Characterization of the two major merlin isoforms and merlin regulation of YAP

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ABSTRACT

Merlin is the protein encoded by the tumor suppressor gene NF2. Deletion or loss-of-function of NF2 leads to neurofibromatosis type 2, a disease characterized by the formation of multiple benign tumors of the nervous system. In addition to the genetic disorder, loss of merlin expression has been found in sporadically occurring schwannomas and meningiomas, as well as in mesothelioma. Merlin has two major isoforms that differ in only one exon at the C-terminal. Previous work hypothesized that isoform II is unable to suppress growth. In this thesis, I show that both of the major merlin isoforms are able to suppress growth in multiple cell lines, including mesothelioma. Merlin has been shown to suppress growth through multiple mechanisms, including upstream regulation of the oncogene YAP through stabilization of the Hippo-pathway kinase Lats, allowing Lats to phosphorylate and inhibit YAP. In this thesis I identify an additional mechanism for merlin regulation of YAP, which occurs independently of the Hippo-pathway-based regulation. I show that mesothelioma cells expressing merlin have lower YAP-driven transcriptional activity and that expression of merlin leads to cytoplasmic localization of YAP. Furthermore, I show that this regulation of YAP is not dependent on the major Lats phosphorylation sites, but does require the YAP WW domains. This thesis provides additional insight into how merlin controls cell growth, and into YAP regulation by merlin.

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Chapter 1: Introduction

The metastatic process consists of the successful completion of a number of steps, termed also the metastatic cascade. Initially, cells need to detach from the primary tumor and enter the blood stream in a process called intravasation. Once in the blood stream, the tumor cells need to survive, arrest, and extravasate from the blood vessel into a new location, where they must initiate and then maintain growth (1). Since invasive behavior is required for metastasis, proteins which control cell polarity, adhesion, and cytoskeletal rearrangements, are an obvious target to examine when searching for proteins that determine the metastatic potential of a tumor.

The proteins of the 4.1 superfamily of proteins, which share a common domain termed the FERM domain, have been shown to be involved in these processes, and my research initially focused on developing a method to screen this family of proteins for enhancers and suppressors of metastasis. While studying the 4.1 family of proteins, I became interested in a well-characterized member of the family, merlin, a tumor suppressor.

Merlin was identified as the protein product of the Nf2 tumor suppressor gene that is lost in the familial neurofibromatosis type II (NF2) syndrome. In NF2, multiple tumors arise in the central nervous system (2). Mutations or biallelic inactivation of merlin has also been shown in other tumor types (3). Mice that are heterozygous for NF2 are prone to a wide variety of tumors and the tumors that arise exhibit a high rate of metastasis (4). Like other FERM domain family members, merlin can be found in a number of spliced isoforms, and although merlin has been studied extensively, the functions of the various isoforms was not clear. I decided to investigate whether a commonly found isoform, isoform II, is able to function in growth suppression and whether there is differential...
regulation between the major isoform, isoform I, and this splice variant. Finally, merlin has been shown to suppress growth through multiple mechanisms, one being regulation of the Hippo tumor suppressor pathway and its target, the oncogene YAP. Our lab is interested in the mechanisms by which YAP enhances tumorigenesis and metastasis (Lamar, Schindler et al. 2012), and the ways in which it can be regulated. In this context, my study of merlin as a FERM domain protein turned towards the mechanism by which merlin regulates YAP, and whether merlin can regulate YAP in a Hippo-pathway-independent manner. This thesis will expand on these three points: (i) the role of FERM-domain proteins in metastasis, (ii) the growth suppressive capabilities of the two major merlin isoforms, and (iii) how merlin regulates the YAP.

1. The 4.1 protein superfamily

The initial family member to be described, 4.1R (6) was originally identified as a critical component of erythrocytes, localizing in the submembranous cytoskeleton and stabilizing the red blood cell’s unique shape. Since 4.1R was identified, the family has grown into more than 50 proteins and is generally classified into five groups through sequence analysis (7), (i) the band 4.1 group; (ii) the ERM family; (iii) talin-related; (iv) the PTPH (protein tyrosine phosphatase) family; and (v) the NBL4 family. The 4.1 superfamily members are characterized by a common conserved N-terminal domain known as the FERM domain (Four-point-one, Ezrin, Radixin, Moesin), which is not only highly conserved among all mammalian species, but is also conserved among eukaryotes, with the fly FERM domain protein ortholog, coracle, containing 65% homology to the human 4.1R (8). The FERM domain has been shown to bind a wide variety of molecules, including phosphoinositols (9), the hyaluronate receptor CD44 (10, 11), ICAM (12), and glycophorins (13) as well as the C-terminal domain of the FERM-domain proteins (14). This variety of binding partners allows the FERM-domain family of proteins to act in
multiple roles that are necessary to the function and maintenance of a multi-cellular organism, explaining their diversity across metazoans. FERM-domain family proteins have been implicated in providing a cytoskeleton-membrane scaffold necessary for assembling multi-protein complexes required for cell signaling, cell junction maintenance, and establishing cell membrane compartments.

2. ERM Proteins: Characterization and regulation

Among the different subgroups of the 4.1 protein superfamily, the ERM group, consisting of ezrin, radixin and moesin has been studied extensively. This subgroup is characterized by a C-terminal actin-binding site in addition to the N-terminal FERM domain, and the protein activity is regulated through intermolecular associations between the N-terminal FERM domain and the C-terminal tail (15). Historically, ERM proteins were described as inactive when found in the “closed” formation, with the C-terminal tail masking other protein-binding regions in the FERM-domain head (16). Following phosphorylation of a C-terminal threonine, the interaction between the head and the tail is weakened enough to allow the FERM-domain head to bind phospholipids (5, 17) and the now “open” conformation allows multiple protein interactions to occur (18-20). The crystal structure of moesin strengthened this model, as it showed a FERM-domain head/tail interaction that could be weakened due to phosphorylation, and that the head/tail interaction masked actin-binding sites on the tail (21). Merlin is closely related to the ERM subfamily, although it has some differences; most importantly, the merlin C-terminal lacks the actin-binding site found in the other family members, ezrin, radixin and moesin, and sequence conservation in the tail is low, the fact that the FERM-domain/tail interface residues are conserved hints that merlin is also regulated in a similar manner through intermolecular interactions between the head and tail and is
Figure 1: The ERM proteins, structure and activation

a. A schematic showing the homology between ERM proteins and merlin. ERM members have a C-terminal actin binding site, whereas merlin does not. Ezrin and radixin share a proline rich region which moesin and merlin do not. The major regulatory phosphorylation (threonine for ERM, serine for merlin) is indicated. C-ERMAD, carboxy-ERM association domain; N-ERMAD, amino-ERM association domain.

b. A model of ERM activation. ERM proteins can form intermolecular associations between the head and tail. Association with PIP$_2$ can recruit ERMs to the membrane, where they can be phosphorylated and persist in an "open" form for additional protein-protein interactions. (From Bretscher, 2002)
dependent on phosphorylation of a serine in the C-terminal tail, and indeed, that this structure is possibly a model for all 4.1 superfamily proteins (15) (Figure 1, from (22)).

3. 4.1 family proteins in cancer and metastasis

4.1 family proteins have been implicated in functions such as cell-cell adhesion, cell signaling, and cell migration that, when altered, can mediate the progression of tumor formation and metastasis (23). Following are a number of examples of 4.1 family proteins which have been shown to play roles in various cancer development (24).

**Ezrin:** Ezrin expression was shown to be elevated in osteosarcomas (25, 26) and prostate cancer (27), as well as being a prognostic marker of metastasis in multiple cancers (28), including osteosarcoma (26) and pancreatic cancer (29). Mutation of the Src-phosphorylation target Y145, a tyrosine residue not conserved in ezrin’s close family members radixin and moesin, reduced cell migration (30). Mutation of an additional Src-phosphorylation target on ezrin, Y477, reduced *in vivo* invasion in mice (31). Ezrin expression was also increased in highly metastatic lung cancer cell lines when compared with poorly metastatic lung adenocarcinoma cell lines and knockdown of ezrin in these highly metastatic lines led to a decrease in proliferation, migration and invasion (32). Furthermore, abnormal ezrin localization from apical regions to the cytoplasm in breast cancer patient samples correlated with poor patient prognosis (33).

**Radixin:** Knockdown of radixin in PANC1 human pancreatic carcinoma cells showed a decrease in proliferation, adhesion and invasiveness *in vitro* as well as a decrease in tumor growth in PANC1 tumor cells implanted in nude mice (34). In addition to role radixin plays in pancreatic cancer, radixin has also been shown to enhance migration of PC3 prostate cancer cells (35).

**Moesin:** Moesin expression has been linked to an increase in tumor size and invasiveness in squamous cell carcinoma (36) as well as to survival of melanoma cells in
a tail-vein assay (37). In addition, moesin has been described as a marker of epithelial-to-mesenchymal transition in pancreatic cancer (38) and breast cancer (39), and moesin was required for actin remodeling in a mouse mammary epithelial cell model of TGF-beta induced EMT (40).

In addition to the roles these 4.1 family members play as enhancers of tumorigenesis and metastasis, two 4.1 family members have been implicated as tumor suppressors. These include merlin, which is the protein product of the NF2 tumor suppressor gene and will be described more extensively below, and 4.1B/DAL, which was identified as a novel gene whose expression was decreased in non-small cell lung carcinoma compared to normal lung tissue (41). Furthermore, 4.1B was downregulated in breast cancer (42) and in metastatic prostate cancer cell lines and shown to be a suppressor of metastasis (43).

**Objectives:** Considering the roles that these proteins play in tumor formation and metastasis, we wanted to see whether other members of the 4.1 superfamily of proteins could also have roles in cancer and metastasis. The dual goal of this research would be to develop a multiplex in-vivo tumorigenesis and metastasis screen, allowing investigation of the involvement of multiple proteins simultaneously through the use of barcoded knockdowns in the context of tumor formation and metastasis, while also discovering previously unidentified 4.1 family members which might also play a role in tumor development.
4. Merlin: A well-characterized tumor suppressor

As we began to investigate the role of FERM-domain proteins in tumorigenesis and metastasis it became clear that there were still questions surrounding the function of merlin in tumor suppression. Merlin had already been studied extensively due to its involvement in human disease, yet questions remained regarding its regulation and its potential role in tumor suppression through the Hippo pathway.

Merlin is an outlier in the ERM subgroup of the 4.1 superfamily of proteins. Although it contains extensive sequence homology to ezrin, radixin and moesin in the FERM domain N-terminal of the protein, it lacks the actin-binding domain that characterizes the ERM protein C-terminal.

Merlin was initially discovered through its role in human disease; genetic mapping of tumors from neurofibromatosis type 2 (NF2) patients revealed a deletion on chromosome 22 (2, 44), which was mapped to the NF2 gene, also called merlin, due to its similarity to the ERM family of proteins (moesin-radixin-ezrin-like-protein).

