inhibitor, MLN8054. In this experiment, we induced Merlin over expression and then, after 24 hours, we gave overnight nocodazole treatment followed by addition of Aruora A specific inhibitor for 45 minutes (MLN8054). Next we harvested mitotic cells and the phosphorylation status of S518 was checked using phosphor-specific antibody and in comparison to extracts from untreated cells. Strikingly, we found very strong decrease (~90%) in the phosphorylation of S518 in presence of Aurora A inhibitor compared to no inhibitor (**Fig 31 B and C**). In this experiment, we confirmed that we inhibited the activity of Aurora A completely as measured by phospho Aurora A signal (**Fig 31 B**). On the other hand, we did not see any effect of Aurora A inhibition on status of T581 phosphorylation (**Fig 32 A and B**). Further, in these experiments we also used Aurora kinase B and Polo-like kinase (Plk) specific inhibitors to see their effect on phosphorylation of Merlin at S518 and T581 sites and we did not find any effects of these inhibitors (date not shown). To further confirm that endogenous Merlin S518 is good substrate for the Aurora A, we performed immunoprecipitation of endogenous Merlin in mitosis with and without inhibition of Aurora A activity and analyzed the S518 phosphorylation levels by western blot. Indeed, we confirmed reduced levels of S518 phosphorylation when we inhibited the activity of Aurora A (**Fig 32 C and D**). Taken together, our results demonstrated that Merlin is phosphorylated by Aurora A on S518 in mitosis. These results further support the idea that Merlin is a good substrate for Aurora kinases and may participate in mitotic progression.

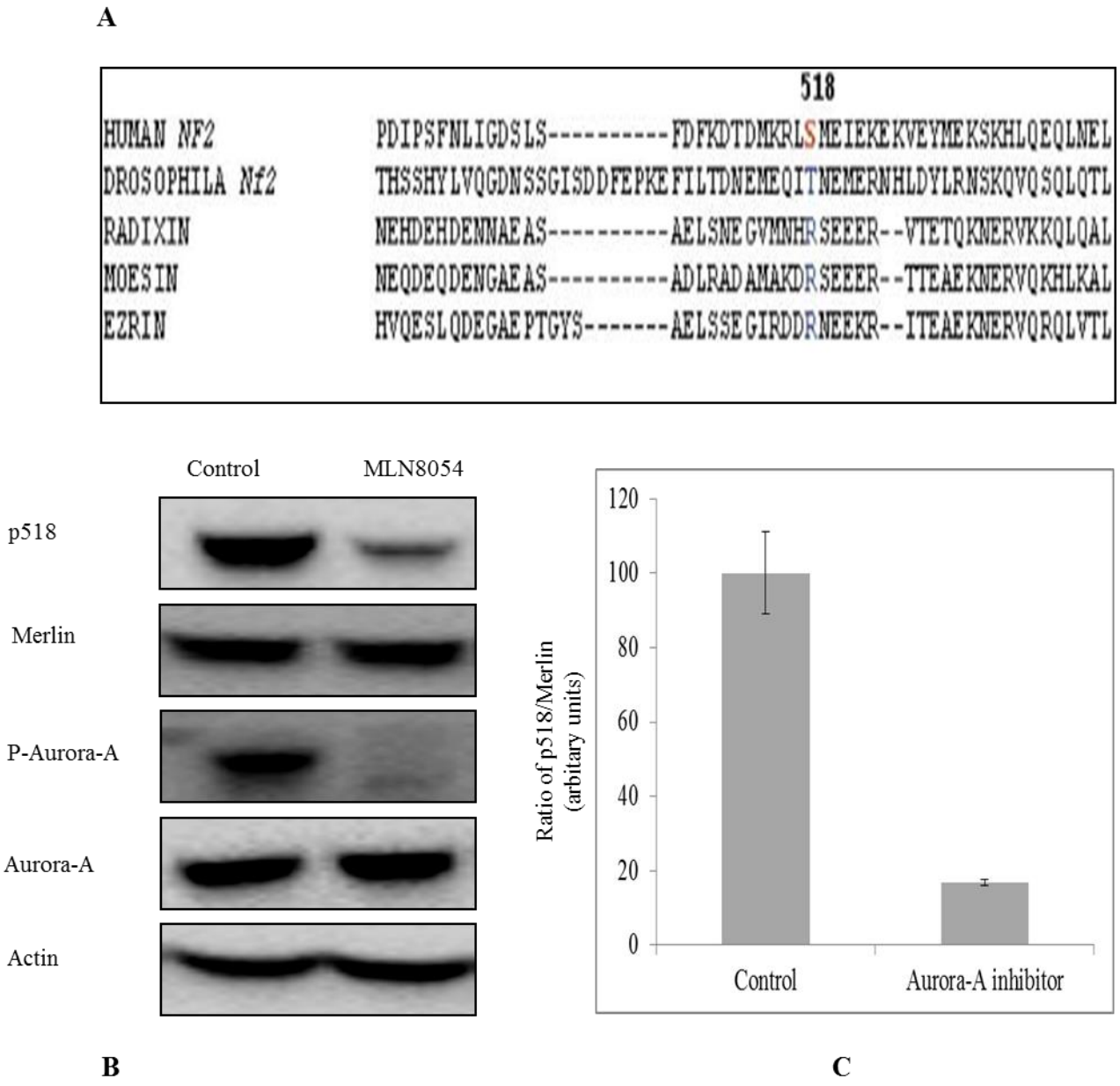


Figure 31 Aurora kinase A phosphorylates Merlin at S518 during mitosis. A. Alignment of human and *Drosophila* Merlin, human Ezrin, Radixin and Moesin C-terminus domain. The S518 site is highlighted with red color. B. Western blot analysis of indicated proteins from lysates of Hela cells over expressing the Merlin that were synchronized in mitosis as discussed previously. C. Quantification of three independent experiments corresponding to experiment B with standard deviations.

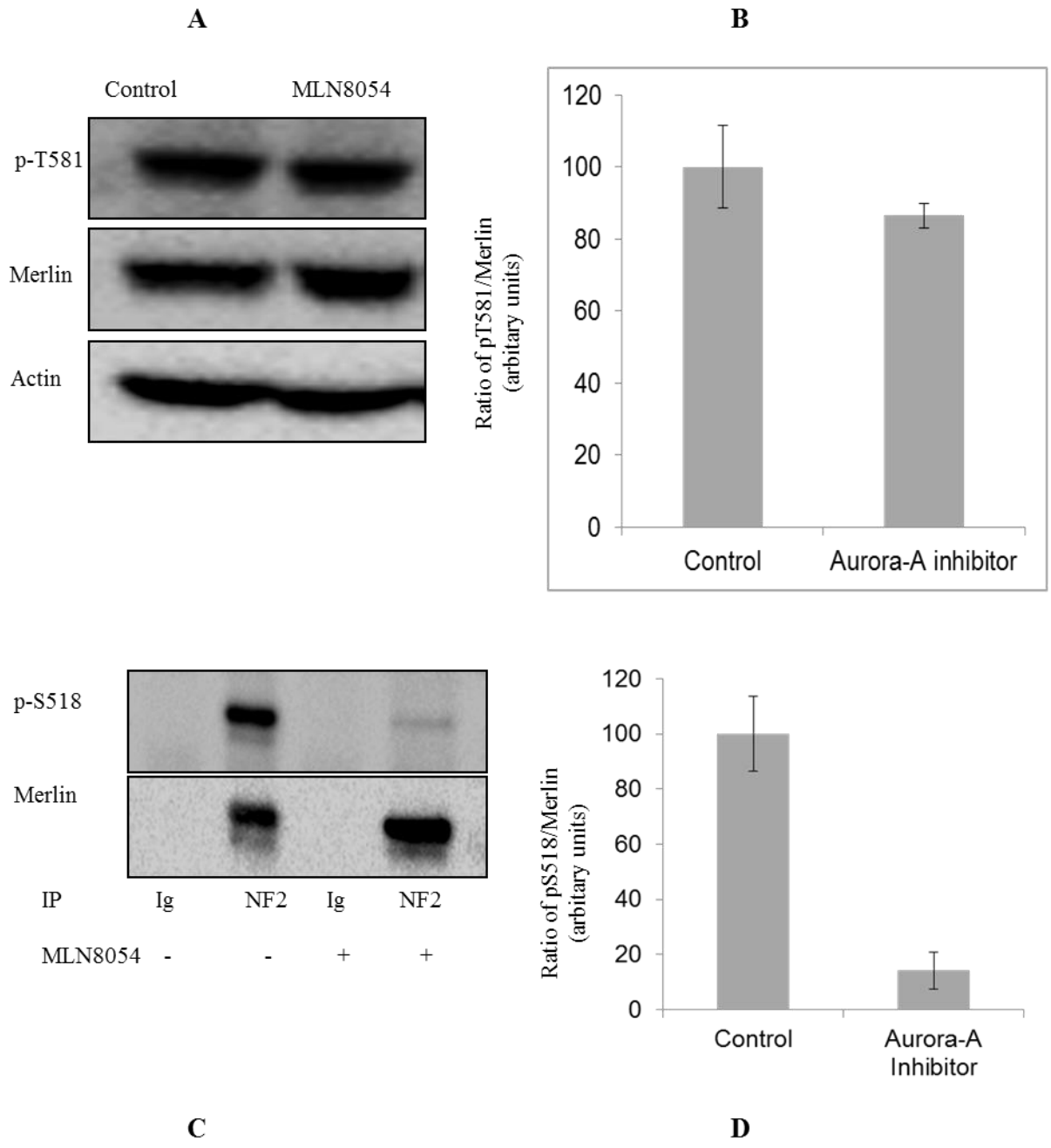


Figure 32. Aurora A phosphorylates Merlin at S518 during Mitosis but not T581. A. Western blot analysis of p581and Merlin proteins from cell lysates of Hela cells over expressing the Merlin that was synchronized in mitosis treated or not with Aurora A inhibitor. B. Quantification of three independent experiments corresponding to blot A with standard deviations. C. Western blots analysis of p518 from cell lysates of Hela after performing the immunoprecipitation of endogenous Merlin in mitotic cells. B. Quantification of three independent experiments corresponding to experiment C with standard deviations.

3.7 Aurora kinase A binds to FERM domain of Merlin

Next we tested whether Aurora A binds to Merlin in order to phosphorylate it. To test this interaction as well as to map the binding region on Merlin, an immunoprecipitation of GFP tagged Merlin constructs (FERM domain of Merlin as wells as C-ter deletion constructs of Merlin) was performed after transfecting them to HEK 293 cells. Interestingly, we found that Aurora A is able to bind Merlin full length but not to the C-terminus constructs. We then showed that Aurora A co-IP with the GFP-FERM domain alone (**Fig33A**). Having mapped Aurora A binding domain on FERM domain of Merlin, we tested whether FERM mutant(▲ 2-3 of Merlin) that harbors a patient deletion from amino acids 39-121 could still interact with Aurora A. We transfected HEK 293 cells with GFP-tagged version of full length Merlin, ▲ 2-3 mutant of Merlin and only FERM domain of Merlin. Interestingly we found that Aurora A binds to wild-type full length Merlin as well as FERM domain of Merlin but not to ▲ 2-3 mutant of Merlin (**Fig 33 B**). Hence, it appears that some of the mutations that affect NF2 patients result in the inability for Aurora A to bind and likely to phosphorylate Merlin.