Neurofibromatosis type 2 is characterized by the development of schwannomas, meningiomas and ependymomas, but merlin loss has also been identified in additional tumor types, including mesothelioma, melanoma, and thyroid cancer (3). NF2 is one of the most frequently mutated tumor suppressors in malignant mesothelioma, and is lost in multiple mesothelioma-derived cell lines (45).

In addition to the benign tumors found in human neurofibromatosis type 2, heterozygous merlin knock out in mice leads to highly metastatic disease (4), including osteosarcomas, fibrosarcomas and hepatocellular carcinomas, suggesting that merlin plays a role in metastasis, and furthermore, that merlin serves as a tumor suppressor in a larger number of cell types.
The 4.1 family is extensively alternatively spliced, and merlin is not different in this regard (46, 47). The NF2 gene contains 17 exons, and the two major splice isoforms differ by inclusion or deletion of exon 16. While exon 16 is lacking in isoform I, it is included in isoform II, which leads to a frameshift involving an early stop codon. This creates an alternative C-terminal (3). Merlin isoform I contains 595 amino acids whereas isoform II contains 590 amino acids. Both share a molecular weight of about 70kDa. Initially, isoform II was described as an inactive form of merlin, due to limited ability of the C-terminal to bind the N-terminal and thus form the “closed” isoform, which appeared to be necessary for merlin activity (48-50). In addition, profiling the changes in splicing following epithelial-to-mesenchymal transition (EMT) showed a change in expression of merlin isoforms (Shapiro, personal communication). In these experiments, the splicing profile of HMLE cells was compared before and after induction of EMT through Twist expression with isoform I of merlin being present prior to EMT in epithelial cells and a switch to isoform II occurring in the mesenchymal cells following Twist expression and EMT (51). This could indicate that the switch to isoform II coincided with an inactivation of merlin during EMT. However, newer data (52) showed that merlin activity was not determined by a simple conformational switch, and furthermore, that the “open” form of merlin (in which there is no intermolecular interaction between the head and tail) was able to suppress growth, thus indicating that the isoform II might be active in suppression of growth as well.

5. Merlin regulation through phosphorylation

Like its close family members, the ERM proteins, merlin can be regulated through phosphorylation at the C-terminal of the protein. This phosphorylation varies in response to growth conditions, with increased confluency, serum starvation, or loss of cell-cell contacts leading to a decrease in the phosphorylated form of merlin (53). Ser518 is
phosphorylated by p21-activated-kinase (PAK1/2) (54, 55), and by cAMP-dependent protein kinase A (PKA) (56). Following phosphorylation on Ser518, merlin can be phosphorylated on multiple other serine and threonines (57, 58). Relying on a similarity to other ERM proteins, and pull-down experiments showing that the unphosphorylated tail of merlin was able to bind the FERM-domain head (50), a canonical model was adopted where phosphorylated merlin was described as being in the “open” form, and unphosphorylated merlin was described as the “closed” form (5). Investigation of merlin’s function determined that phosphorylated merlin was unable to suppress growth, and that mutating the predominant phosphorylation target Ser518 lead to constitutively active merlin (59). However, this model indicated that the “closed” form of merlin was the active state, which seemed paradoxical since the closed form would have less exposed sites available for binding mediators of merlin function. Further study showed that indeed the conformation of merlin was more fluid, and not altogether “open” or “closed”. In this model, the conformationally “open” state of merlin was the growth suppressive state, and phosphorylation tipped the protein into adopting a more closed (but not entirely) closed state (52). In addition, FRET studies show that Merlin’s N-terminal FERM-domain head and C-terminal tail are constitutively found in close proximity and only undergo subtle changes following phosphorylation (60).

Objectives: Recent research determining that a “closed” conformation isn’t necessary for merlin activity indicates that previous assumptions regarding the inability of isoform II to inhibit growth were mistaken. We decided to compare the function of the two major merlin isoforms, in order to determine whether both of the major merlin isoforms were able to regulate growth, and further, in what context does this happen. The role of isoform I in growth regulation during contact inhibition has been described, but the role of isoform II in growth inhibition remains unclear. In addition, the regulation of isoform I
through phosphorylation has been investigated, but it is unknown whether regulation of isoform II occurs in a manner similar to isoform I.

6. Multiple mechanisms for merlin-derived suppression of growth

Merlin is able to suppress cell growth through many different mechanisms (Figure 2), due to the ability of merlin to bind numerous transmembrane and intercellular proteins (reviewed by (61)). In some cases, contradictory evidence raises questions about the context and relevance of these different mechanisms to various biological models. For example, merlin has been implicated in suppressing proliferation through contact inhibition via control of actin organization. Although merlin lacks the C-terminal actin-binding domain found in the ERM proteins, it contains an actin-binding site in the N-terminal (62). Schwannoma cells lacking merlin have been shown to have disorganized stress fibers, altered cell spreading, and increased membrane ruffling, all related to abnormal actin organization (63), which can be reversed by the expression of merlin (64). In addition, expression of merlin in mesothelioma cells lacking endogenous merlin leads to decrease of phospho-FAK (65). This decrease in FAK phosphorylation leads to a downstream decrease in Src activation, either directly, since merlin has been shown to form a complex with FAK (66), or indirectly, through inhibition of Rac/PAK signaling (67). Furthermore, merlin is able to block recruitment of Rac to the membrane and loss of merlin can lead to increased Rac activity, lamellipodia formation, and increased cell motility (68).

The mechanism behind merlin’s ability to regulate Rac can be explained by Merlin’s interaction with angiomotin. Angiomotin, a scaffolding protein that localizes to tight junctions, was shown to bind merlin. This interaction is competitive with angiomotin binding Rich, which is then able to inactivate Rac (69). However, both unphosphorylated merlin (S518A) and a phosphomimetic form of merlin (S518D) were shown to bind
Figure 2: Multiple mechanisms for merlin regulation of growth
1. Negatively regulates CD44 (Bai et al. 2007).
2. Regulates the distribution, aggregation and availability of receptor tyrosine kinases (McClatchey et al. 2009).
3. Translocates to the nucleus and inhibits the ubiquitin ligase CRL4DCAF (Li et al. 2012).
4. Stabilizes adherens junctions through an interaction with α-catenin (Gladden et al. 2010).
5. Interacts with angiomotin to suppress Rac activity (Yi et al. 2011).
6. Recruits the Lats kinases to the plasma membrane and coordinates their activation by Mst1, driving phosphorylation and inhibition of YAP/TAZ (Yin et al. 2013).
angiomotin, which raises the question of how merlin regulation through phosphorylation can be explained in this context, and in what contexts does this mechanism for Rac regulation through angiomotin confer biological relevance.

Merlin has also been shown to stabilize adherens junctions in mouse embryonic fibroblasts and keratinocytes (70), through an interaction with α-catenin (71).

Aside from mediating contact inhibition through stabilization of adherens junctions, merlin has also been shown to mediate contact inhibition through an interaction with CD44, the hyaluronan receptor. CD44 is upregulated in several cancers, and is an indicator of poor prognosis (reviewed by (72)). Using a rat schwannoma cell line, Morrison et al. found that at high cell densities, when contact inhibition was required, a hypophosphorylated form of merlin bound CD44, and that treatment with hyaluronic acid or with anti-CD44 antibody led to a merlin-dependent decrease in cell proliferation (10).

In addition to its role in mediating contact inhibition, presumably through adherens junction and CD44 interactions, merlin can also regulate cell growth through control of the distribution, aggregation and availability of receptor tyrosine kinases (RTKs) in the plasma membrane. This function of merlin as a regulator of cell growth has been shown both in mammals (73, 74) and in Drosophila (75).

Finally, merlin has also been suggested to localize to the nucleus and inhibit cell growth through an inhibitory interaction with the E3 ubiquitin ligase CRL4DCAF1 (76). CRL4 ligases have been implicated in histone remodeling and can mediate a generally cell-proliferative gene expression pattern, as well as directly ubiquitinating and inhibiting a major component of the canonical Hippo pathway, LATS1/2 (77), providing an additional, nuclear and transcriptionally-mediated role for merlin-dependent growth suppression. However, since the merlin FERM-domain alone is able to suppress growth in this manner, and the literature supports a role for C-terminal domain being required to suppress growth as well (Lallemand et al. 2009), it is clear that merlin does not sustain
its major effects through nuclear localization and activity alone, and that the biological context and other participants in the system described must be carefully considered.

7. The Hippo pathway in flies and mammals

Regulation of the Hippo pathway in Drosophila

Although multiple mechanisms and functions have been proposed for merlin as an explanation for its role as a tumor suppressor, there remains one pathway, the Hippo pathway in which merlin has been described as a member, and yet a mechanism for merlin’s participation in this pathway remain unclear; this will be one topic of interest in this thesis. The Hippo pathway has recently been described as an important tumor suppressor pathway in both flies and mammals, with mammalian orthologs for the canonical components of the Drosophila pathway. In Drosophila the Hippo pathway serves to control organ size through regulation of proliferation, cell growth and apoptosis (78, 79), by limiting imaginal disc size. The core components of the pathway include the serine/threonine kinases Warts (mammalian LATS1/2) and Hippo (mammalian MST1/2), and two adaptor proteins, Salvador (Sav), a scaffolding protein that interacts with Hippo, and Mats, which binds Warts. When the kinase cascade is activated, Hippo, in a complex with Sav, phosphorylates and activates Warts, bound to its co-factor Mats. Activated Warts phosphorylates the transcriptional co-activator Yorkie (mammalian YAP/TAZ) at multiple sites, inhibiting its function (80). This inhibition occurs through cytoplasmic sequestration of Yorkie, as the phosphorylation on Ser168 (Ser127 in the mammalian ortholog YAP) acts as a recognition site for 14-3-3 binding, which retains Yki in the cytoplasm and prevents its activity as a transcriptional co-activator. In the nucleus, Yki promotes a transcriptional profile that is growth-permissive and anti-apoptotic through interaction with the TEAD/TEF family transcription factor Scalloped (81, 82). In
flies, Hippo-independent regulation of Yki can occur through the FERM-domain protein
Expanded, which can suppress Yki activity directly through binding and cytoplasmic
retention (83-85) (Figure 3).

**Mammalian regulation of the Hippo pathway**

In mammals, multiple upstream regulators have been identified that can control the
Hippo pathway or alternatively YAP activity directly, independently of the Hippo pathway.
A major component of YAP regulation involves mechanical stress (86), with the
extracellular matrix regulating YAP/TAZ activity as a function of varying stiffness.
Dupont et al. found that on stiff ECM YAP localized to the nucleus regulated through
Rho and actin cytoskeleton activity, whereas on a soft matrix YAP localized to the
cytoplasm. Furthermore, knockdown of LATS was unable to rescue cells plated on a soft
matrix, suggesting that the stiffness-mediated regulation is independent of the Hippo
pathway. However, contradictory evidence showed that LATS was activated by cell
detachment and that LATS knockdown can prevent mechanical-stress-induced YAP
phosphorylation (87, 88).

In addition to YAP regulation through matrix stiffness, G-protein receptors have also
been identified as regulators of the Hippo pathway (89) through LATS inhibition by
G12/13 or Gq/11-coupled receptors, which leads to YAP activation, or LATS activation
through Gs-coupled receptors which leads to an inhibition of YAP.

The mammalian orthologs of Yki, YAP and TAZ, were discovered to be Yes-associated
proteins, and were the first WW-domain containing proteins to be identified (90). YAP
contains one or two WW domains, depending on the splice isoform involved, and TAZ
contains one WW domain. These domains contain two conserved tryptophans (WW),
and bind proline-rich motifs. In addition to the WW domains, YAP and TAZ also contain
a coiled-coil domain and a PDZ-binding motif, which regulate YAP localization and
function (91). There are five HXRXXS LATS target sequences in YAP/TAZ (92), with two
Figure 3: The Hippo pathway in *Drosophila* and mammals

The corresponding proteins are indicated by matching colors. Direct biochemical interactions are shown with a solid line, genetic interactions are shown with a dashed line. An hypothesized interaction is indicated with a grey line.
of these sites appearing most important for regulation (80). Phosphorylation at Ser127 in YAP (Ser89 in TAZ) creates the 14-3-3 binding site which mediates YAP cytoplasmic sequestration, and phosphorylation at Ser381 mediates YAP ubiquitination and degradation (93).

YAP is implicated in human disease as an oncogene amplified in multiple tumor types (94), and YAP expression and nuclear localization correlate with poor patient outcome in several cancers (80, 95-98). Furthermore, YAP overexpression in multiple cell lines, as well as transgenic activation in mice, has been shown to promote tumor growth (99, 100). Our lab has demonstrated an important role for YAP activity in metastasis (101).

8. Merlin regulation of YAP

Merlin regulation of the Hippo pathway in flies

Merlin has been implicated as an upstream regulator of the Hippo pathway in Drosophila. Mutations in merlin and another FERM-domain family protein, expanded, lead to overgrowth in multiple adult tissues in Drosophila, phenocopying mutations of Hippo pathway components. In addition, mer;ex double mutants had higher levels of the Yki target gene diap1, an anti-apoptotic gene. Interestingly, Hpo mutants could reverse the reduced-eye-size phenotype caused by loss of Expanded, placing Expanded upstream of Hippo (102), which is in contrast to later findings that Expanded is able to regulate Yki directly without relying on the kinase cascade for phosphorylation and regulation of Yki (83). However, the epistasis experiments could not rule out an additional model of regulation in which the Hippo pathway is bypassed altogether, and the experiments in Drosophila that illustrate how loss of merlin leads to EGFR trafficking defects (75) demonstrate that merlin may play multiple roles through various mechanisms depending on the cellular context and the developmental stage.
Since merlin and expanded appeared to synergize (103) and were assumed to be redundant to each other in *Drosophila*, these genetic experiments which placed expanded upstream of the Hippo pathway were considered to place merlin upstream of the Hippo pathway as well. Due to the presence of a highly conserved Hippo kinase cascade in mammals, and the relevance of the pathway to human disease, there has been a focus on identifying the role of merlin in regulation of YAP, the Yki homolog, primarily as an upstream regulator of the Hippo pathway.

**Merlin regulation of YAP in mammals**

In mammalian cells, merlin has been shown to regulate the expression and localization of YAP in meningioma cells (104) and mesothelioma cells (105), although neither of these studies convincingly implicated the Hippo pathway in this regulation of YAP. In the search for a tractable mammalian model, that could be compared to the fly model of the Hippo pathway regulating organ size, the liver was selected as an appropriate example, since mammalian liver can undergo dramatic changes in organ size. When YAP was inducibly overexpressed in the adult liver this led to an increase in liver size, which was reversed when YAP overexpression was removed (99) Similar overgrowth phenotypes were observed when the mammalian Hippo orthologs MST1 and MST2 were depleted in embryonic livers using a Cre-albumin promoter, which acts on embryonic liver progenitors (106). These results show that YAP, as regulated by the Hippo pathway, appears to be an important player in liver regeneration. However, attempts to determine whether merlin plays a role in this regulation led to contradictory results. Work by Zhang et al. showed that elimination of *NF2* using an albumin-cre model led to expanded liver progenitor growth, which could be suppressed by heterozygous deletion of YAP, linking YAP regulation to merlin tumor-suppressor activity (107). But research by Benhamouche et al. contradicted this experiment. When merlin was knocked out using the same model of embryonic liver conditional knockout with...
albumin-cre, there was an enlargement of the liver. However, there was no evidence of Hippo pathway activity or YAP activation in these experiments, and the phenotype observed following loss of merlin was explained through EGFR pathway regulation and could be suppressed by treatment with erlotinib, an EGFR inhibitor (108). Considering that there is crosstalk between YAP and downstream components of the EGFR signaling pathway, where Raf can prevent MST2 interaction with Lats1 (reviewed by (109)), and on the other hand, the EGFR ligand amphiregulin is upregulated through YAP-mediated transcription (100), the intertwined nature of EGFR activity and YAP activity can further complicate the picture when trying to tease out regulatory mechanisms.

**Merlin can stabilize Lats**

One possible role for merlin in regulation of the Hippo pathway is through direct stabilization of Lats. Merlin has been shown to bind Lats through the FERM-domain, recruiting Lats to the membrane, which allows it to be phosphorylated by MST1/2, activating it and allowing it to phosphorylate and inhibit YAP (110). Although this is a mechanism that allows regulation of YAP through attenuation of the Hippo pathway, it does not rule out other mechanisms for merlin regulation of YAP. The question remains whether, in addition to the role of merlin in LATS stabilization, merlin is able to regulate YAP independently of the Hippo pathway.

**Objective: examining Hippo pathway independent regulation of YAP by merlin**

An example of Hippo pathway-independent regulation by merlin occurs in *Drosophila*. In flies, expanded can regulate Yki without Yki phosphorylation by the Hippo kinase cascade, through binding of the PPxY motifs of expanded to the WW domains of Yki (83). Could a similar mode of regulation be available in mammals? Mammalian expanded is shortened and lacks the PPxY motif found in *Drosophila*, however one Hippo pathway-independent regulatory function involves the scaffolding protein angiomotin binding YAP through a WW domain/PPxY motif interaction (111, 112) and...
sequestering it in the cytoplasm without prior phosphorylation of YAP by the Hippo kinase cascade. Since merlin has been shown to bind angiomotin through its role in Rac inhibition (69), could it play an additional function in YAP inhibition as well? And what are the cellular contexts that mediate merlin suppression of growth through the multiple avenues that appear to be available for this suppression? In order to attempt to address these questions we decided to investigate the ability of merlin to regulate YAP in a mesothelioma model, and determine whether the ability of merlin to regulate YAP was dependent upon the Hippo pathway.

9. Summary
This thesis aims to investigate multiple aspects of the FERM domain family of proteins. The subsequent chapters will describe the following:

In Chapter 2 I attempt to establish an in vivo screen to determine whether members of the FERM domain family are enhancers or suppressors of metastasis. In order to do this we chose ezrin, a known enhancer of metastasis, as well as close family members radixin and moesin, and 4.1B, a known metastasis suppressor, as preliminary targets for screening.

In Chapter 3 I describe the FERM domain family member merlin’s two major splice isoforms, isoform I and isoform II, and establish that both of the isoforms are able to suppress growth in multiple cell lines. I further show that similarly to the well characterized isoform I, isoform II also suppresses cell growth in a contact inhibition-dependent manner, and is regulated by phosphorylation. However, there appears to be differential regulation of the two isoforms, and isoform II not only is able to suppress growth, despite being described previously as an inactive form of merlin, but is also more stable when expressed in culture.
In Chapter 4 I investigate the mechanism by which merlin regulates the Hippo pathway target YAP. I show that in addition to regulation through the Hippo pathway, which has been described previously, merlin is able to regulate YAP in a Hippo-pathway-independent manner, which requires the WW domains of YAP.

In Chapter 5 I summarize the work described in this thesis, the unanswered questions that arose from this study, and possible avenues for future research into these questions.

References


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Chapter 2. FERM-domain proteins in tumorigenesis and metastasis

Introduction

FERM-domain proteins have been implicated in various cancers (1), both as tumor suppressors (2, 3) and as promoters of metastasis (4, 5). This is not surprising, since FERM-domain proteins have been shown to play roles in cell polarization, cell-cell and cell-ECM contact, and participate in cell signaling (6-10), all factors which play a role in a tumor’s ability to metastasize.

In order to further determine the role these proteins may play in metastasis, I developed a screen wherein FERM-domain proteins can be knocked down or overexpressed in a murine mammary carcinoma metastasis model and the resulting metastases examined for enrichment or depletion of FERM-domain proteins.

Results

1. 4.1 family expression in murine mammary carcinoma cells

In order to examine the effects of FERM-domain proteins on metastasis I used a panel of murine mammary carcinoma cell lines, which have varying metastatic potential (11)(Table 1). 4T1 is described as the most aggressive, showing metastasis to the lungs when injected into the tail vein or into the mammary fat pad. The 67NR and 168FARN cell lines were described to have low to no metastatic potential when used in this manner. Therefore, 67NR and 168FARN cells could be used to identify proteins which when knocked down that increase metastatic potential, whereas 4T1 cells could be used to assay for a decrease in metastatic potential.
<table>
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<tr>
<th></th>
<th>67NR</th>
<th>168FARN</th>
<th>4T07</th>
<th>66cl4</th>
<th>4T1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumorigenic</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>Intravasation:</td>
<td></td>
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<td></td>
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</tr>
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<td>very few</td>
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</tr>
<tr>
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<td>yes</td>
<td>yes</td>
<td>yes</td>
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<tr>
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<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>(Cells found in lungs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Metastatic tumors:</td>
<td></td>
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</tr>
<tr>
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</tr>
<tr>
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</table>

Table 1: The 4T1 panel of cell lines as described by Asklason and Miller ranked in order of metastatic potential. The columns marked in red denote the cell lines I chose to use for the screen: the poorly metastatic cell line 67NR and the highly metastatic cell line 4T1.
Of the FERM-domain family proteins, we initially chose to investigate the ERM proteins (ezrin, radixin and moesin) and a number of the 4.1 family proteins; 4.1B, 4.1G, 4.1N and 4.1R, and examined their expression in these cell lines. Ezrin, a FERM-domain protein which acts as a membrane-cytoskeletal linker and has previously been shown to be highly expressed in multiple types of invasive human cancers (12-14) had increased expression levels in the highly metastatic 4T1 cell line compared to the poorly metastatic 67NR cell line, although there was no significant difference in expression of ezrin between the highly metastatic 4T1 cell line and the 168FARN cell line, which has been described as having a low metastatic potential. Radixin and moesin, two closely related proteins, did not show much change in expression in the various cell lines (Figure 1a).