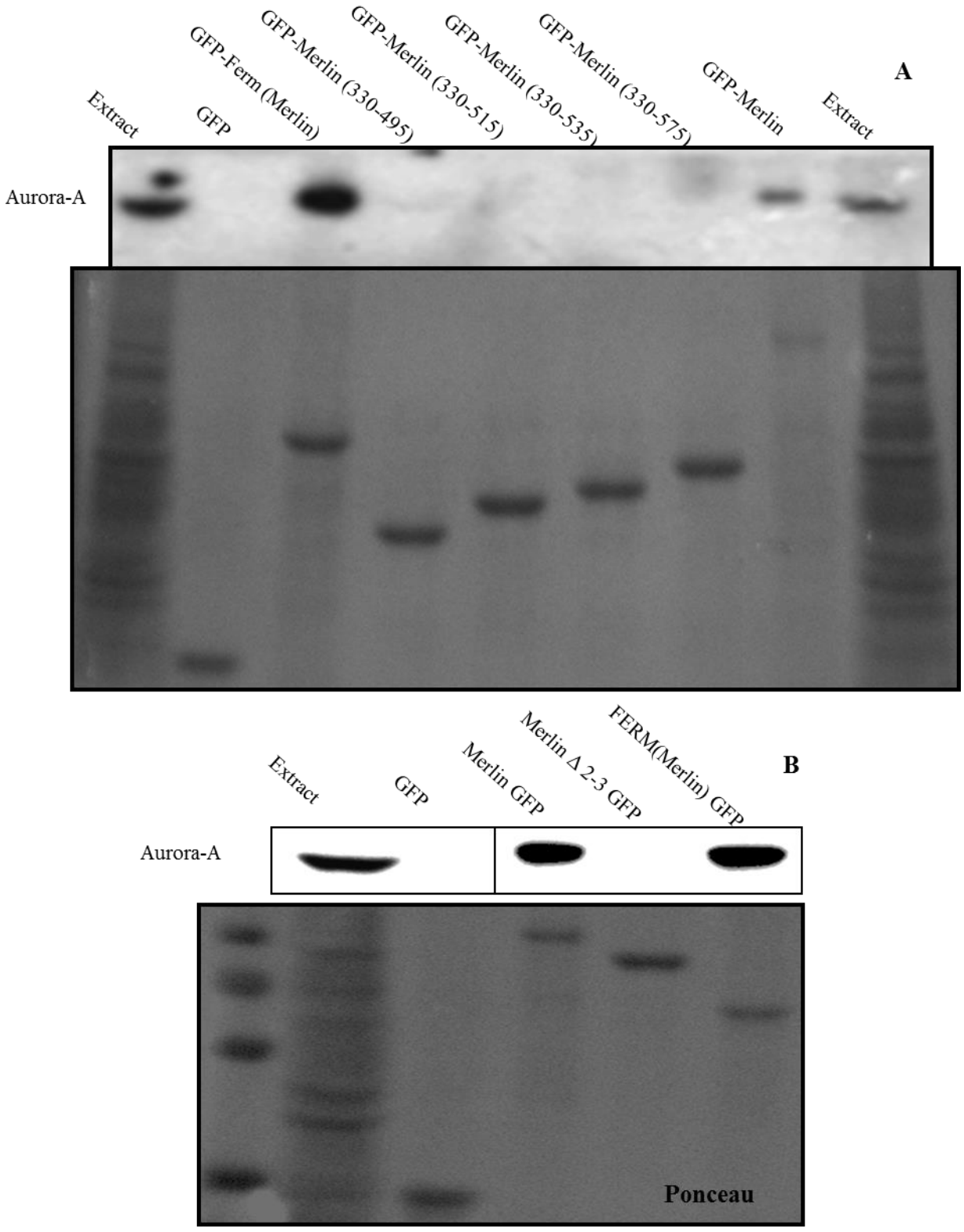


Figure 33. Aurora Kinase A binds to FERM domain of Merlin but not to FERM mutant (▲ 2-3 mutant) of Merlin. A. Western blot for Aurora A after performing the immunoprecipitation (IP) of GFP- Merlin constructs from HEK 293 cell lysates. B. IP of indicated GFP-tagged Merlin constructs were performed and analyzed for Aurora A using specific antibody.

3.8 Mitotic kinases (LATS, SLIK) and PKA, PAK do not seem to phosphorylate Merlin at 581 during mitosis.

In order to identify the kinase that phosphorylates the Merlin at T581 during mitosis, first we focused on the canonical mitotic kinases such LATS and Slik. Previously it has been shown that LATS 1 is a part of mitotic exit network kinase and its depletion results in mitotic defects, including failure of centrosome maturation and spindle organization and cytokinesis defects (Bothos et al., 2005; Yabuta et al., 2007). In addition, other studies also have shown that Aurora Kinase-A phosphorylates LATS 2 upon mitotic entry which in turn is important for the mitotic progression (Yabuta et al., 2011). In this context, we have observed that LATS binds to FERM domain of Merlin in our immunoprecipitation of experiments**.** Based on these results, we hypothesized that Merlin might be a potential substrate for the LATS during mitosis. To test this hypothesis, we co-transfected HEK 293 cells with vectors expressing LATS 2 and Merlin, and given nocodazole treatment for overnight. Whole mitotic cell extracts were prepared and analyzed for the phosphorylation status of Merlin at T581 compared to cells not transfected with LATS vector. In this condition, we found that LATS 2 is not phosphorylating Merlin at T581 (**Fig 34A**). In a further step, we checked if Slik could phosphorylate T581. It has been shown that, in *Drosophila,* Slik coordinately phosphorylates Merlin and Moesin (Hughes and Fehon, 2006b). However, the phosphorylation site on D-Merlin does not correspond to T581 in human sequence which has no clear equivalent in *Drosophila*. Using two different siRNA, we knocked down the endogenous Slik in Hela tet-on Merlin cells and extracts were prepared after giving an overnight nocodazole treatment to collect the cells in mitosis. Then we looked for T581 phosphorylation status by western blot and we found that knocked down of Slik did not affect the T581 phosphorylation compared to control siRNA indicating that Merlin is likely not a substrate for Slik during mitosis (**Fig 34B**). Finally we also tested whether two known protein kinases for Merlin in interphase, PKA and PAK, might phosphorylate it during mitosis. Using the kinases specific inhibitor (PKA inhibitor fragment 14-22 and IPA3), we did not find any effect on T581 phosphorylation status compared to mock treated cells (data not shown**).** Collectively these results suggest that LATS, Silk, PKA and PAK do not phosphorylate the Merlin at T581 under the conditions we used. Identifying the protein kinase which phosphorylates Merlin at T581 position during mitosis is still under progress and we hope that we will catch the kinase that is playing the hide and seek game with us in a future life.

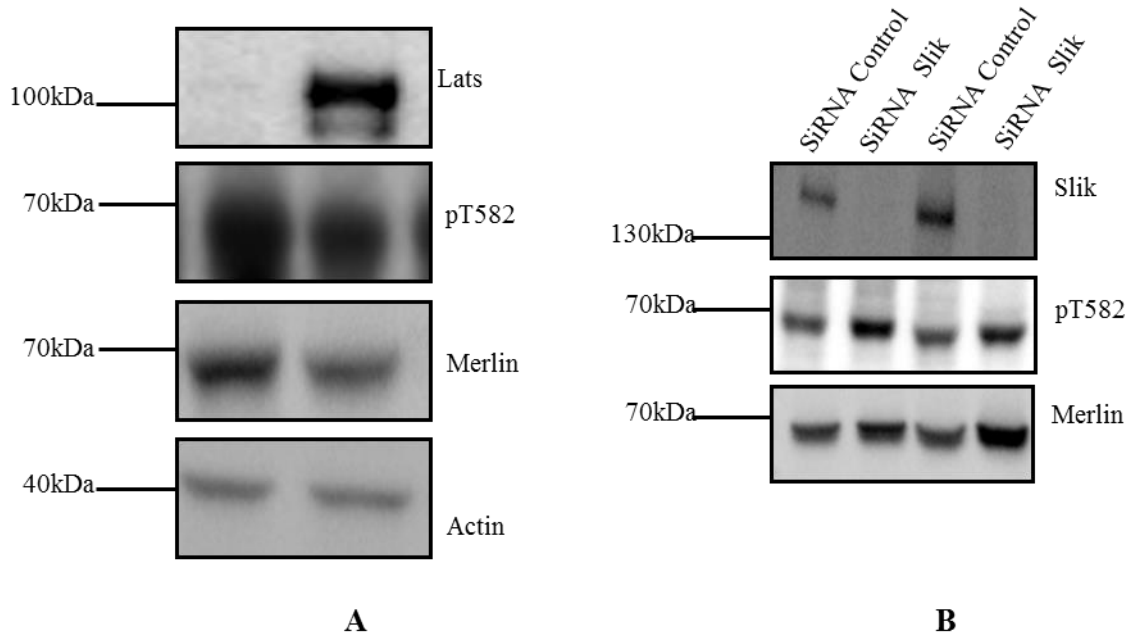


Figure 34. LATS and Slik don't phosphorylate Merlin at T582. A. HEK 293 cells co-transfected either with GFP and Merlin expression vectors or with LATS and Merlin expression vectors. Cells were synchronized in mitosis and analyzed for pT581 and Merlin by western blot. B. Slik expression is knocked down in Hela cells over expressing Merlin using two different siRNA and analyzed for pT581 and Merlin by western blot.

3.9 Phosphorylation of S518 facilitates the phosphorylation of T581 during mitosis

Since we identified the new phosphorylation in the close proximity of S518, we tested whether there is any crosstalk between S518 and T581 phosphorylation of C-terminus Merlin. To test the interplay between these two sites, we used Hela Tet On cell lines expressing the phospho-mutants of S518 site (Merlin 518A and Merlin 518D) and T581 site (Merlin 581A and Merlin 581D). Using these cell lines we tested the effect of A or D mutation of one site on the phosphorylation of the second site in Merlin during mitosis. Overnight nocodazole treatment was given after 24 hours of induction of Merlin phosphomutants expression. The phosphorylation status of each site was evaluated by western blot. We found that the phospho-mimetic form of Merlin S518D showed significant increase in the phosphorylation of T581 by four fold when compared to S518 A (**Fig 35A and B**). In contrast, when we expressed the T581 mutants (Merlin 581A and Merlin 581D) we did not find any difference on the phosphorylation status of S518 of Merlin during mitosis (**Fig 35 C and D**). Hence, we show that phosphorylation of S518 facilitates the phosphorylation of T581 during mitosis. Our results are reminiscent of previous observations showing that S518 phosphorylation increases the phosphorylation of S10 at N-terminus of Merlin (Laulajainen et al., 2011). Whether this

affect is due to a change in conformation facilitating interaction with the kinase that phosphorylates T581 deserves further investigation.

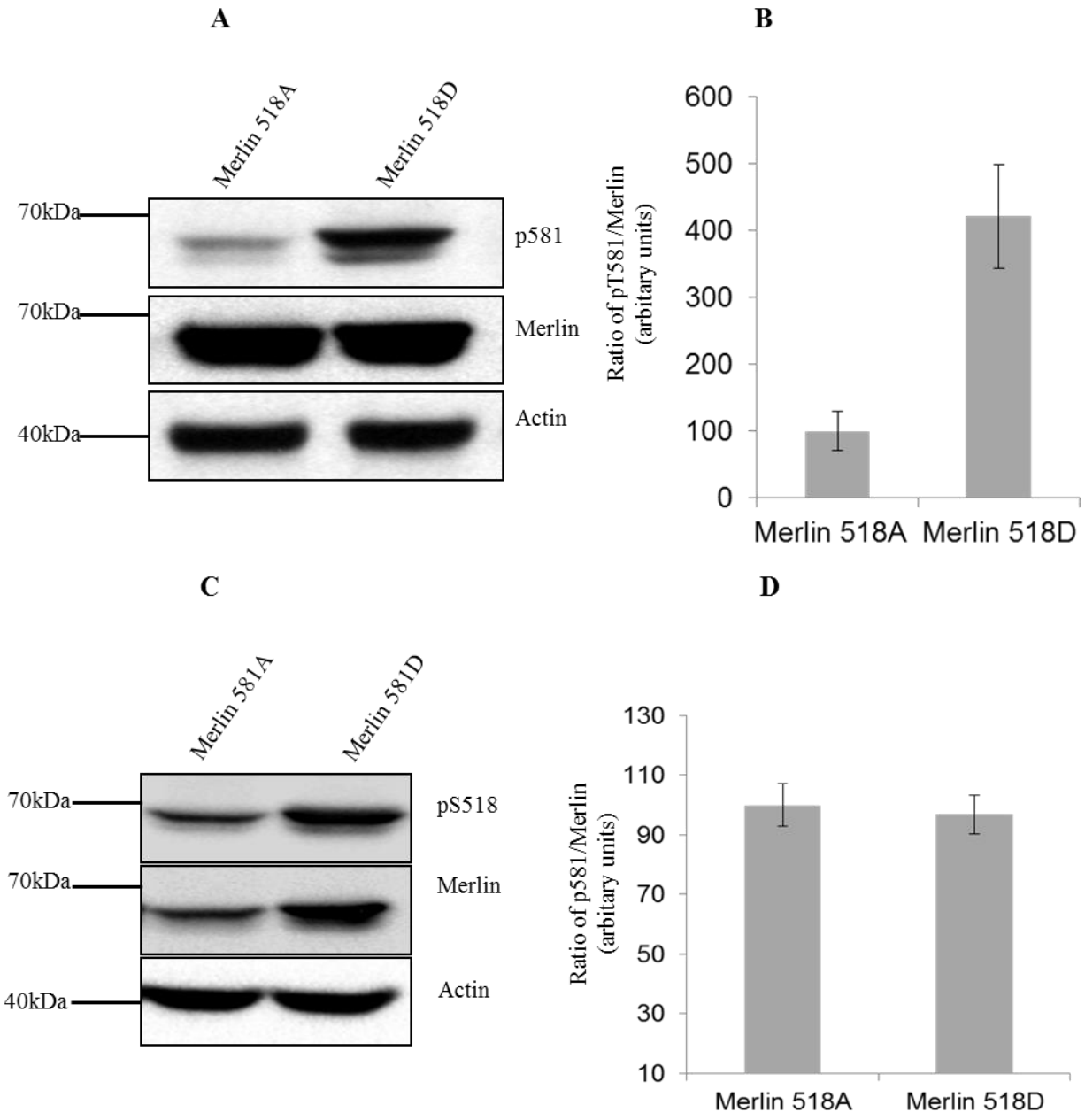
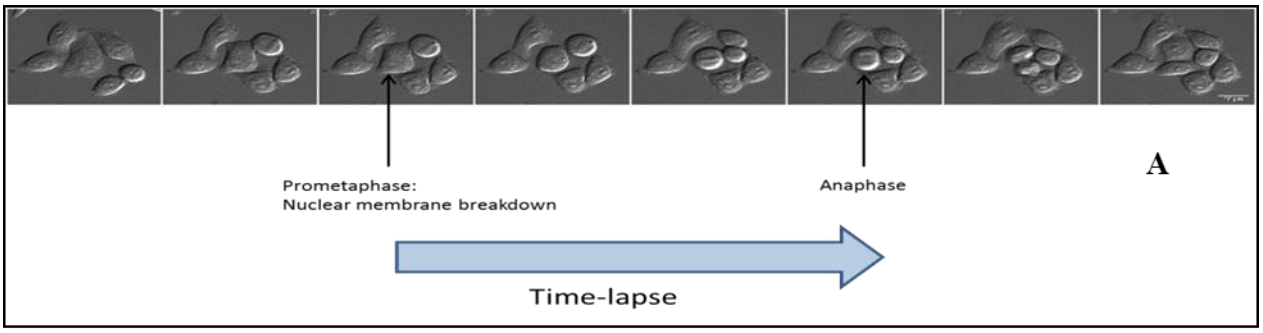
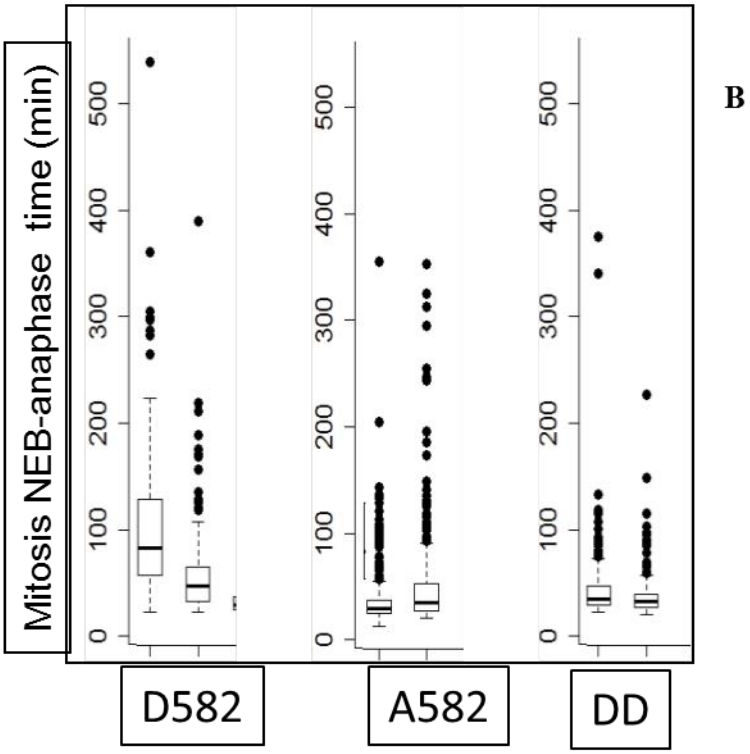


Figure 35. Phosphorylation of Merlin at S518 favors the phosphorylation of T581 but not vice versa. A. Hela cells overexpressing indicated Merlin phosphomutants and analyzed for pT581 and Merlin. Cells were synchronized in mitosis as described previously. B. Quantification of three independent experiments corresponding to experiment A. C. Hela cells overexpressing indicated Merlin mutants and analyzed for pS518 and Merlin. Cells were synchronized in mitosis as described previously. D. Quantification of three independent experiments corresponding to blot C.

3.10 Coordinated phosphorylation of Merlin at S518 and T581 residues is important for timely mitotic progression

To investigate if phosphorylation of S518 and T581 have a role during mitosis, we measured the time required from nuclear envelope breakdown (mitotic entry) to sister chromatid separation (Anaphase) in Hela Tet-on cells over-expressing the T581 phosphomutants alone (581A or 581D) or in combination with S518 phosphomutants (518A581A or 518D581D). To test the effect of these mutants on mitotic progression, we synchronized the cells in S phase using thymidine block and after 16 hours, released the cells from the block by changing the media to let them progress quite synchronously toward mitosis. Next we investigated the mitotic progression of these cells using video microscopy. In these experiments, we found that overexpression of T581D lead to a delay (from 58 minutes to 101 minutes) in mitotic progression compared to no expression of this mutant (**Fig 36**). In contrast, over expression of a non-phosphorylatable form of S581 (581A) shortened (from 58 minutes to 38 minutes) the time required for the mitotic progression compared to no expression of this mutant (**Fig 36**). In addition, we also overexpressed phosphomimetic double mutant of Merlin on S518 and T581 (518D581D) to see its effect on mitotic progression and interestingly we found no significant change in the time required for the mitotic progression compared to no expression (**Fig 36**). Finally, double A mutant for S518 and T581 lead to slightly longer mitosis. In the end, it appears that DD mutant (518D581D), that mimics normal double phosphorylation during mitosis does not alter mitotic progression. On the contrary, AA (518A581A) delays mitotic progression. Interestingly, single mutants of T581 strongly alter mitosis suggesting that coordinated phosphorylation of Merlin at S518 and T581 is crucial for proper progression of mitosis.





Doxycycline	+	-	+	-	+	-
Duration	101	58	36	58	45	37
P value	2^{e}-16		2^{e}-16		0,006	
Δ	+43 min		-22 min		+8min	

Figure 36. Coordinated phosphorylation of Merlin at S518 and T581 residues are important for timely cell cycle progression. A. Schematic representation showing the window from nuclear breakdown to anaphase we used to test the effect of Merlin phosphorylation at T581 on mitotic progression (see the text for more details). B. Quantification of time for mitotic progression when phosphomutants are synchronized in mitosis. Expression of phosphomutants in Hela cells are induced by addition of Dox (+). (-) not induced. Duration, P value and D indicates time difference between with (+Dox) and without (–Dox).

3.11 Phosphorylation of Merlin at Threonine 581 inhibits Merlin and Ezrin heterodimerization

In order to understand the possible molecular mechanism of the mitotic delays, we hypothesized that phosphorylation of T581 residue may affect Merlin interaction with its partners involved in cell cycle progression. To test this, first we focused on Merlin interacting partner Kibra, because it has been shown that Kibra is also phosphorylated by Aurora A at Serine 539 during mitosis and that event affects the interaction with Merlin (Xiao et al., 2011). To investigate the effects of T581 phosphorylation, we transfected the HEK293 cells with Kibra and GFP tagged phosphomutant forms of T581 (581A or 581 D). Then we employed GFP trap of tagged phosphomutants of T581 and looked for Kibra by western blot. In this experiments we observed that Kibra was able to bind equally to both phosphomimetic form (581D) as well as to non-phosphorylatable form (581A) showing that phosphorylation of T581 does not affect the interaction between Merlin and Kibra (**Fig 37A**).

Next we investigated the role of T581 phosphorylation on the interaction between Merlin and ERM. It has been shown that Moesin is phosphorylated by Slik and its activation is important for the cortical stability and its remodeling, and microtubule organization in mitotic cells (Kunda et al., 2008; Roubinet et al., 2011). However the cross talk between Merlin and ERMs in mammalian cells remains to be elucidated during mitosis. Very recently it was demonstrated that Merlin is indirectly involved in the positioning of interphase centrosome by regulating Ezrin localization. Based on these results, we hypothesized that phosphorylation of T581 might plays a role in Merlin and Ezrin heterodimerization. Previously it has been shown that the C-terminus of Merlin interacts with the FERM domain of Ezrin (Grönholm et al., 1999; Lallemand et al., 2009a). We then investigated whether phosphorylation of T581 affects heterodimerization between Merlin and Ezrin *in vitro*. To test this we used immunoprecipitated GFP-tagged C-terminus Merlin constructs mutated at S518 (518D and 518A) or T581 (581D and 581A) alone, and also in combination (518D581D and 518A581A).

We incubated bound immunopurified C-terminus mutants constructs of Merlin with purified myc tagged N-terminus Ezrin (myc). Following extensive rinses, binding capacity was revealed by western blot using an antibody against myc. Interestingly, we found that phosphomimetic from of T581 (581D) binds much less efficiently to Ezrin compared to non-phosphorylatable form of T581 (581A) (**Fig 37**). In addition introducing another mutation at S518 (518D581D) weakens the interaction with Ezrin compared to non-phosphorylatable double mutant form (518A581A) (**Fig 37**). In contrast, we did not see any strong effect of S518 phosphomutants alone (518A or 518D) on Merlin interaction with Ezrin contrasting with earlier reports (**Fig 37**). Collectively these results demonstrated that the new phosphorylation which we identified at T581 residue plays a major role in Merlin heterodimerization with Ezrin. While we were performing these experiments we also confirmed that the C-terminal half of Merlin isoform 2 does not bind to Ezrin FERM domain. In a sense, the phosphorylation of Merlin isoform 1 on T581 converts it to an isoform 2 like isoform in terms of Ezrin binding.