4.1B, another FERM-domain family protein and a member of the 4.1 subgroup, has been described as a suppressor of prostate-cancer metastasis (3) and was not expressed in 4T1, 66Cl4 or 4T07, the cell lines with the higher metastatic potential, but was expressed in the poorly metastatic cell lines 168FARN and 67NR (Figure 1b).

Using qPCR to determine the transcript levels of 4.1G (Figure 1c), 4.1N (Figure 1d), 4.1R isoform I (Figure 1e) and 4.1R isoforms II/III (Figure 1f), we did not detect any significant trend of expression changes that correlated with the metastatic potential of the cell lines.
a. Ezrin expression: Western blot showing levels of ezrin expression in the 4T1 panel of cell lines. The cell lines are in order of metastatic potential from left to right.
b. 4.1B expression:
Western blot showing levels of 4.1B expression in the 4T1 panel of cell lines. n=2
c. 4.1G expression:
qPCR showing normalized expression levels of 4.1G in the 4T1 panel of cell lines. n=2
d. 4.1N expression:
qPCR showing normalized expression levels of 4.1N in the 4T1 panel of cell lines. n=2
e. 4.1R expression:
qPCR showing normalized expression levels of 4.1R isoform I in the 4T1 panel of cell lines. n=2
f. 4.1R isoform II/III expression:
qPCR showing normalized expression levels of 4.1R isoform II/III in the 4T1 panel of cell lines. n=2
2. Barcode-containing knock down of ERM proteins for Luminex-based assays

Following verification of expression of the ERM proteins and 4.1B in the 4T1 and 67NR cell lines, and a correlation of expression levels of ezrin and 4.1B with metastatic potential in accordance with their description in the literature as a metastasis enhancer and suppressor respectively, we then established in vivo models of metastasis and tumorigenesis that would enable us to screen multiple FERM-domain proteins simultaneously for a role in metastasis or tumorigenesis.

In this system, MSCV-retroviral vectors including both a mir30-based shRNA targeting the gene of interest and an oligonucleotide barcode unique to each hairpin were used to knock down the genes of interest while labeling the cells with a barcode unique to the identity of the knockdown. A mixed population of cells carrying different knockdowns with their corresponding barcodes was then used in both experimental tail-vein metastasis assays and orthotopic mammary transplants. While an experimental metastasis assay involves tail-vein injection of the cell mixture and analyzes the number of lung metastases that form, thus focusing primarily on survival, extravasation and growth, the orthotopic experiment includes all stages of tumorigenesis and metastasis and can therefore more closely recapitulate the metastatic cascade. DNA isolated from the tumor or metastases was isolated and analyzed quantitatively to determine relative increases or decreases of the barcode populations in the primary tumor or metastases. When normalized to the barcode signal in the primary tumor, an increase or decrease in a knockdown barcode signal compared to the control barcode signal indicated an increase or decrease in metastatic potential of the cells in which we had knocked down the gene in question.
To optimize and establish the assay we used two approaches. In one, the highly metastatic 4T1 cell line was used to determine whether there is a decrease in metastatic potential following knockdown of the metastasis enhancer ezrin and its close homologs radixin and moesin. In the second approach, the poorly metastatic cell line 67NR was used to determine whether there is an increase in metastatic potential following knockdown of the metastasis suppressor 4.1B.

Two separate barcoded hairpins were used for each gene and decreased expression of the ERM proteins following knockdown was validated in the 4T1 cell line (Figure 2a) and of 4.1B protein levels in the 67NR cell line (Figure 2b).
Figure 2: Knock down of FERM-domain-containing proteins in murine mammary carcinoma

a. Knock down of ERM proteins in 4T1:
Western blot showing knockdown of ezrin, radixin or moesin in the highly metastatic
4T1 murine mammary carcinoma cell line using a Luminex-barcoded retroviral vector
containing a mir30-based shRNA for each of the ERM proteins or a control.
Two separate hairpins targeting each gene are shown.

b. Knock down of 4.1B in 67NR:
Western blot showing knock down of 4.1B in the poorly metastatic 67NR cell line
using a Luminex-barcoded retroviral vector containing a mir30-based shRNA for 4.1B or
a control. Two separate hairpins targeting 4.1B are shown.
3. Orthotopic and tail vein assays following loss of ERM proteins

Once we established knockdown of the FERM-domain proteins ezrin, radixin or moesin in the 4T1 cell line we initially performed both orthotopic transplants and tail-vein injections in the traditional manner investigating only the loss of one protein per experiment. In these experiments, mice (n=5) were injected either via the tail vein or orthotopically into the mammary gland with 4T1 cells in which ezrin had been knocked down using a barcoded mir30 shRNA vector containing a fluorescent label or expressing a control vector containing a fluorescent label, and the numbers of lung metastases were counted. There was no significant difference between the control and the ezrin knockdown cells in the number of metastases following tail-vein injection in an experimental metastasis assay, or the number of metastases following an orthotopic mammary transplant. In addition, there was no significant difference between the control and the ezrin knockdown in the size of the tumor formed in the orthotopic transplant (Figure 3a). We next performed the experiment as a screen using a mix of multiple barcoded knockdowns and a control injected simultaneously into the same mouse. In this way, we could control for any internal variability during the injection or transplant, screen multiple genes at the same time and limit the number of mice required for the experiment. In these experiments the mice were injected via tail vein (n=6), or orthotopic transplant (n=20) with a mixture of 4T1 cells containing barcoded knockdowns for ezrin, radixin, moesin, or a control. Two different validated hairpins were used for each gene of interest. There were no significant differences in metastasis between the control cells and cells where ezrin, radixin or moesin had been knocked down assayed either by tail-vein injection (Figure 3b) or following orthotopic transplant (Figure 3d). There were also no significant differences between the control and the knockdown cells in contribution towards establishing the primary tumor in the orthotopic transplant (Figure 3c, cohorts were sacrificed and tumors analyzed at 3 separate time points).
Metastasis assays for 4T1 cells with Ezrin knockdown:

**a. Tail vein lung metastases**

Orthotopic primary tumor

Orthotopic lung metastases

Luminex experimental metastasis assay for 4T1 cells with ERM knockdown:

**b.**

Luminex orthotopic transplant experiment, primary tumor:

**c.**

Luminex orthotopic transplant experiment, metastases:

**d.**
Figure 3

a. Metastasis assays for 4T1 cells with Ezrin knockdown:
Ezrin was knocked down in 4T1 cells using two separate validated hairpins in an MSCV retroviral vector. Cells were injected into the tail vein (n=5) or transplanted orthotopically into the mammary gland (n=5) and metastases counted. For the orthotopic transplant the resulting primary tumor was weighed.

b. Luminex experimental metastasis assay for 4T1 cells with ERM knockdown:
Ezrin, radixin, or moesin knockdown cells along with 2 barcoded control lines were mixed and injected into the tail vein. (n=6) Lungs containing metastases were collected and the total lung DNA analyzed for the knockdown or control vector barcodes. The average relative signal generated from the barcodes associated with each hairpin was quantified as described.

c. Luminex orthotopic transplant experiment, primary tumor:
Ezrin, radixin or moesin knockdown cells along with 2 barcoded control lines were mixed and injected orthotopically into the mammary gland. Primary tumors were collected on day 5 (n=4), day 15 (n=4) and day 30 (n=10) and the DNA analyzed for knockdown or control vector barcode contribution. The average relative signal generated from the barcodes associated with each hairpin was quantified as described.

d. Luminex orthotopic transplant experiment, metastases:
Ezrin, radixin or moesin knockdown cells along with 2 barcoded control lines were mixed and injected orthotopically into the mammary gland. Total lungs containing metastases or tumor cells were collected on day 5 (n=4), and day 30 (n=10) and the DNA analyzed for knockdown or control vector barcode contribution. The average relative signal generated from the barcodes associated with each hairpin was quantified as described.

4. Migration and invasion *in vitro* following loss of ERM proteins

As with the *in vivo* experiments, *in vitro* proliferation (Figure 4a), migration (Figure 4b,c) and invasion (Figure 4d) experiments showed no change in migration or invasion following knockdown of ERM proteins individually in the 4T1 cell line.
Figure 4

a. Luminex cell proliferation in vitro of ERM knockdown 4T1 cells:
Two separate ERM knockdown 4T1 cell lines and 2 control lines were mixed and plated. DNA was isolated and analyzed from the culture at timepoints post-plating to determine contribution of each cell line to the culture. The average relative signal generated from the barcodes associated with each hairpin was quantified as described.

b. Luminex cell migration experiment with ERM knockdown 4T1 cells:
Two separate ERM knockdown 4T1 cell lines and 2 control lines were mixed and plated in a Transwell culture system. DNA was isolated and analyzed from the cells which had migrated 16 hours post-plating to determine contribution of each cell line to the migrating cells. The average relative signal generated from the barcodes associated with each hairpin was quantified as described.

c. Migration of 4T1 cells with ERM knockdown or control:
4T1 cells with stable knockdown of ezrin, radixin, moesin or control were plated in Transwell plates in media lacking serum, and migration into 10% serum was counted 16 hours later.

d. Invasion of 4T1 cells with ERM knockdown or control:
4T1 cells with stable knockdown of ezrin, radixin, moesin or control were plated in Matrigel-coated Transwell plates and cells that had invaded through the Matrigel were counted 16 hours later.
5. Compensation and redundancy of ERM proteins

There is a possibility that these close homologs can compensate for each other and that all three proteins, ezrin, radixin and moesin need to be knocked down simultaneously in order to achieve a change in the metastatic potential of the cell. In work by Takeuchi et al. (15) they show single knockdown of ezrin, radixin or moesin in thymoma cells using antisense phosphorothioate oligonucleotides (PONs). Knockdown of these proteins singly had no effect on the presence of microvilli on the cell surface. Only the disruption of all three proteins simultaneously could eliminate the microvilli on the cell surface.