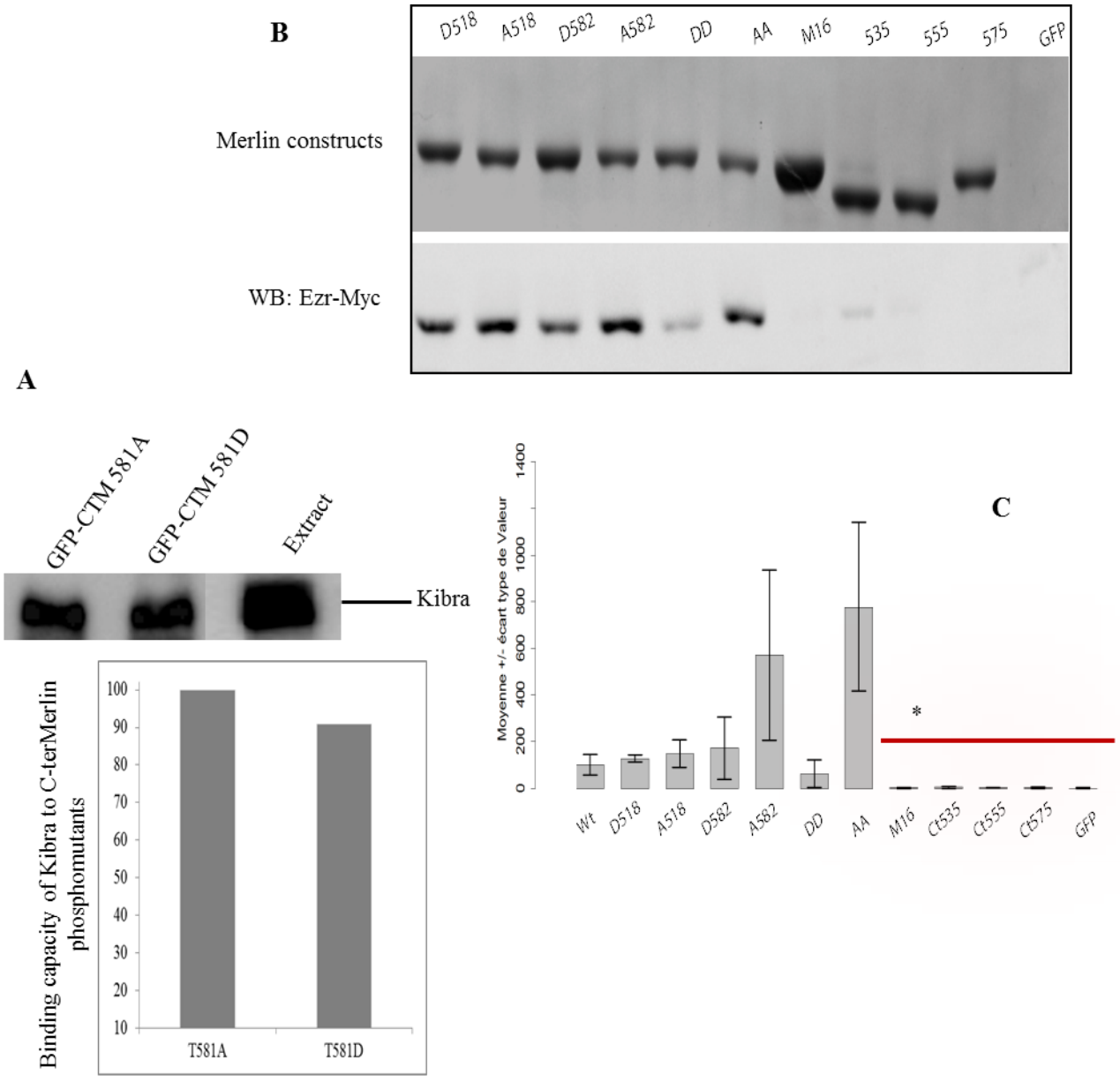


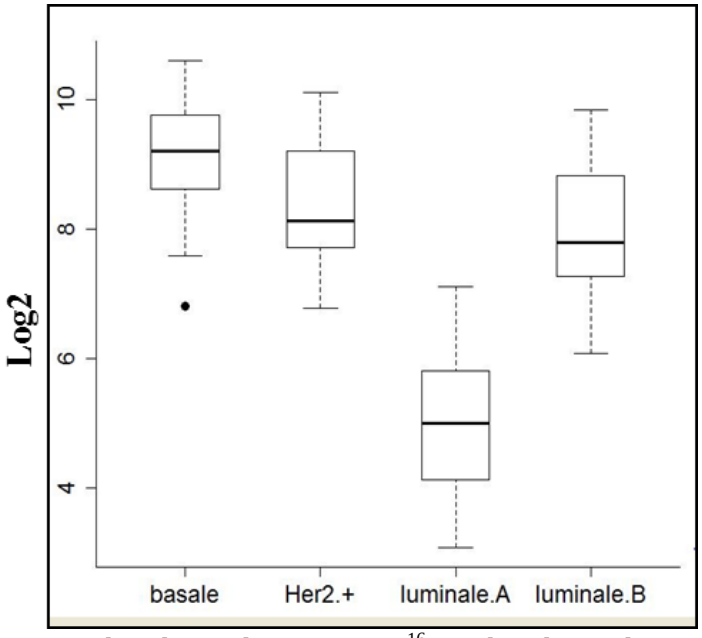
Figure 37. Phosphorylation of Merlin at Threonine 581 inhibits Merlin and Ezrin heterodimerization. A. HEK 293 cells transfected with GFP-C-ter Merlin constructs and IP of GFP performed and analyzed for Kibra by western blot. B. HEK 293 cells were transfected with indicated GFP tagged Merlin constructs. These constructs were immunoprecipitated using GFP. Purified constructs were incubated with recombinant FERM domain of Ezrin tagged with myc and analyzed for the binding capacity for Ezrin using myc antibody by western blot. C. Quantification of three independent experiments corresponding to blot A. * denotes background of level of binding.

3.12 Aurora A phosphorylates Merlin in interphasic breast cancer cells: implications for tumor cell proliferation.

As we discussed previously, Aurora A activity is up regulated in various cancers including breast cancer. In addition, we have shown that Aurora A phosphorylated Merlin at 518 during mitosis. This suggests that Aurora A could also phosphorylate Merlin during interphase when its activity is abnormally elevated. Aurora A activity is known to be strongly up-regulated in various malignancies, notably breast cancer. Curie Institute is a reference center for the treatment of breast cancer and disposes of a huge bio-bank of breast cancer tissues. In a first step, we carried out a transcriptional analysis of Aurora A on a set of 150 breast tumors to evaluate Aurora A expression in function of the tumor subtype. This was done from transcriptomic analysis data already available at the institute. Interestingly, we observed that the more aggressive breast cancer subtype, the basal like subtype, expresses higher levels of Aurora A transcripts. Interestingly, up-regulation is significant when compared to HER2 ($P = 0.0049$), to luminal A ($P < 2*10^{-16}$) and to luminal B subtypes ($P < 3.3*10^{-5}$) (**Fig 38**). To evaluate an eventual cross talk between Aurora A and Merlin in breast cancer cells, we tested, by western blot, the levels of Aurora A, p-Aurora A, Merlin and p-Merlin (p-S518) in a set of 13 breast cancer cell lines of basal subtype. Remarkably, 10 out of 11 cell lines showed a parallel pattern of expression levels between p518 and p-Aurora A proteins (**Fig 39**). Two additional cell lines did not express Merlin. In addition, in these experiments we found that the levels of p-Aurora A matched those of Aurora A suggesting that the levels of activity of Aurora A (evaluated by p-Aurora staining) are essentially a consequence of the variations of Aurora A expression levels in these cell lines. These results suggest that, in breast cancer cell lines, overexpression of Aurora A may lead to higher levels of pS518 Merlin.

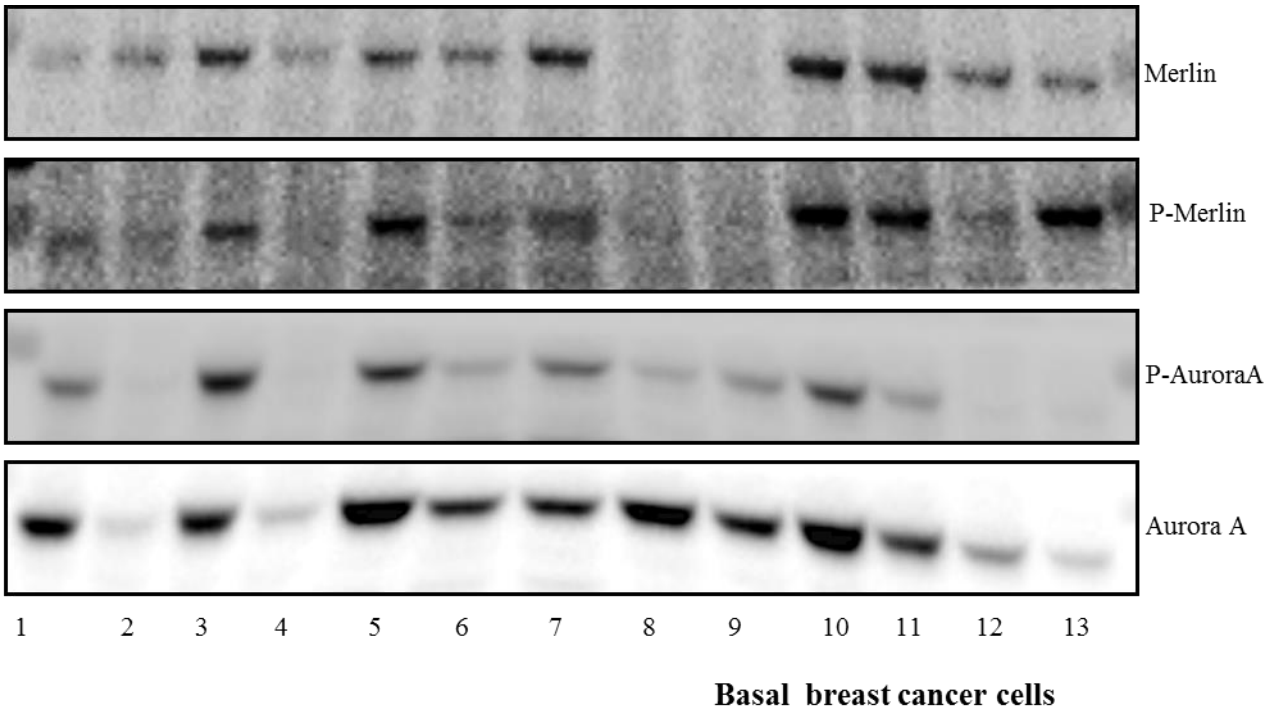
Previously, we have shown that pharmacological inhibition of Aurora A results in decreased phosphorylation of Merlin at S518 during mitosis. Similarly, we decided to test the effect of the inhibition of Aurora A activity on S518 during interphase in breast cancer cell lines. To do so, we took two cell lines (BT20 and HCC 1937- highlighted in red in **Fig. 39**) which are basal breast cancer cell lines expressing significant levels of Aurora A, and inhibited the activity of Aurora A with specific inhibitor MLN8054. Interestingly, we found that the

inhibition of Aurora A activity decreases the phosphorylation of Merlin on S518 significantly compared to mock control cells as analyzed by western blot (**Fig 40A-D**). However, we did not see complete inhibition of phosphorylation of Merlin on S518, suggesting that during the interphase Merlin might also be phosphorylated by other kinases such as PAK and PKA as described previously. In conclusion, in breast cancer cell lines, the overexpression of Aurora A results in hyperphosphorylation of Merlin at S518. As the phosphorylation of Merlin on S518 has been reported to inhibit its growth suppressive activity, we propose that elevated Aurora A levels lead to a decrease in Merlin activity.



Basal vs. Her2 P=0, 0049. Basal vs. luminal A $P< 2*10^{-16}$ Basal vs. luminal B $p<3, 3 * 10^{-5}$

Figure 38. A Transcriptional analysis of Aurora A in various breast tumors subtypes. Quantification of Aurora A transcripts in basal breast cancer subtypes as indicates. P-value is given in comparison to Basal as it is the most aggressive subtype (Kruskall-wallis test).



1	**BT20**
2	HCC70
3	HCC1143
4	HCC1187
5	**HCC1937**
6	MDA-MB-468
7	HCC38
8	MDA-MB-231
9	MDA-MB-436
10	MDA-MB-157
11	BT-549
12	Hs 578T
13	MCF-10A

Figure 39. Effect of Aurora A activity on Merlin phosphorylation status at S518. A. Expression analysis of Aurora A, p-Aurora A, p-S518 and Merlin expression levels in a serie of 13 breast cancer cell line extracts was performed by western blot. The different cell lines are mainly derived from the basal subtype tumors and are shown in the table below.

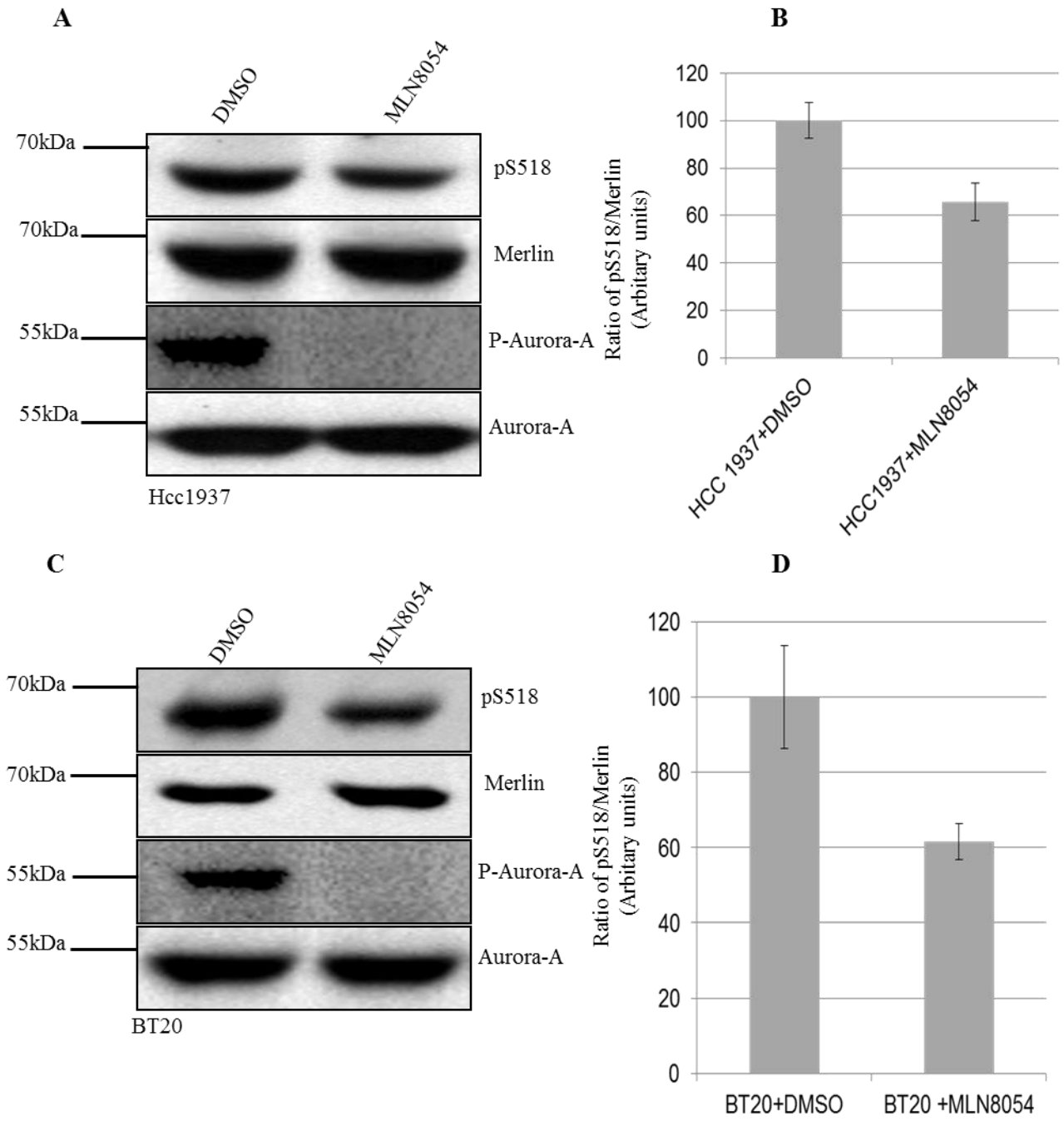


Figure 40. Aurora kinase A phosphorylates Merlin at S518 *in vivo.* A. Cell lysates from HCC1937 cells were analyzed for the indicated proteins with and without Aurora A inhibitor (MLN8054). Actin is used as a loading control. B. Quantification of three independent experiments corresponding to blot A. C. Cell lysates from BT20 cells were analyzed for the indicated proteins with and without Aurora A inhibitor (MLN8054). Actin is used as a loading control. D. Quantification of three independent experiments corresponding to blot C.

3.13 Aurora kinase A co-localize with Merlin in the cytosol as well as in the nucleus in BT20 cells.

Next, we investigated where, in interphasic BT20 cells, Merlin and Aurora A might co-localize. To answer this question, first we looked at the localization of Aurora A when it is overexpressed. In this experiment we transfected Hela cells with GFP tagged Aurora A and checked its localization by immunofluorescence. Interestingly, we found a major portion of Aurora A localized in the nucleus. Then we turned to endogenous Aurora A, which is overexpressed in BT20 cells and observed a similar pattern of distribution with a clear staining in the nucleus (**Fig 41 A**). Furthermore, we also observed that a pool of Aurora A also localizes in the cytosol. To evaluate where Merlin and Aurora A might interact, we employed Duolink technique as described previously. By confocal microscopy, we found that these proteins interact both in cytosol and nucleus with higher levels of detection in the nucleus (**Fig 41B**). All together our results indicate that Merlin and Aurora A co-localize both in cytosol and nucleus of interphasic BT20 cells, with a larger pool in the nucleus. In accordance with our results, one study showed that the capacity of Aurora A to transform cells when overexpressed was directly correlated to its presence in the nucleus (Tatsuka et al., 2009).

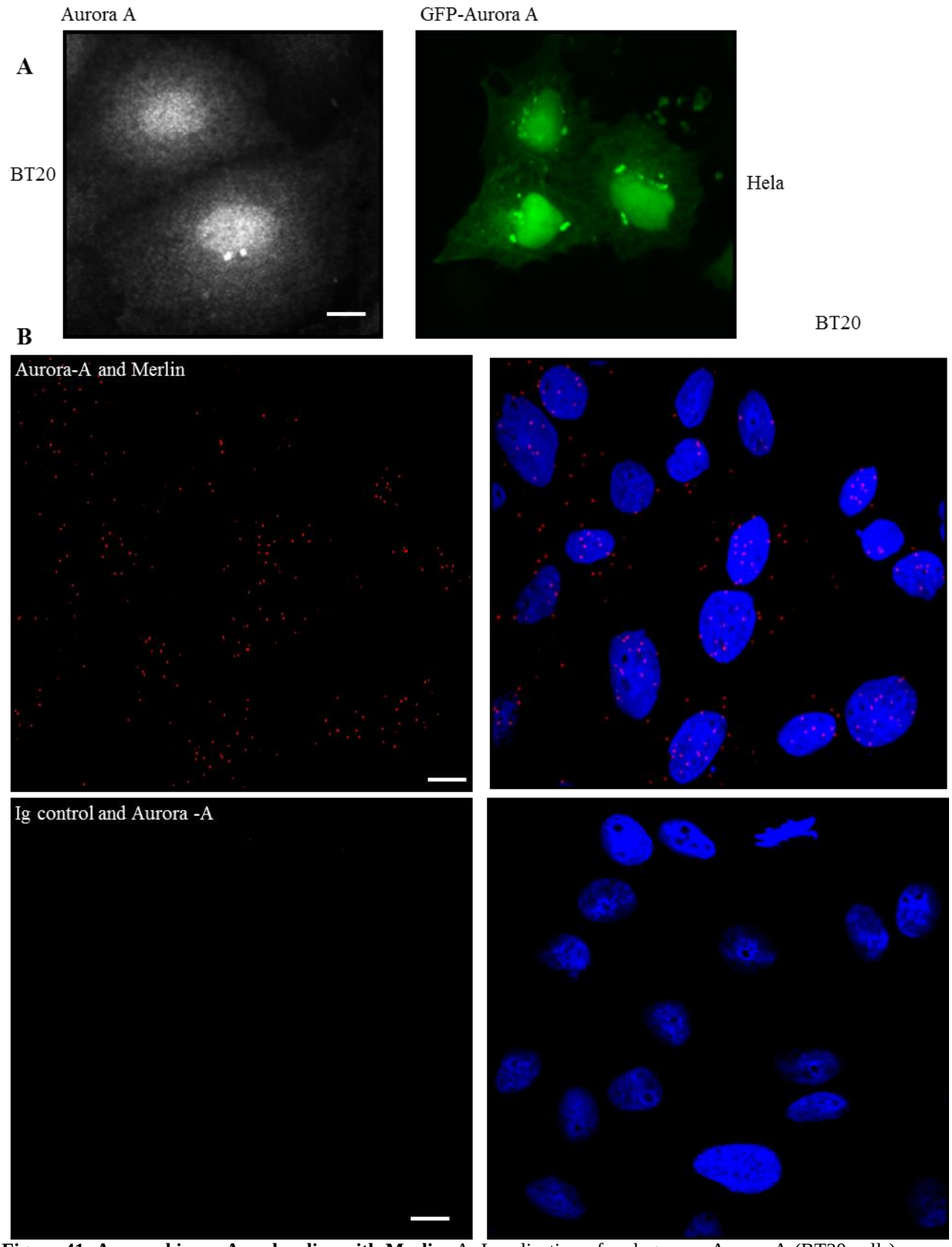


Figure 41. Aurora kinase A co-localize with Merlin. A. Localization of endogenous Aurora A (BT20 cells) and overexpressed GFP Aurora A in Hela cells. B. Co-localization of endogenous Merlin and Aurora A by Duolink experiment. Images were acquired by confocal microscopy. Scale bar=10um.

3.14 Aurora kinase A phosphorylates Merlin at S518 residue inside the nucleus

To identify the cellular compartment where Aurora A phosphorylates S518 of Merlin, we performed subcellular fractionation of BT20 cells. In this experiment, we have grown the BT20 cells up to confluence in order to minimize phosphorylation by other kinases such as PAK or PKA and then we added the Aurora A specific inhibitor to the media for an additional 16hrs. BT20 cells were then subjected to subcellular fractionation and the purity of the fractions was tested by Histone H3 (nuclear marker) and pYAP 127 (cytosol). Proteins from the nuclear (NF) and nonnuclear (CM; cytosol + crude membranes) fractions were analyzed by western blot to evaluate the status of S518 phosphorylation with and without Aurora A inhibitor using the specific antibodies. Interestingly, we found that levels of phosphorylated 518 (p-S518) decreased upon MLN8054 treatment and that total levels of Merlin increases in the nucleus in presence of Aurora A inhibitor (**Fig 42**). Collectively these results suggest that in the presence of active Aurora A, Merlin is phosphorylated at S518 inside the nucleus and in the cytosol. It has been proposed that hypophosphorylated Merlin translocates into the nucleus whereas phosphorylated Merlin on S518 cannot (Li et al., 2010). This could explain the elevated levels of Merlin in the nucleus when cells are treated with Aurora A inhibitor (**Fig 42**). Phosphorylation of Merlin may exist in the nucleus by direct phosphorylation mediated through Aurora A in this compartment. Inhibition of Aurora A also decreases the phosphorylation of Merlin in the cytosol and possibly at the plasma membrane. Total Merlin levels decrease in the cytosol which could be due to increase in translocation of up-regulated Merlin to the nucleus (**Fig 42**). In support of our results, one study showed that phosphorylated Merlin at S518 (p518) could be found in the nucleus but however, the underlying mechanism involved in its nuclear localization was not explained (Muranen et al., 2005). We show that Aurora A possibly phosphorylates Merlin at S518 inside the nucleus.