We attempted to knock down these three proteins simultaneously using two different approaches. In one approach, we infected the cells sequentially with MSCV retroviral vectors containing the mir30 shRNA hairpin which had been successful in the single protein knockdown experiment (Figure 5a). In another approach, we infected cells with an MSCV retroviral vector containing concatemerized mir30 shRNA hairpins for all three proteins (Figure 5b). Both of these approaches were unsuccessful in knocking down all three proteins simultaneously.
Figure 5

a. Sequential knockdown of ezrin, radixin and moesin in the 4T1 cell line:
Western blot showing an attempt at knockdown of ezrin, radixin and moesin simultaneously in the highly metastatic 4T1 murine mammary carcinoma cell line using Luminex-barcoded retroviral vectors infected at single copy levels containing a mir30-based shRNA for each of the ERM proteins or a control. The cells were infected sequentially with each vector, then lysates were collected to examine knockdown efficiency. A control which was infected only once at single copy level with a hairpin targeting one gene is shown to illustrate single knockdown efficiencies.

b. Simultaneous knockdown of ezrin, radixin and moesin in the 4T1 cell line:
Western blot showing an attempt at knockdown of ezrin, radixin and moesin simultaneously in the highly metastatic 4T1 murine mammary carcinoma cell line using a Luminex-barcoded retroviral vector, containing three concatemerized mir30-based shRNAs targeting each of the ERM proteins, as depicted in the illustration. (Ezr indicates shRNA targeting ezrin, EM indicates shRNA targeting ezrin and moesin, and ERM indicates shRNA targeting ezrin, radixin and moesin)
6. Analysis of metastasis suppressor 4.1B knockdown

Knockdown of the metastasis suppressor 4.1B in the poorly metastatic cell line 67NR did not increase metastasis in experimental tail-vein metastasis assays, either in a single-cell-line experiment (Figure 6a, n=10, p=0.35) or using a barcoded mixture of knockdown and control vectors (Figure 6b n=4, p=0.28). Furthermore, metastasis of the control cell line indicates that the 67NR cell line did not act in this system as previously described (11). In an orthotopic experiment the control line failed to form a tumor (Figure 6c), however, the cell line has been described as able to form tumors in an orthotopic experiment and the failure to form tumors in this experiment (n=5) might be due to a technical issue or an immune response to the fluorescent label ZS-Green. The knockdown cells which also expressed ZS-Green in addition to the barcoded mir30 shRNA targeting 4.1B showed a lower expression of ZS-Green compared to the control (data not shown). In vitro experiments to determine whether loss of 4.1B expression in the 67NR cell line affected growth (Figure 6e) or migration (Figure 6f) again showed no significant difference between the knockdown line and the control.
a. Tail vein assay

b. Luminex tail vein assay

c. Orthotopic experiment- primary tumor

d. Orthotopic experiment- metastases

e. Luminex cell proliferation

f. Migration
Figure 6
a. **Experimental metastasis assay for 67NR cells with 4.1B knockdown:**
4.1B was knocked down in 67NR cells using two separate validated hairpins in an MSCV retroviral vector. Cells were injected into the tail vein (n=5) and lung metastases were counted.

b. **Luminex experimental metastasis assay for 67NR cells with 4.1B knockdown:**
4.1B knockdown cells along with 2 barcoded control lines were mixed and injected into the tail vein. (n=4)
Lungs containing metastases were collected and the total lung DNA analyzed for the knockdown or control vector barcodes. The average relative signal generated from the barcodes associated with each hairpin was quantified as described.

c. **Orthotopic transplant experiment, primary tumor:**
Two separate 4.1B knockdown 67NR cell lines and a control cell line were injected orthotopically into the mammary gland. Primary tumors were collected on day 30 (n=5) and tumor weight recorded.

d. **Orthotopic transplant experiment, metastases:**
Two separate 4.1B knockdown 67NR cell lines and a control cell line were injected orthotopically into the mammary gland. Lungs were collected on day 30 (n=5) and number of metastases recorded.

e. **Luminex cell proliferation in vitro experiment:**
Two separate 4.1B knockdown 67NR cell lines and two control cell lines were mixed and plated.
DNA was isolated and analyzed from the culture at timepoints post plating to determine contribution of each cell line barcode to the culture. The average relative signal generated from the barcodes associated with each hairpin was quantified as described.

f. **Migration of 67NR cells with 4.1B knockdown or control:**
67NR cells with stable knockdown of 4.1B or control were plated in transwell plates in media lacking serum, and migration into 10% serum was counted 16 hours later.
Discussion

FERM domain proteins act as linkers between the actin cytoskeleton and the cell membrane, and regulating the attachment of the membrane to cytoskeletal actin has been implicated in many cellular processes including cell adhesion and migration, membrane protein localization and transport, and signal transduction. A number of proteins belonging to the larger 4.1 superfamily have been implicated in tumorigenesis and metastasis, and in these experiments I attempted to establish a screen in order to determine whether other proteins in the FERM domain family of proteins are also relevant to roles in suppressing or promoting tumorgenesis and metastasis. In order to do so, I initially chose to look at the ERM proteins, ezrin, radixin and moesin. Ezrin has already been established as a metastasis promoter in osteosarcoma (5) and is expressed at higher levels in numerous human cancers, as well as being correlated with a poor outcome in breast cancer (16).

In addition, some other FERM domain proteins have been implicated in tumor or metastasis suppression, merlin being the most extensively studied in this category. Merlin, the product of the Nf2 gene which is lost in neurofibromatosis type 2, a dominantly inherited disease which leads to the formation of multiple tumors in the nervous system. Furthermore, mice which have lost merlin expression can develop a variety of highly metastatic tumors, indicating a role for merlin not only in tumorgenesis but also in metastasis (17). Another FERM domain protein implicated in metastasis suppression is 4.1B/DAL-1, which has been described as a growth suppressor *in vitro* in non-small cell lung carcinoma (18) and as a metastasis suppressor in prostate cancer (3).

In a microarray analysis of existing microarrays (19) from the 4T1 panel of murine mammary carcinoma cell lines ezrin and another FERM domain protein, Epb4.114b (Ehm2) (described previously as being expressed in highly metastatic murine melanoma...
cells (20)), were at least 2-fold higher in the highly metastatic 4T1 cell line compared to the cell line described as poorly metastatic, 67NR. FERM-domain-containing proteins that showed at least 2-fold lower expression in the highly metastatic 4T1 cell line compared to 67NR, and might be acting as metastasis suppressors included Epb4.113 (4.1B/DAL-1), FRMD4b, Talin2, and Epb4.111 (4.1N).

Considering the roles of FERM domain proteins as both metastasis suppressors and enhancers, the FERM domain family of proteins appeared to be good candidates to use in developing an in vivo screen for metastasis. In this screen, cells which have sustained a knock-down of the proteins of interest are mixed with control cells. Both the knock-down and control cells stably express a unique DNA-based barcode which correlates with either the control or a knock-down vector. This mixture is then either plated as an in vitro experiment or introduced into mice through orthotopic injection or tail-vein injection. As tumors or metastases grow, the contribution of the cells to the tumors or metastases can be measured by the contribution of the barcoded vector, with cells that have undergone a knock-down which results in decreased metastasis showing a decreased contribution of the barcode in the resulting metastases, compared to cells which have undergone a control treatment, and cells in which the knockdown results in an increase in metastasis showing an increase in the barcode contribution compared to a control barcode. This method of in vivo screening was established successfully in our lab and used to determine the metastatic potential of breast cancer cells expressing various mutated forms of the oncogene YAP (21). However, when this system was implemented as a screen for FERM domain proteins we did not see a change in the contribution of ezrin, radixin or moesin knockdown 4T1 cells to metastasis when compared to control cells. Since ezrin and radixin had been previously described to enhance metastasis we expected to see a decrease in metastasis in 4T1 cells in which these proteins were knocked down. In both the in vivo screen and in vitro migration and invasion experiments...
we did not see a change in the cells’ metastatic potential or ability to migrate or invade in culture following knock down.

The literature tells us that these proteins may have a functional redundancy, and that knocking down one of them merely leads to compensation through one of the other close family members (15). This led to the next step, of attempting to knock down all three family members, ezrin, radixin and moesin, simultaneously, to see if elimination of these proteins could result in a decrease in metastasis in 4T1 cells. However, despite trying both successive knockdown in the cells and simultaneous knockdown using a concatemerized vector including all three hairpins, we were not able to successfully eliminate expression of all three proteins in the 4T1 cells. An alternative method to attempt this now would be by using the Crispr-Cas9 genome editing system to knock out all three genes simultaneously, assuming cells are able to survive without all three ERM proteins.

Along with experiments in which FERM-domain proteins described as metastasis enhancers were knocked down, where we expected a decrease in metastasis, we also knocked down a FERM domain protein described as a metastasis suppressor, 4.1B, in a cell line described as poorly metastatic. We hoped to establish FERM-domain family controls of both metastasis enhancers and suppressors before screening additional family members whose functions in metastasis are currently unknown. However, when 4.1B was knocked down in the 67NR cell line, which was described as poorly metastatic, we did not see a difference in metastasis between the knockdown line and the control. Surprisingly, we saw metastasis in the control despite the fact that the cell line had been described as poorly metastatic. We also attempted to repeat these experiments in a different murine mammary carcinoma cell line panel of varying metastatic potential, the
D2 panel (22), however in our hands the D2.1 cell line which had been described as poorly metastatic still was able to form lung metastases upon orthotopic injection into the fat pad, which had not been described in the original literature regarding these cells. We saw no significant differences in expression in other 4.1 family proteins (Figure 1, c-f), and decided not to pursue the screen any further.

In summary, although ezrin and its close homologs radixin and moesin have been implicated in promoting metastasis, we could not show that loss of either of the proteins singly effected metastasis in a murine mammary carcinoma model. In addition, loss of the FERM-domain family metastasis suppressor 4.1B could not promote metastasis in the murine mammary carcinoma cell line 67NR, which proved to be more metastatic in our hands than originally described. A wide-scale screen of FERM-domain-containing proteins to establish whether other FERM-domain proteins can enhance or suppress metastasis was not performed, and more recent literature (Valderrama, 2012) suggests that although some of these proteins can play a role in tumorigenesis and metastasis, they do not necessarily play a role in breast cancer metastasis and a screen specific for a mammary carcinoma model may not be entirely useful in elucidating the roles of these proteins in metastasis.

References


Chapter 3. Both major merlin isoforms regulate cell growth

Introduction

Merlin (NF2) is a well-characterized FERM-domain protein, identified as the protein product of the Nf2 tumor-suppressor gene which is lost in the familial neurofibromatosis type II (NF2) syndrome. In addition, mice that are heterozygous for NF2 are prone to a large variety of tumors, which show a high rate of metastasis. Merlin has two major isoforms (1) which I will refer to hence as isoform I (NF2a) and isoform II (NF2b). These two isoforms differ only by one exon at the C-terminus. Isoform I contains exons 1-15, and exon 17, whereas isoform II contains an alternative exon, exon 16, instead of exon 17, which introduces an alternate reading frame and an early termination. Isoform II is therefore 16 amino acids shorter than isoform I and contains a different C-terminus (Figure 1a.). Early literature in the field regarded isoform II as unable to suppress growth both in vitro and in vivo. In an experiment where rat schwannoma cells were injected subcutaneously into mice cells expressing isoform I failed to grow, but cells expressing isoform II were not suppressed (2). This inability to suppress growth was attributed to an inability of isoform II to achieve or maintain a closed conformation, due to a reduced ability of the isoform II C-terminus to bind the N-terminus (3). FERM-domain proteins, such as merlin, are classically described as switching between a closed conformation, in which the C-terminus binds the N-terminus, and an open conformation. This switch is regulated by a phosphorylation event. In Merlin, a major phosphorylation event that controls growth suppression occurs at S518 and is mediated by PAK (4) or PKA (5). Phosphorylation of Merlin results in loss of contact inhibition and increased proliferation (6). Initial research suggested that phosphorylated merlin was unable to form intramolecular associations and was in an open conformation, and therefore
suggested that the open conformation of merlin was inactive in suppressing growth (7). However, further investigation revealed that phosphorylated merlin could be preferentially found in the closed conformation, and that the switch between open and closed conformations appears to be a lot more dynamic than originally considered (8). Considering that the intramolecular associations between the N-terminus and the C-terminus of merlin are dynamic and not necessarily indicative of merlin being “active” or “inactive” in growth suppression, this raises the question of whether isoform II is indeed unable to suppress growth merely due to a decreased ability to form those intramolecular associations. Thus, we investigated whether isoform II is able to suppress growth, and whether there is a possible differential regulation of these two isoforms.