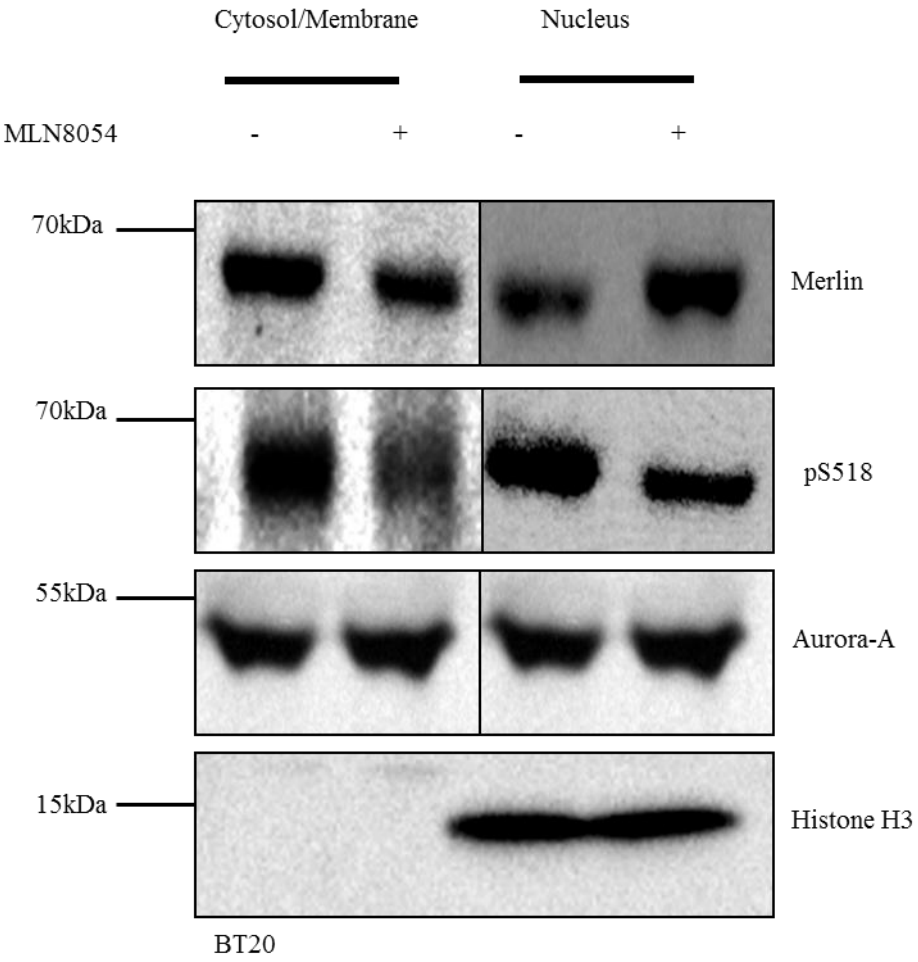


Figure 42. Merlin is phosphorylated inside and outside the nucleus by Aurora kinase A. BT20 cells were subjected to subcellular fractionation. Proteins from the nuclear (NF) and non-nuclear (CM; cytosol + crude membranes) fractions were subjected to immunoblotting with antibodies to the indicated proteins. Aurora A is used as internal control for loadings.

3.15 Consequences of Merlin phosphorylation by Aurora A kinase on Yap activity

Merlin inhibits YAP activity by facilitating its phosphorylation on S127, hence leading to cytosolic retention by 14-3-3 in the cytosol (Zhao et al., 2007). We tested total Yap and p-YAP levels, in nuclear and non-nuclear compartment after treating BT20 cells with Aurora A inhibitor and compared to untreated cells. By western-blots, we observed that the levels of p-YAP increase slightly in presence of Aurora A specific inhibitor in the cytosol without change in total levels of YAP (**Fig 43**). In this context, we looked for the levels of total YAP in nucleus in presence of inhibitor and we found that total YAP levels decreased slightly compared to control (**Fig 43**). Based on these results we think that inhibition of Aurora A activity in the cytosol leads to increased levels of un-phosphorylated Merlin (concerning S518 at least) and which in turn results in the activation of Hippo pathway to phosphorylate the co-transcription factor YAP. This phosphorylation of YAP inhibits its translocation into nucleus. For this reason we saw more p-YAP in the cytosol and less active un-phosphorylated YAP in the nucleus in presence of Aurora a specific inhibitor. Concerning the nuclear localization and function of Merlin, one study elegantly demonstrated that hypo-phosphorylated Merlin localized to nucleus in confluent cells and binds to an E3 ubiquitin ligase known as DCAF1 leading to the inhibition of cell proliferation. Based on these preliminary results we think that Aurora kinase A inhibits the growth suppressive function of Merlin in two subcellular compartments. First Aurora A binds to the FERM domain of Merlin and phosphorylates S518 residue in the nucleus, which in turn abolishes the binding of Merlin to DCAF, an E3 ubiquitin ligase directly involved in cell proliferation (Li et al., 2010). Experiments are still in progress to demonstrate that phosphorylation of Merlin at S518 by Aurora A inhibits the interaction between Merlin and DCAF1 in the nucleus. We also think that Aurora kinase A phosphorylates Merlin at S518 in the cytosol, which results in the inactivation of Hippo pathway. This in turn increases the pool of active YAP that enters into nucleus to promote the cell proliferation.

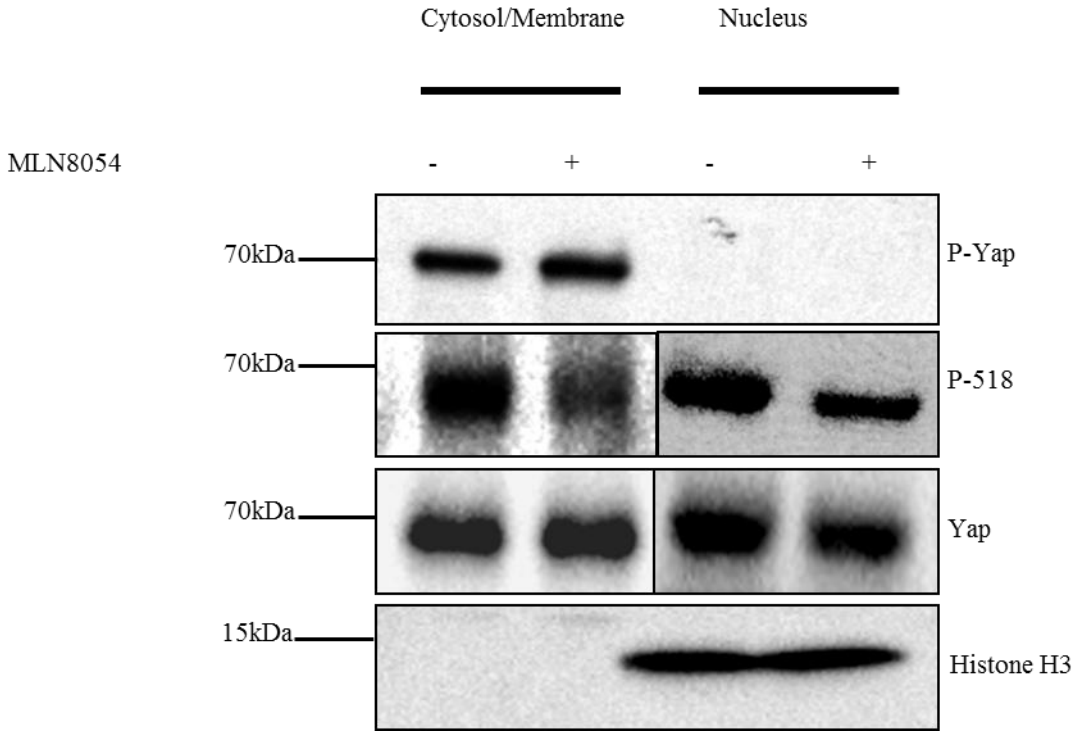


Figure 43. Inhibition of Aurora activity leads to Yap phosphorylation and Yap decrease in the nucleus. BT20 cells were subjected to subcellular fractionation. Proteins from the nuclear (NF) and nonnuclear (CM; cytosol + crude membranes) fraction were analyzed by immunoblotting with antibodies to the indicated proteins.

In conclusion, our data show that the overexpression of Aurora A that is observed in various type of cancers can lead to the phosphorylation of Merlin on S518, hence reducing its activity as a proliferation inhibitor. We propose evidences that this regulation of Merlin directly affects the activity of the Hippo effector Yap and also likely the ubiquitin ligase DCAF activity. Our study therefore shows that Merlin may play a role in cancers that present no mutations of the *NF2* gene. It also demonstrates a link between two previously unrelated signaling pathways such as Hippo and Aurora A pathways and their importance for cancer.

DISCUSSION AND PERSPECTIVES

Emerging tumor suppressor mechanisms of Merlin via its C-terminus domain

Biallelic inactivation of *NF2* tumor suppressor gene is responsible for the development of intracranial tumors in humans such as schwannomas, meningiomas and ependymomas. Although NF2 is a rare disease, population studies suggest that these tumors represent the second most frequent type of intracranial tumors following gliomas. In addition several studies demonstrated that loss or inactivation of *NF2* results in other malignancies such as mesotheliomas (Sekido, 2011), gliomas (Lau et al., 2008), breast cancers (Morrow et al., 2011) and melanomas (Murray et al., 2012). The major therapeutic strategy to cure the NF2 disease is the surgical removal of the tumors and is associated with high morbidity and mortality due to their close proximity to the brain. Till date, there is no chemotherapy based treatment for NF2 disease. This is due to the poor knowledge in the understanding the mechanisms of tumorigenesis associated to the loss of *NF2* expression. In this scenario, defining the physiological function of *NF2* gene product, Merlin, in detail and understanding the molecular mechanisms involved in the *NF2* related cancers is therefore needed to improve prevention and treatment of these cancers.

Our work was based on the observation that the C-terminal domain of Merlin localized beyond amino acid 532 was essential for its tumor suppressor function. This observation lead us to search for new interactors and new post-translational modifications of this domain and to try to characterize their importance for Merlin tumor suppressor function.

1. Merlin: A Magnet of the Hippo pathway.

Using co-immunoprecipitation and mass spectrometry, we found surprisingly a small number of specific interactors. We essentially identified one family of proteins, the Angiomotin family.

The importance of this interaction will be discussed later. However, based on publications that were contemporaneous to our discovery, it quickly appeared that Angiomotins were linked to the Hippo pathway and interacting with the co-transcription factor Yap. Thus, Merlin is interacting with another component of this pathway, after being shown to interact with Kibra. Interestingly, whereas it is clear that Merlin loss of expression affects the activity of the

downstream Hippo effector Yap (Chan et al., 2011; Zhao et al., 2011b), the mechanisms of this regulation are largely unknown.

Hence, we started to GFP trap, a very efficient coimmunoprecipitation technique, to try to better characterize the "interactome" of the Hippo pathway, with a specific emphasis on Merlin and the newly discovered Amot protein. We found several new interactions and some of which have been published since we observed them. We could show that Merlin co-immunoprecipitate with Yap and Lats, as well as Kibra. We also observed co-immuprecipitation of Kibra with AmotL1.

It is yet still quite difficult to make a clear sense of these many interactions, however, given what is known from the literature about the function of the proteins, some interesting speculations can be made for the role of Merlin in these interactions.

A. Merlin interacts with LATS and YAP

Merlin interacts with Lats via the FERM domain and with YAP through C-terminus domain. DJ Pan's laboratory has shown that LATS phosphorylates co-transcription factor YAP both in Drosophila and mammals mediated by Hippo pathway activation (Zhang et al., 2010a). This event was shown to be mediated by a direct interaction between the WW domains of Yap and the PPXY motifs of Lats1/2. In this context, the possibility exists that Merlin could play a role of a scaffolding protein that would facilitate the phosphorylation of Yap by Lats through interacting with both proteins and bringing them in close proximity. This result is supported by several observations showing that in the absence of Merlin expression, Yap is less heavily phosphorylated and hence translocate to the nucleus more efficiently. It is also possible that Merlin may bring the two proteins together at specific locations in the cells such as cell-to-cell contact or in the nucleus in response to various environmental conditions such as confluency.

In addition, we have observed that a mutant of FERM domain, Merlin Blue Box, is not binding to Lats. The Blue Box mutant of Merlin acts as a dominant negative and is able to trigger proliferation in the fly (LaJeunesse et al., 1998) and transformation of NIH3T3 fibroblasts (Johnson et al., 2002). This raises the possibility that Merlin Blue Box mutant is a dominant negative because it does not bind to Lats and therefore cannot promote Yap

phosphorylation. This hypothesis has not yet been tested but we hope to have an answer in the very near future.

The direct binding of Merlin to Yap also raises the possibility that Merlin can sequester it in the cytosol or at the plasma membrane, preventing nuclear entry, in a similar way to the regulation that was proposed for Yap by Amot proteins (Zhao et al., 2011b). Our observations suggest that Merlin could regulate Yap activity independently of the canonical Hippo cascade by sequestering it but also via the more classical Mst-Lats-Yap axis by bridging together Yap and Lats. Finally, our results show that Merlin is involved in the regulation of the Hippo pathway and its effector Yap at many more levels than were previously expected.

B. Merlin, Kibra and AmotL1 are interacting

Merlin, Amot and Kibra are all defined as scaffolding or platform proteins. Therefore, their mutual interactions suggest that they could form large complexes at specific locations in the cell. Interestingly, all three proteins have been shown to be implicated in cell migration and they have been seen at the leading edge of cells. Kibra and Amot proteins are seen as promigratory but Merlin was shown to inhibit cell migration. The Kibra (kidney and brain protein) is a scaffold or an adaptor-like protein and shown to play many cellular processes such as cell migration (Duning et al., 2008) and mitotic progression (Xiao et al., 2011). Concerning the role of Kibra in cell migration, Rosse et al. have shown that Kibra play an important role in the migration of normal rat kidney (NRK) cells. In their study the authors have shown that Kibra mediates the interaction of aPKC (a protein kinase) with exocyst, a complex of eight proteins also known to be required for exocytosis, by acting as a scaffold protein. This interaction facilitates the localization of this complex at the cell leading edge which in turn results in activation of mitogenic kinases such as JNK (c-jun Nterminal kinase and ERK (extracellular regulated kinase). These protein kinases further activates the paxillin, a component of focal adhesion, by phosphorylation and the final results is more dynamics in focal adhesion, and hence migration. Interestingly inhibition of any of the complex activity results in defects in migration of NRK cells suggesting the importance of this coordinated function of the complex at the leading edge of the cell (Rosse et al., 2009). No link has yet been made between this mechanism and Amot or Merlin functions, however although they both are able to regulate the JNK and ERK pathways. Only point mutations that would disrupt

the binding of Merlin to Kibra or the binding of Kibra to Amot would allow us to tell if these interactions are essential for the regulation of migration.

C. Merlin and AmotL1

The recently discovered interaction between Merlin and the Amot family has been investigated in the context of the cell growth inhibition activity of Merlin. Indeed, Yi et al 2011 have demonstrated that Amot functions downstream of Merlin to promote the growth of Schwann cells by acting as a positive regulator of Rac1. They have shown that Merlin and Amot interact in vitro and in vivo in myelinating peripheral nerves of mice. The interaction masks the binding site of the Rac GAP, Rich1, on Amot. Released Rich1 acts by converting active Rac1 (Rac1-GTP) to inactive Rac1 (Rac1-GDP). Instead, under growth promoting conditions, Merlin becomes inactive and uncouple from the Amot. This in turn allows Amot to bind to Rich1 and finally results in the activation of Rac1 to promote the growth (Yi et al., 2011).

However, the Amot family has been shown to promote cell migration suggesting it could play a role in the migratory phenotype that was observed in cells upon *NF2* inactivation. This phenotype has rarely been investigated owing to the fact that NF2 related tumors are essentially benign schwannomas and meningiomas that do not invade adjacent tisuue or producing distant metastasis. However, *NF2* is inactivated in mesotheliomas that are malignant tumors and the levels of Merlin were shown to drop in various aggressive tumor types.

In this regard, we uncovered a new role for Merlin and AmotL1 interaction in cell migration in breast cancer cells. We have shown that Merlin regulates the AmotL1 expression at several levels. First we have shown that Merlin and AmotL1 levels appear to be inversely correlated in a series of breast cancer cell lines. When we inactivated Merlin expression in one of these cell lines, we observed a strong up-regulation of the AmotL1 protein levels. This up-regulation is due to increased transcription induced by elevated Yap activity following Merlin inhibition but also by a decrease in the degradation AmotL1 that we showed is mediated via its interaction with Merlin.

Hence, we have shown that Merlin is an essential regulator of AmotL1 expression which levels directly impact on the migratory capacity of the cells. Furthermore, we have shown that Merlin also regulates the subcellular localization of AmotL1 by removing the protein from the leading edge. Taken together, our results show that Merlin is an essential regulator of cell migration and potentially invasion by inhibiting AmotL1 pro-migratory functions.

Altogether, Merlin interaction with Amot proteins impacts cell proliferation and migration. In the context of the NF2 disease, the role in proliferation appears to be predominant although the consequences on cell movements certainly deserve further investigation in this pathological context.

2. Role of Merlin in cell migration and in cancer

Our observation points to an important role for Merlin in cell migration and possibly invasion in non-NF2 context. Interestingly, mice that are heterozygous for *Nf2* inactivation succumb to tumors of very different type than those seen in human patients. In this model, hepatocellular carcinomas, fibrosarcomas and osteosarcomas developed. McClatchey et al have shown that these tumors are highly aggressive and metastatic strengthening the idea that Merlin plays an important role in tumor invasion and dissemination (McClatchey et al., 1998). At the cellular level, Gutmann et al. showed that Merlin organizes the actin cytoskeleton. In addition our lab and others showed that Merlin null schwann cells harbor many altered cytoskeletal phenotypes such as increased membrane ruffling, disorganized stress fibers and altered spreading (Gutmann et al., 1999). In this context, our lab also demonstrated that the inhibition of cell proliferation by Merlin was independent of its function of regulation of actin cytoskeleton architecture, suggesting that impact on proliferation and migration might be due to unrelated mechanisms (Lallemand et al., 2003, 2009a). However, this study showed that Merlin regulation of actin cytoskeleton was dependent of a region of the FERM domain that binds actin. Therefore, it is possible that the effects of Merlin on proliferation and on migration both necessitate Amot interaction but that the latest requires binding of Merlin to actin to be completed.

Our work has focused more specifically on breast cancer where Merlin clearly inhibits AmotL1 function in migration. Interestingly AmotL1, but not Amot and AmotL2, is highly expressed in ER- tumors compared to ER+ tumors, suggesting curial role for AmotL1 in

breast cancers as well as specificity of AMOT family proteins in breast cancers. In this context, it is interesting to note that loss of tight junction (TJ) molecules such as ZO-1 and 3, and occludin are frequently observed in breast tumors. The loss of these TJ molecules is associated with aggressiveness of tumors (Jiang et al., 2006; Martin et al., 2004, 2010). Very interestingly, we have seen a negative correlation between AmotL1 and ZO-3, and Occludin mRNA levels (**please refer paper manuscript for more details**). Altogether, we proposed the following model: loss of TJ integrity during tumor progression is associated with a down regulation of Merlin expression and lead to elevated levels of AmotL1 expression, and its re-localize from TJ, resulting in AmotL1 accumulation at the leading edge where it stimulates the small GTPase activity, hence migration.

However, alternative mechanisms have been proposed to explain the variations of Merlin levels in breast tumors. Morrow et al have shown the inverse correlation between Merlin and osteopontin expression as demonstrated by immunohistochemistry in 77% (58 of 75) of patient breast tumor tissues analyzed. Osteopontin is a cytokine which signals through several receptors such as integrins and CD44, and activates NF-κB, PI-3 kinase and AKT signaling and enhances tumor progression and metastasis. In this context, authors have shown that osteopontin activates the Akt signaling to phosphorylate Merlin at serine 315 which in turn results in its degradation via the proteasomal pathway. Interestingly the expression of degradative resistant mutant of Merlin results in the inhibition of tumor growth in a xenograft model even in presence of osteopontin. Finally the authors have shown that expression of Merlin results in inhibition of tumor invasion, migration, motility, and impeded tumor (xenograft) growth in immunocompromised mice (Morrow et al., 2011). Our results then provide the mechanisms by which this regulation of Merlin can lead to more aggressive tumors.

Strong variations in Merlin expression levels have been observed in other type of cancers. Murray et al have shown that loss of expression or functional inactivation of Merlin results in increased melanoma cell proliferation, migration, invasion in vitro and in subcutaneous melanoma in mice, suggesting a role for Merlin in inhibiting the melanoma growth by controlling the cell migration and invasion. This study has shown that this anti-melanoma capacity of Merlin is mediated by MTS1/2 kinases (Hippo core components) as over-

expression of Merlin results in increased activation of MTS1/2 kinases in melanoma cells (Murray et al., 2012). Furthermore Lau et al showed that reduced expression of Merlin resulted in more malignant gliomas and re-expression of Merlin in these cells inhibited the glioma development in vivo. In this study, authors have observed that Merlin mediates its glioma suppressive function by activating the LATS1/2 and inhibiting Wnt signals (Lau et al., 2008).

It would be very interesting to evaluate the role of the Amot family of proteins in these tumor types in relation to Merlin loss. Altogether, including our work, suggest that variations in Merlin levels certainly play a role in many tumor types even in the absence of gene mutation. The importance of that role cannot be precisely weighted without an extensive evaluation of Merlin expression levels in large cohorts of tumors. This is a work that our lab has started to undertake for breast cancer and we hope to develop in the context of other major malignancies.

3. Merlin in mitosis

The role of Merlin during mitosis is not yet been investigated intensively. Report by Muranen et al indicated that Merlin localized to mitotic spindle but the consequences of it were not explored. Other observations suggest that Merlin may be involved in mitosis. Meningiomas exist in two groups, those expressing Merlin and the second group harboring *NF2* inactivation. Interestingly, it was shown that this second group harbors a much more pronounced genomic instability (Goutagny et al., 2007) suggesting that Merlin might be involved in proper chromosome distribution in daughter cells. However, no mechanism has been proposed to explain this observation. Nevertheless, very recently, A. McClatchey group has provided a very interesting insight on how Merlin could impacts cell division. In this study, authors have shown that restricted distribution of Ezrin in a cap structure is important for the positioning of centrosome and correct orientation of mitotic spindles. They have shown that Merlin plays a crucial role in regulating the distribution of Ezrin during cycle progression in single epithelial Caco cells in 3D cultures. They also have shown that Merlin is important for the formation of Ezrin cap which in turn is important for the orientation of mitotic spindles. The loss of Merlin expression results in a more uniform distribution of Ezrin without formation of a define cap. Finally they have shown that absence of Merlin expression

results in centrosome un-clustering and aberrant mitotic spindle formation. Interestingly, these defects are observed in *NF2* deficient human mesotheliomas and expression of Merlin in these tumors corrected the centrosome and spindle orientation defects. In conclusion, Merlin modulates Ezrin localization before the onset of mitosis and this in turn impacts mitotic progression and likely genomic stability (Hebert et al., 2012).

Our work provides complementary information to this process as it has focused on what happens to Merlin during mitosis. In these experiments, we have shown that Merlin is remarkably hyperphosphorylated during the mitosis at S518 and at a novel phosphorylation site T581 which we identified in our laboratory. We also showed that phosphorylation of Merlin at S518 by Aurora kinase A favors the phosphorylation of Merlin at T581 by a yet unknown protein kinase. Interestingly we found that this coordinated phosphorylation of Merlin at S518 and T581 is important for the timely mitotic progression as non phosphorylatable double mutant of Merlin (518A581A) delayed the mitotic progression. In contrast, the double DD mutant (518D581D) which in a sense mimics the «normal» mitotic phosphorylation on both sites does not modify mitosis length significantly. We have not found yet the possible molecular mechanisms which are involved in this phenotype. However, very interestingly we found that phosphorylation of Merlin at S518 and T581 abolishes the interaction between Merlin and Ezrin. Ezrin was the sole interactor we tested which binding was sensitive to this phosphorylation. In our 2D cell model, the DD or the AA mutants did not seem to affect Hela cells proliferation. We did not see any specific impact on Ezrin localization during interphase or mitosis. But in this system, no Ezrin cap is visible (data not shown). We are currently investigating the consequence of the two Merlin mutants on Ezrin localization in Caco cells in 3D.