Results

1. Expression of two major merlin isoforms in multiple cell lines

In order to determine whether expression of either of the two major merlin isoforms (Figure 1a) is able to suppress growth, and whether phosphorylation of merlin influences its growth-suppressive ability, we generated MSCV-based retroviral vectors to stably deliver each merlin isoform, as well as merlin phospho-mutants and phospho-mimetics for each isoform. In the phospho-mutant expression vector the main regulatory phosphorylation site, Ser518, is mutated into an alanine. The phospho-mimetic consists of the Ser518 residue mutated into an aspartate, both mutants described previously (9). These constructs were then stably transduced into several cell lines, including normal epithelial mouse mammary gland cells (NMuMG, Figure 1b) and human mammary carcinoma cells (MDA-MB-231, Figure 1c), both of which express low endogenous levels of merlin, and two human mesothelioma cell lines which lack endogenous merlin (Meso428 and Meso59, Figures 1d and 1e). As expected, when the phospho-mutant and phospho-mimetic versions of merlin were expressed they were not recognized by an
Figure 1: Merlin isoform a and b expression in multiple cell lines using an MSCV-based retroviral vector

a. Illustration of the two major merlin isoforms with main phosphorylation sites noted.
b. Western showing expression of both major merlin isoforms and phospho-mutants or empty vector control in the normal mouse mammary gland epithelial cell line NMuMG.
c. Western showing expression of both major merlin isoforms in the MDA-MB-231 human mammary carcinoma cell line
d. Western showing expression of both major merlin isoforms or an empty vector control in the human mesothelioma cell line Meso428
e. Western showing expression of both major merlin isoforms in the human mesothelioma cell line Meso59
antibody specific for the phosphorylated Ser518 residue. Surprisingly, isoform II showed low phosphorylation of Ser518 when compared to isoform I in MDA-MB-231 and NMuMG cells, and showed a corresponding hypophosphorylated band when immunoblotting with a general merlin antibody. This initial result indicated that there could be differential regulation between the two isoforms.

2. Both major merlin isoforms can suppress growth

We next wanted to test the ability of each merlin isoform to suppress growth. For these experiments, cells were infected with either a retroviral vector expressing merlin and GFP, or a control vector expressing GFP alone. The percent of GFP-positive cells after culture following infection was determined, and all future measurements were normalized to the starting point. The cultures were followed over the course of 25-30 days post-infection, with cells being split post-confluency but kept at near confluent levels throughout the experiment. This was done to maintain merlin activity, since merlin-dependent growth suppression has been shown to occur upon contact inhibition of cells (10). In both immortalized mouse fibroblast cells (Figure 2a) and NMuMG cells (Figure 2c), both merlin isoforms were able to suppress growth, as shown by a decrease of GFP-positive (i.e. merlin positive) cells in the culture compared to the starting point, whereas the percentage of GFP-positive control cells (GFP alone) remained stable over time. In 4T1 cells (Figure 2b), which have a high amount of endogenous merlin, although both isoforms showed a suppression of growth, the decrease was only significant in the cells expressing isoform I. This might be due to endogenous merlin already acting in these cells, so expression of additional merlin does not significantly increase the amount of merlin-derived regulation.

Next, merlin was expressed in mesothelioma cells that have lost endogenous merlin. Malignant mesothelioma frequently shows a loss of NF2 (11) and therefore human
mesothelioma cell lines were selected as a biologically relevant model in which to examine rescue of merlin function. In two separate mesothelioma cell lines expression either of the two major merlin isoforms resulted in a significant suppression of growth, as measured by total cell number over time (Meso59, figure 2d, Meso428, figure 2e).

Figure 2: Both major merlin isoforms are able to suppress growth in multiple cell lines

Cells were infected with a merlin-GFP expressing retroviral vector or a control GFP+ vector at low titer, resulting in a mixed population of GFP+ cells expressing merlin, and GFP- cells not expressing merlin. The cells were normalized to the starting point and loss of GFP+ merlin expressing cells was measured over time, indicating a disadvantage in growth for merlin expressing cells. For the mesothelioma cell line experiments a standard growth curve was measured by counting cells over time post plating.

- A: Both major merlin isoforms can suppress growth in MEF cells, n=3.
- B: Merlin isoform a can suppress growth in 4T1 cells, n=3
- C: Both merlin isoforms can suppress growth in NMuMG cells, n=3
- D: Both merlin isoforms can suppress growth in Meso59 human mesothelioma cells, n=3
- E: Both merlin isoforms can suppress growth in Meso428 human mesothelioma cells, n=4

Astrisks denote significance at <0.05(*), <0.01(**), and <0.001(***). p-values were taken at the end point of each experiment.
3. Growth suppression requires contact inhibition and is regulated by phosphorylation

Next, we wanted to determine whether suppression of growth by isoform II was contact inhibition-dependent, and phosphorylation-dependent, as described by Shaw et al., 1998 for isoform I. Proliferation experiments as described above were carried out in cells expressing each merlin isoform or a control, or a merlin S518A phospho-mutant, and maintained at near confluency or at non-confluent levels. At confluency, cells are contact inhibited and therefore merlin should suppress growth, whereas cells maintained at non-confluency should not be suppressed by merlin.

When NMuMG cells were maintained at non-confluent levels and the percentage of GFP-positive cells was measured over time, there was no change in the percentage of merlin expressing cells when compared to the starting point for either merlin isoform I (Figure 3a) or isoform II (Figure 3b). This indicates that, at subconfluent levels, there is no growth disadvantage to merlin-expressing cells. However, when NMuMG cells were maintained at confluent levels, both isoform I (Figure 3c) and isoform II (Figure 3d) showed a decrease in GFP-positive, merlin-expressing cells over time. This decrease was potentiated when a merlin mutant (of either isoform) that could not be phosphorylated on serine 518 was expressed. It is interesting to note that in subconfluent cells, a presumably constitutively active unphosphorylated merlin is still unable to significantly suppress growth. Thus it appears, that similarly to isoform I, isoform II is able to suppress growth and is active in growth suppression when cells are contact-inhibited. Furthermore, a mutant in which the regulatory phosphorylation event on Ser518 can not occur suppresses growth even further in confluent cells, but has no effect on non-confluent cells in both isoforms, indicating that both merlin isoforms are active as growth suppressors only on contact inhibited cells, and even a constitutively
Figure 3: Both merlin isoforms regulate cell growth through a contact-dependent mechanism

a. NMuMG cells infected with GFP +NF2a, NF2a S518 phosphorylation mutant, or a GFP+ control were maintained at low density and proliferation of merlin-expressing cells was measured by analyzing retention of GFP+ cells in the culture, normalized to the starting point. n=2

b. NMuMG cells infected with GFP +NF2b, NF2b S518 phosphorylation mutant, or a GFP+ control were maintained at low density and proliferation of merlin-expressing cells was measured by analyzing retention of GFP+ cells in the culture, normalized to the starting point. n=2

c. NMuMG cells infected with GFP +NF2a, NF2a S518 phosphorylation mutant, or a GFP+ control were maintained at high density and proliferation of merlin-expressing cells was measured by analyzing retention of GFP+ cells in the culture, normalized to the starting point. n=2

d. NMuMG cells infected with GFP +NF2b, NF2b S518 phosphorylation mutant, or a GFP+ control were maintained at high density and proliferation of merlin-expressing cells was measured by analyzing retention of GFP+ cells in the culture, normalized to the starting point. n=2
active form will not significantly suppress growth of non-confluent cells.

4. Isoform I and isoform II are differentially regulated and differ in stability in culture

As seen from these results, both isoforms of merlin are able to suppress growth and this suppression of growth relies on contact inhibition and can be regulated through phosphorylation of S518. However, in both NMuMG cells and in MDA-MB-231 cells, we observed that phosphorylation of S518 was reduced in isoform B (Figure 1b and 1c). This indicated that there could be a difference between the two isoforms in upstream regulation through phosphorylation.

Merlin is subject to multiple post-translational modifications. In addition to a PAK-driven phosphorylation on S518, merlin is phosphorylated on additional serine and threonine residues by both PKA and Akt (12) and these phosphorylation sites are present in both merlin isoforms (Figure 1a).

Merlin isoform I (NF2a) expressed in NMuMG cells migrates on SDS-PAGE as a doublet. This doublet has been interpreted previously (10) to indicate that the slower migrating band is a hyperphosphorylated form of merlin and, indeed, immunoblotting with an antibody that recognizes phosphorylated S518 detects this band (Figure 1b). However, in NMuMG cells expressing isoform II (NF2b) merlin does not migrate as a doublet, but only as the hypophosphorylated form even though it does show some phosphorylation on serine 518. Similarly, in MDA-MB-231 cells, isoform I does not migrate as a doublet, but only as the hyperphosphorylated slower migrating band, (Figure 1c), while isoform II migrates exclusively as the faster migrating band, showing significantly less Ser518 phosphorylation when immunoblotted with the phospho-specific antibody. As a major regulatory event that determines merlin activity, the fact that isoform II is less prone to phosphorylation indicates that isoform II could not only be an active form of merlin, but have increased activity over isoform I depending on the
context.
Meso428 cells did not show a difference in S518 phosphorylation (Figure 1d), further indicating that cellular context could play a role in determining the differences in regulation between the isoforms. However, when Meso428 mesothelioma cells expressing both major merlin isoforms were plated for a proliferation experiment, both isoform I and isoform II showed high levels of the slower migrating hyperphosphorylated band, with isoform I migrating almost exclusively as the slower band while isoform II appeared as a doublet (Figure 4a, day 3 lysates). By day 12 the lysates showed a loss of isoform I (Figure 4a, day 12 lysates) and, along with that loss, the cells expressing isoform I continued to proliferate despite reaching confluence. This indicated a difference in protein stability in culture between isoform I and isoform II, even in a case where both isoforms showed initially similar levels of inhibitory phosphorylation.

MDA-MB-231 cells, which show differential phosphorylation between the two isoforms (Figure 1c) showed a similar lack of similar lack of stability for isoform I as seen in the mesothelioma cell lines. In a lysate collected shortly after infection of MDA-MB-231 cells with the MSCV-based expression vectors of either isoform I or isoform II, isoform I is present in the lysate almost completely in the slower migrating (i.e. phosphohorylated) form, while isoform II is present in the faster migrating form (Figure 4b, left). In a lysate collected from cells cultured for several weeks post-infection, isoform I is completely lost from the culture while isoform II is still present (Figure 4b, right).