The new T581 site alone abrogates binding to Ezrin when phosphorylated. However, it is localized in a region that is specific to isoform I of merlin. Isoform II differs from isoform I from amino acid 579 to the end. Interestingly, we confirmed that the C-terminal end of Isoform II doesn't bind to the FERM domain of Ezrin. Recent reports suggest that Isoform II also is a tumor suppressor as efficient as isoform I. Indeed, mice that express only isoform II are alive and well. Therefore, this suggests that mitosis proceeds normally when only isoform II is expressed and that binding to Ezrin is not required. In fact, our observations that the DD mutant has very little impact on mitosis whereas the AA mutants strongly delays mitotic progression indicate that dual phosphorylation of Isoform I seems necessary during mitosis to

uncouple Merlin from Ezrin. Hence, these dual phosphorylations in a sense convert Merlin Isoform I into Isoform II with respect to Ezrin binding. This conversion is interesting in the context of the NF2 disease. In this case, patients are heterozygous for *NF2*. These cells usually harbor one wild-type allele of *NF2* and one mutant allele. It is generally believed that this situation leads to frequent tumor development due to the random loss of the wild-type allele.

Our experiments showed that the FERM mutant of Merlin (▲ 2-3 mutant Merlin) do not bind to Aurora kinase A. It is therefore possible that this type of mutants are still expressed but cannot be phosphorylated properly during mitosis. This could result in a situation where cells would express a wild-type Merlin together with a mutant Merlin improperly regulated during mitosis. We can only speculate about the consequences of this situation but our results suggest that it could lead to abnormal mitosis and genomic instability in the end, therefore favoring tumor development. It would also mean that the type of first hit on *NF2* could impact on the rate of tumor formation at the heterozygous stage. Indeed, several studies have shown that the severity of the disease is linked to the type of mutation of *NF2* (Baser et al., 2004, 2005; Parry et al., 1996; Ruttledge et al., 1996)

4. Merlin is a target of Aurora kinase A

Our study of Merlin phosphorylation during mitosis reveled that it can be a substrate for Aurora A. Several studies have shown that over expression of Aurora A is involved in increased cell migration and tumor development in many cancers such as HCC, colon, breast cancers and ovarian cancers (Gritsko et al., 2003; Jeng et al., 2004; Li et al., 2003; Takahashi et al., 2000; Tanaka et al., 1999). Hence, we explored the possibility that Merlin might be a target of Aurora A during interphase of cancer cells that express abnormally high levels of the kinase. Hence, we indeed showed that Merlin is partly phosphorylated by Aurora A in breast cancer cells, on S518 residue.

Previously (Tatsuka et al., 2009) Tatsuka et al. have shown that transformation capacity of Aurora A is linked to its nuclear localization. Interestingly we found phosphorylated Merlin on S518 in the nucleus of BT20 cells. Giancotti group have shown that hypophosphorylated form of Merlin localized to nucleus and binds to DCAF1 to inhibit its growth promoting capacity (Li et al., 2010). However they did not see any phosphorylated Merlin in the nucleus

and their study postulates that indeed, phosphorylated Merlin do not enter in the nucleus. In contrast Carpen group has observed the p-S518 in the nucleus of U251 glioblastoma cells but they did not explain the molecular mechanism involved in the phosphorylation of Merlin in the nucleus (Muranen et al., 2005). Nevertheless, it has been shown that these cells have high levels of Aurora A and it is therefore possible Merlin is phosphorylated in the nucleus by this kinase (Lehman et al., 2012). We also showed that inhibition of Aurora A activity results in increased levels of total Merlin and decreased levels of p-518 in the nucleus. Based on our results, we propose that Aurora A mediated phosphorylation of Merlin in the nucleus could abolish the interaction between Merlin and DCAF1, and inhibit cell proliferation. Experiments are in progress to test whether Aurora A indeed disrupts the interaction between Merlin and DACF1

DCAF1 is obviously not the only target regulated by Merlin. DJ Pan Group has shown that YAP co-transcription factor is a downstream target of Merlin and its activity is negatively regulated following the activation of the Hippo pathway (Zhang et al., 2010b). In this context, we have shown another level of regulation of Merlin function. In our subcellular fractionation experiments, we have shown that inhibition of Aurora A activity results in increase of p-YAP in the cytosol and decrease of nuclear localization of YAP. This allowed us to believe that Aurora A also phosphorylates Merlin in the cytosol. Inactivation of Merlin leads to decrease Hippo signaling that in turn results in YAP nuclear translocation. Indeed, in the presence of Aurora A inhibitor, we observed less Yap in the nucleus. We are in the process of developing experiments to asses if this regulation of Yap by Aurora A via Merlin impacts the expression of target genes. We also plan to inactivate *NF2* in BT20 cells and to verify if then, inactivation of Aurora A has no impact on Yap localization. This would demonstrate the fact that the regulation of Yap by Aurora A goes entirely through Merlin.

In the end, my results link two previously unconnected cancer pathways, Aurora A dysregulation and Hippo signaling. It could mean that strategies using inhibitors of Yap activities could prove useful for the treatment of tumors with high Aurora A activity. Clearly more work is necessary to prove this hypothesis.

Altogether, during my PhD, I was able to uncover new regulations of Merlin, during interphase and also during mitosis. These new regulations appear to be important in the context of the role of Merlin in non-NF2 tumors, and they reinforce the idea that Merlin is important in various cancers where there is no mutation of the gene. They may also be important for patient suffering from Neurofibromatosis if we can, in the future, more formally demonstrate that specific mutations of the FERM domain act at the heterozygous stage.

MATERAILS AND METHODS

More details of the materials and methods are described in publication which is attached at the end of the thesis.

Cell lines

Human embryonic kidney (HEK) 293T cells, Hela cells, MDA-MB-468, BT20, BC52, Schwann cells (*NF2*$^{+/+}$ and N*F2*$^{-/-}$) were cultured in DMEM medium (Gibco, Life technologies, France) supplemented with 10% FBS (Gibco, Life technologies), penicillin (500 units/mL) and streptomycin (500 ug/ml) antibiotics (Gibco, Life technologies, France). HCC1937 cells were cultured in RPMI medium supplemented with 10% FBS (Gibco, Life technologies), penicillin (500 units/mL) and streptomycin (500 ug/ml) antibiotics (Gibco, Life technologies, France). BT20 and HCC1937 basal breast cancer cell lines were obtained from ATCC. BC52 cell lines was developed at Laboratory of pre-Clinical Investigation in Curie Institute and kindly provided by D. Decaudin. All the cell were cultured at +37^{o} C in a 5% CO_2 humified incubator. All transient transfections were performed using JetPEI solution (Polyplus transfection, France) according the manufacturer's instructions.

GFP trap

Cell lysates were combined and incubated overnight with GFP-Trap (ChromoTek, Deutschland) following manufacturer's instructions and the different GFP-tagged constructs were immunoprecipitated. Co-precipitated proteins were then detected using the appropriate antibody.

Quantification analysis of blotting images

All signal intensities of blotting images were analyzed by imaging software (Bio-Rad Image lab v 4.0.1) using manufacturer’s procedure. Briefly, the same size of pixel area was selected and signal intensity calculated by subtraction the background signal. Each signal was normalized with reference to standard control signals, e.g. Actin or total protein (when analyzed for p-proteins), and a signal/control ratio was calculated.

Duolink

BT20 or Hela cells were grown on 12-well chamber slides until 100% confluency. Cells were then washed with PBS, fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.2% Triton X-100 for 10 min, and blocked with Duolink blocking solution (Olink Biosiences, Uppsala, Sweden). After blocking, cells were incubated with combination of primary antibodies (indicated in figure legends) for overnight at 4^0C. Cells were then washed with Duolink washing buffer and incubated with the PLA probes diluted 1:5 in antibody diluent (Olink) in a humidified chamber for 1 h at 37°C. Subsequent hybridization, ligation, amplification, and detection were performed using manufacturer's instruction (Olink). Fluorescence images were acquired using a Nikon A1R confocal microscopy.

Luciferase reporter assay

$1{,}5x10^5$ BC52 cells were seeded in a 25 mm dish. 24 hours later, transfection was performed in the following conditions: 1µg of either 8xGTIIC-Luc vector (kindly given by Stefano Piccolo) or pδ51-Luc and 0.5 µg of pRL-TK vector (from Promega) were transfected using Jet PEI reagent according the manufacturer protocol. Measurements were performed 48 hours later using the Dual luciferase reporter assay system from Promega, according to their recommendations, and the results are normalized on pRL-TK transfected cell measures. Each experiment was performed in triplicate.

Mitotic shake-off

Hela cell expressing the either wild type or mutant Merlin was seeded on 6cm2 dishes. After adhesion (overnight), cells were induced with doxycycline (1uM) and after 24 hours nocodazole (1uM) was added to the cells for 16 hours. Then HeLa cells were incubated with the respective inhibitors such as MLN8054 (1uM), ZM447439 (2uM) along with MG132 (1 uM, Sigma) for 45 min and mitotically arrested cells were collected by shake-off. The control cells were incubated with 1uM MG132 and DMSO or water, depending on the solvent the respective kinase inhibitor was dissolved. Collected cells were lysed in RIPA (50mM Tris pH 7.4, 150mM NaCl; 1mM EDTA; 1% Triton X100; 4% SDS; 1mM PMSF; Phosphatase inhibitor (sigma) and protease inhibitor (sigma)). Supernatant were collected after spun at 8,000 rpm for 10 min and 5X Laemmli buffer added to supernatant. Samples were analyzed by SDS-PAGE and western blotting.

Mitotic time lapse experiment

Quantification of mitosis duration was done on inducible Hela Tet-on cells expressing either wild-type or phosphomutants of Merlin. Cells were cultured in DMEM medium (Gibco, Life technologies, France) supplemented with 10% FBS (Gibco, Life technologies), penicillin (500 units/mL) and streptomycin (500 ug/ml) antibiotics (Gibco, Life technologies, France). 4 days before time lapse video microscopy, cells were passed with or without doxycycline (1ug/ml). After 3 days, cell again passed with and without doxycycline in in fluorodish in 2ml. After adhesion (6h), cells were synchronized in S phase by thymidine block (20uM) for16h. Then cells were rinsed in DMEM medium with or without doxycycline and after 7 to 8 hours, time lapse video microscopy analysis was started to measure the mitotic duration. We used classical Nikon video microscopy (40 X DIC oil objective; 5% CO2, 37°C chamber). One image was taken at every 2 min. Images were analyzed with imageJ software and duration was calculated with tracking plugin. A statistical analysis was done on R Software.

Subcellular fractionation

Nuclear and non-nuclear (crude membranes and cytosol) fractions were prepared as follows. Cells were grown up to 100% confluency and then cell washed with PBS for three times. Scraped cells were centrifuged at 10,000 rpm for 1 min and lysates were prepared in cytosolic buffer (20mM Tris-HCl pH 7.4, 150mM KCl; 1mM DTT; 1mM PMSF; 0.5% Nonidet P-40). Resuspended the cells and kept in ice for 20 min. After 20 min take few ul of lysate on cover slip and observed under light microscope to make sure that suspension was clear and cells lysed properly. Then lysates were centrifuged at 4,000rpm for 5 min to separate non-nuclear and nuclear fractions. These steps were repeated for two times to get pure non-nuclear fraction (supernatant). The nuclear pellet then dissolved in buffer-A (10mM HEPES pH 7.9; 10mM KCl; 1.5mM MgCl2; 0.34 M Sucrose). Resuspended the nuclear pellet slowly with 200 ul pipette and kept on ice for 5 min. After, cell lysate was centrifuged at 4,000rpm for 5 min. After repeating same steps for one more time and nuclear fractions (pellet) were pooled and supernatant was discarded. Finally the nuclear fraction was extracted with RIPA buffer and added 5X Laemmli buffer. Fractions were analyzed for indicated proteins by western blotting.

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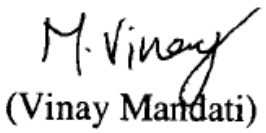
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