There is precedence for differential phosphorylation of isoforms I and II. Tang et al., (12) show that isoform I is phosphorylated by Akt on residues T230 and S315. Furthermore, they show that this phosphorylation leads to merlin ubiquitination and degradation. In a pull down experiment to establish that Akt binds merlin directly, Akt binds the N-terminal fragment or C-terminal fragment of isoform I, indicating that Akt acts upon the "open" form of merlin. However, Akt only weakly binds the C-terminal fragment
**Figure 4: Isoform I and isoform II of merlin differ in stability in culture**

a. Left: Proliferation curve of mesothelioma Meso428 cells in culture. Cells reached confluence at day 10. Right: Western blots of mesothelioma cell lines corresponding to proliferation at early vs. late time points. Isoform I is lost at the later time point, which corresponds with a loss of growth suppression.

b. Western blot of early culture vs. late culture MDA-MB-231 lysates.

c. Meso428 lysates expressing isoform I or isoform II were treated with CIP or control, and immunoblotted for pS518 and merlin.
of isoform II. Since this modification leads ultimately to merlin degradation, this could explain how phosphorylation of isoform I, but not isoform II, on S518 might lead to increased accessibility of isoform I to further Akt phosphorylation, resulting in ubiquitination and degradation. This might explain both the results from the NMuMG and MDA-MB-231 cells, and the mesothelioma cells. In mammary epithelial or carcinoma cells, isoform I is phosphorylated on Ser518, leading to additional phosphorylation events and a gel shift to the hyperphosphorylated position. Isoform II, however, experiences less phosphorylation on Ser518 and therefore is not a target for additional phosphorylation events and therefore runs in the hypophosphorylated position on an SDS-PAGE gel. In mesothelioma cells, both isoforms are phosphorylated on Ser518. However, isoform II might be protected from additional phosphorylation, ubiquitination, and ultimate degradation due to decreased accessibility to the phosphorylation sites even when phosphorylated on S518. This protection could lead to increased stability of isoform II in culture. To investigate whether there was decreased accessibility to the Ser518 site on isoform II, compared to isoform I, Meso428 lysates expressing a hyperphosphorylated isoform I and isoform II were treated with calf intestinal protease (CIP). CIP treatment led to elimination of the pS518-recognized bands in isoform I, but not in isoform II (Figure 4c). This could explain how despite being hyperphosphorylated, isoform II is protected from further modifications and is not able to be ubiquitinated and degraded, leading to a persistence of isoform II in culture when compared to isoform I. Surprisingly, in the non-CIP treated sample we noted that both the slower and faster migrating bands of isoform I were recognized by the pS518 antibody, which could indicate that phosphorylation of S518 alone does not lead to the slower migrating band, but rather additional phosphorylation events or other post-translational modifications lead to the hyperphosphorylated state indicated by the slower migrating band.
Discussion

Merlin has been studied extensively due to its role in human disease, yet some confusion remains in the field regarding the regulation of merlin. Initially discovered to have a high homology to the ERM proteins ezrin, radixin and moesin (13), merlin was assumed to have a conformational regulation similar to the canonical model introduced for the ERM proteins (14), where the protein can be activated or inactivated through a phosphorylation event that induces conformational change. The conformational change described for ERM proteins involves a switch between a “closed” conformation, mediated by an intramolecular interaction between the FERM domain and the C-terminal domain of the protein, and, following phosphorylation, loss of this intramolecular interaction and acquisition of an “open” conformation. In the context of ERM proteins this phosphorylation and switch to an open conformation leads to activation of the protein, since the open conformation allows multiple intermolecular interactions at the membrane-cytoskeleton interface. In merlin it had long been thought that the open conformation was an inactive conformation, since phosphorylation of merlin proved to lead to an inability of merlin to suppress growth and it was posited that, similar to the canonical ERM proteins for which merlin was named, phosphorylation led to an open conformation (2). However, recent studies (8) show that, in the case of merlin, this is not as clear cut. The unique C-terminal of merlin is able to fit in a groove of the N-terminal FERM-domain when merlin is phosphorylated (15), leading to a closed conformation despite phosphorylation. Furthermore, the switch between an “open” and “closed” conformation in merlin appears to be much more fluid.

When merlin was described and characterized, it was noted that it could be found as one of two major isoforms, products of alternative splicing. Due to decreased intramolecular interaction in isoform II, and a reported failure of isoform II to mediate growth inhibition in
vivo or in vitro (2) or to impair cell migration (16) in rat schwannoma cells it was generally assumed that isoform II was unable to act as a tumor suppressor. We have shown that isoform II is able to suppress growth, and similarly to isoform I, to suppress growth in the context of contact inhibition. Only when cells were maintained at a confluent level was isoform II able to suppress growth.

Phosphorylation of merlin on serine 518 has been described as inhibitory (4) and, following this phosphorylation, merlin is more susceptible to additional phosphorylation events (12) which leads to eventual ubiquitination and degradation (Okada 2009). Hyperphosphorylation leads to an increase in intramolecular associations between the N-terminal domain (NTD) and the C-terminal domain (CTD), and isoform I is more likely to be found in a closed conformation when phosphorylated, whereas isoform II is found in an open conformation more frequently, whether phosphorylated or not (8). When serine 518 is mutated to an alanine, both isoforms I and II exhibit increased suppression of growth, indicating that isoform II can be regulated through phosphorylation in a manner similar to isoform I. However, both NMuMG and MDA-MB-231 cell lysates show a difference between isoform I and isoform II in migration on SDS-PAGE. While isoform I migrates as a doublet with a slower band indicating hyperphosphorylation (10), isoform II migrates as a faster band, level with the bands from lysates of cells expressing phospho-mutant forms of merlin. This suggests that there could be differential regulation between the isoforms despite the fact that both can be inhibited by phosphorylation on S518. In MDA-MB-231 cells and in mesothelioma cells, isoform I was lost in culture over time; in mesothelioma cells this occurred along with a resumption of cell growth that coincided with loss of merlin. Isoform II was maintained in culture although the majority was present as the hyperphosphorylated slower migrating band. When lysates of mesothelioma cells were treated with CIP, the upper bands on the immunoblot of merlin
were reduced, and those recognized by an antibody specific for S518 disappeared, but only for isoform I. In the case of isoform II the phosphorylated band remained despite treatment with CIP. A possible explanation for this difference between the isoforms could be that the conformation of isoform II C-terminal domain protects the phosphorylated residues from phosphatase treatment.

Tang et al. addressed the phosphorylation of merlin by Akt, and showed that both merlin isoforms, when expressed as full-length proteins, were unable to bind Akt. However, the N-terminal domain (NTD, which is shared between the isoforms) and the C-terminal domain (CTD) of isoform I were able to bind Akt in a pull-down assay. The CTD of isoform II was unable to pull down Akt. Since phosphorylation by Akt is followed by ubiquitination and degradation, this could explain a mechanism where differential phosphorylation of the isoforms by Akt can lead to differences in protein stability between the isoforms. The inaccessibility of the isoform II CTD to phosphatase treatment could also explain its inaccessibility to Akt, and to subsequent phosphorylation events, ubiquitination and degradation, explaining how isoform II is maintained in cells longer than isoform I, despite being phosphorylated on S518. In order to examine this hypothesis additional experiments are needed. Initially, it remains to be seen whether isoform II can be phosphorylated on residue S315 by Akt, using the anti-phospho S315 antibody developed by Tang et al. In addition, lysates from cells expressing isoform I or II at time points post infection with the merlin expressing vector can be used to examine the change in merlin protein levels over time. Merlin phosphorylation and ubiquitination levels could also be assayed to determine whether there is an increase in ubiquitination in isoform I concomitant with an increase in phosphorylation, that is not present in isoform II. Finally, treatment with an Akt inhibitor or mutating the Akt target serine 315 should allow for persistence of isoform I in culture in a manner similar to isoform II.
In summary, both of the major merlin isoforms are able to suppress growth in multiple cell lines. Both of the isoforms are dependent on contact inhibition in order to suppress growth, and can be regulated through an inhibitory phosphorylation on S518. However, isoform II is able to persist in culture longer than isoform I, despite being hyperphosphorylated in some contexts although not in others and depending on cell type. This indicates a differential regulation between the isoforms that might be related to accessibility of the isoforms to Akt phosphorylation and subsequent ubiquitination and degradation.

References


Chapter 4. Merlin regulation of YAP

Introduction
Yes-associated protein (YAP) and its close paralog, TAZ (WWTR1), are transcriptional co-activators that mediate expression of proliferation and anti-apoptotic genes through interaction with TEAD transcription factors (1).

YAP and TAZ can be regulated by multiple upstream inputs, including extracellular factors (2), cell-cell adhesions (3), and mechanotransduction (4). Since YAP/TAZ are homologs of the Drosophila transcription factor Yki, much of the original research into the regulation of YAP/TAZ focused on the canonical Hippo pathway (Figure 1), which regulates Yki/YAP/TAZ as described in flies (5), and conserved in mammals. The serine-threonine kinase Hippo (MST1/2 in mammals) phosphorylates Warts (Lats1/2 in mammals) (6) which in turn phosphorylates Yki, promoting cytoplasmic sequestration of Yki and preventing transcription mediated by the interaction of Yki with the TEAD transcription factor family member, Scalloped (7). In Drosophila, the FERM-domain protein Merlin (NF2) links the Hippo kinase cascade to signals from the cell membrane (8). However, the role of merlin regulation of YAP in mammals is not as clear-cut.

Many of the studies involving mammalian regulation of YAP by merlin focus on the function of these proteins in the liver. YAP-deficient livers show impaired function and bile-duct deficiencies (9), while Merlin-deficient livers show corresponding bile-duct hamartomas and hepatocellular carcinomas in older mice (9) and hyperplasia of undifferentiated liver progenitors, termed oval cells, in younger mice (10). However, whether the YAP-deficient liver phenotype is a direct result of merlin activity is unclear.
Figure 1: The canonical Hippo pathway and possible avenues for regulation by merlin
Zhang et al. (9) showed that heterozygous deletion of YAP was able to suppress the merlin-deficient overgrowth phenotype. However, considering merlin’s multiple roles and mechanisms in suppressing cell growth, merlin might only be indirectly involved in YAP regulation through the Hippo pathway in the liver, as merlin is also able to control liver growth through its role in controlling EGFR signaling (10). Complicating these results is the fact that crosstalk exists between EGFR signaling and YAP activity, both through upstream regulation of YAP by EGFR via regulation of MST2 (11) or Lats (12) and downstream since the EGFR ligand AREG is a transcriptional target of YAP (13).

Aside from its role in the developing liver, the question of how merlin might regulate YAP in mammals has also been investigated in mesothelioma. Merlin is frequently mutated in mesothelioma (14) and this tumor model has also shown high-copy amplification of YAP(15). Furthermore, transfection of a merlin-null mesothelioma cell line with merlin and YAP showed that merlin was able to relocate YAP from the nucleus to the cytoplasm, and showed an increase of Lats-phosphorylation of YAP on serine 127 (15).

Since our lab had been studying the role of YAP in metastasis (16) and the role of FERM domain proteins in metastasis, we decided to investigate further how the two major merlin isoforms, described in the previous chapter, might regulate YAP, and decided to focus on merlin regulation of YAP in the context of mesothelioma.

Results

1. Merlin expression decreases nuclear localization of YAP in mesothelioma cells

In order to determine whether merlin expression was able to regulate YAP, we first investigated nuclear localization of YAP following expression of merlin. YAP promotes
Figure 2: Merlin expression decreases nuclear localization of YAP

a. Meso59 cells stably expressing merlin or a control were fixed at confluency and stained for YAP. On the left, a representative image is shown. On the right, the bar graph illustrates a quantification of YAP nuclear localization. (n=8 fields, in a total of 2 separate experiments)

b. Meso59 cells stably expressing merlin or a control were infected with a retroviral WT YAP construct, fixed at confluency and stained for YAP. (n=8 fields in a total of 2 separate experiments)

c. Image depicts how cells were scored and quantified in the above experiments for nuclear or mostly nuclear YAP compared to cytoplasmic YAP. Asterisks denote significance at <0.05(*), <0.01(**), and <0.001(***).
growth through nuclear localization, where it acts as a transcriptional co-activator. We acquired two merlin-null mesothelioma cell lines, Meso59 and Meso428 (a kind gift from Dr. Jonathan Fletcher) for these experiments. In confluent merlin-null mesothelioma cells, YAP localized to the nucleus, however, upon expression of merlin, YAP localized predominantly to the cytoplasm (Figure 2a). Furthermore, when wild-type YAP was overexpressed in these mesothelioma cells, merlin expression was still able to relocate YAP to the cytoplasm (Figure 2b).

2. Merlin expression decreases TEAD reporter activity

Next, we wanted to determine whether YAP relocalization to the cytoplasm following merlin expression also reflected a decrease in YAP transcriptional activity. To do this we used a YAP/TAZ-responsive luciferase reporter (Mahoney et al. 2005) to directly assess YAP/TAZ transcriptional activity (see illustration, Figure 3a, left). In Meso59 mesothelioma cells stably expressing either merlin or a control vector, and transiently transfected with the reporter, we saw a significant decrease in reporter activity following expression of either of the two major merlin isoforms (Figure 3a). These results were corroborated with an additional merlin-null mesothelioma cell line, Meso428. Again, mesothelioma cells stably expressing the two major merlin isoforms were transiently transfected with the reporter and expression of either merlin isoform showed a decrease in TEAD reporter activity. Furthermore, this decrease in TEAD activity was not associated with an increase in an inhibitory phosphorylation of YAP on the major Lats target serine 127 (Figure 3b).

In order to determine whether merlin expression was able to regulate YAP in additional non-mesothelioma contexts we examined the impact of merlin expression on HMLER cells, a ras-immortalized human mammary epithelial cell line which has very low levels
Figure 3: Merlin expression decreases TEAD reporter activity

a. Expression of either major merlin isoform in Meso59 cells decreases TEAD reporter activity. The illustration depicts the TEAD reporter vector used in these experiments. For NF2a n=20, for NF2b n=6. Astrisks denote significance at <0.05 (*), <0.01 (**), and <0.001 (***)

b. Expression of either major merlin isoform in Meso428 cells decreases TEAD reporter activity (n=10) but with no change in YAP phosphorylation on the LATS target S127 when immunoblotted for pYAp S127.

c. Expression of merlin in HMLER cells decreases TEAD reporter activity. Cells were infected with a retroviral vector expressing Merlin-IRES-GFP or a GFP alone control and sorted for GFP-positive cells. (n=4)

d. Knockdown of merlin in HMLE cells increases TEAD reporter activity (n=4, two separate hairpins tested, labeled shRNA1 and shRNA2, inset shows Western depicting knockdown)
of endogenous merlin. Expression of either merlin isoform in HMLER cells significantly decreased YAP activity as measure by our TEAD reporter assay (Figure 3c). Next we knocked down merlin in the HMLE cell line, a human mammary epithelial cell line which expresses merlin; when merlin was knocked down in HMLE cells, there was a concomitant increase in YAP/TAZ transcriptional activity compared to the merlin-expressing control (Figure 3d).

In summary, merlin expression in multiple cell lines decreases YAP nuclear localization and transcriptional activity, however it does not affect the levels of Hippo-pathway mediated phosphorylation of YAP.

3. Merlin can regulate a LATS-insensitive YAP mutant

In Drosophila, merlin has been implicated as an upstream regulator of the Hippo pathway. However, when merlin was expressed in mesothelioma cells, there was no increase in phosphorylation of the major Lats phosphorylation site, serine 127 (Figure 3b). This suggests that merlin may be regulating YAP in a Hippo-pathway-independent manner. To determine whether Lats phosphorylation was necessary for merlin regulation of YAP we established Meso59 mesothelioma cells which stably expressed a mutant form of YAP in which either one or two major Lats phosphorylation sites, S127 and S381, had been mutated to alanines, along with merlin or an empty vector control. Surprisingly, even when YAP is not regulated by the Hippo pathway through the two canonical Lats phosphorylation sites, merlin is still able to suppress YAP activity, as indicated by a decrease in YAP/TAZ reporter activity (Figure 4a). Furthermore, in confluent Meso59 cells stably expressing both merlin and a Lats-insensitive mutant form of YAP, YAP is localized primarily in the cytoplasm, suggesting that merlin promotes cytoplasmic localization of YAP independent of LATS phosphorylation on S127 and S381 of YAP (Figure 4b).
Figure 4: Merlin expression decreases TEAD reporter activity and YAP nuclear localization in presence of LATS-insensitive YAP

a. Merlin expression decreases TEAD reporter activity in Meso59 cells expressing either WT YAP, a YAP S127A mutant or a YAP S127A;S381A mutant. (For isoform a n=6, for isoform b n=4 except in YAP S127A mutant where n=2.)
Astrisks denote significance at <0.05 (*), <0.01 (**), and <0.001 (***)

b. Merlin expression decreases YAP nuclear localization in Meso59 cells expressing a YAP S127A;S381A mutant. On the left, a representative image. On the right, quantification of immunofluorescently stained YAP in confluent mesothelioma cells.
(n=8 fields from 2 separate experiments)
4. Hippo-pathway-independent regulation of YAP by merlin requires the WW domains of YAP

In the canonical model of YAP regulation described in Drosophila and homologous in mammals, a kinase cascade leads to YAP phosphorylation by LATS, which in turn leads to YAP cytoplasmic sequestration and degradation. In mammals, however, there are additional regulatory arms controlling YAP that do not involve the canonical Hippo kinase cascade. In addition to mechanotransduction being able to determine YAP localization in a Hippo-independent manner (4), YAP/TAZ can be sequestered in the cytoplasm either in a complex with β-catenin (17) or in a complex with Angiomotin-like 2 (AMOTL2) (18), without LATS phosphorylation being necessary for this cytoplasmic sequestration; instead, the YAP WW domains interact with the AMOTL2 PPxY motif. In order to investigate whether the WW domains of YAP are necessary for suppression of YAP activity by merlin, we generated a YAP mutant in which the tryptophans in both of the YAP WW domains were mutated to phenylalanines, as described in (19). When Meso59 cells stably expressing merlin or a control were infected with wild-type YAP or a LATS-insensitive YAP mutant, YAP/TAZ reporter activity decreased, as described previously (Figure 4). However, cells expressing wild-type YAP or LATS-insensitive YAP with mutated WW domains maintained high YAP/TAZ transcriptional activity when merlin was expressed (Figure 5a), suggesting that mutation of the YAP WW domain renders it insensitive to merlin.

Furthermore, mRNA levels of a YAP/TAZ target gene, CTGF, were significantly decreased by merlin expression in Meso59 cells expressing wild-type YAP or LATS-insensitive YAP mutants (YAP^{S127A} or YAP^{S127A,S381A}), but not in cells expressing the same constructs with mutated WW domains. This suggests that merlin is unable to suppress YAP activity when YAP WW domains are mutated (Figure 5b). These experiments establish a function for merlin in inhibiting YAP/TEAD-mediated transcription that does
Figure 5: The YAP WW domains are required for merlin regulation of YAP

a. Merlin expression is unable to regulate TEAD reporter activity in Meso59 cells expressing YAP with mutated WW domains. (n=7)

Astrisks denote significance at <0.05 (*), <0.01 (**), and <0.001 (***)

b. Merlin expression is unable to regulate the YAP target gene CTGF expression in Meso 59 cells expressing YAP with mutated WW domains. (n=2)
Figure 6: The YAP WW domains are required for YAP cytoplasmic relocation by merlin
Above, representative images of Meso59 cells expressing WW-mutant YAP and merlin
or an empty vector control. Below, quantification of nuclear localization.
(n=8 fields, from a total of 2 separate experiments)
not require Hippo pathway signaling but relies instead on direct interactions of merlin with the WW domains of YAP.

Note, however, that merlin is still able to suppress the activity of wild type (LATS-sensitive) YAP with mutated WW domains, presumably through merlin’s ability to stabilize LATS at the plasma membrane and activate the canonical Hippo pathway (20). Therefore, merlin has at least two independent mechanisms to regulate YAP/TEAD transcription, one LATS-independent and one dependent on LATS-mediated phosphorylation of YAP.

Finally, as shown previously, merlin expression can lead to cytoplasmic localization of YAP, including LATS-insensitive YAP mutants. However, in confluent Meso59 cells expressing merlin and a LATS-insensitive, WW domain-mutated form of YAP, merlin is no longer able to mediate cytoplasmic localization of YAP, and the majority of YAP is found in the nucleus as shown through immunofluorescence imaging (Figure 6).

To determine whether merlin’s suppression of YAP nuclear localization and activity, as evidenced through a decrease in reporter activity and CTGF expression, was able inhibit cell growth, we measured mesothelioma cell proliferation over time using a standard assay in which cell numbers were counted over time post-plating. As expected, merlin expression in mesothelioma cells resulted in a decrease in cell proliferation over time (Figure 7a). This decrease was also seen when mesothelioma cells were infected with wild-type YAP vector (Figure 7b) or with a LATS-insensitive YAP vector (Figure 7c). However, merlin expression was not able to decrease proliferation of mesothelioma cells infected with a LATS-insensitive YAP vector in which the WW domains were mutated (Figure 6d). This is further evidence that Hippo-independent regulation of YAP by merlin requires the WW domains of YAP in order to mediate suppression of YAP activity.
Figure 7: Merlin expression decreases proliferation of mesothelioma cells in presence of YAP, but not YAP WW domain mutants

Astrisks denote significance at <0.05 (*), <0.01 (**), and <0.001 (***)

a. Cell growth of Meso59 cells expressing Merlin or a control vector, n=4
b. Cell growth of Meso59 cells expressing Merlin or a control vector and wild type YAP, n=4
c. Cell growth of Meso59 cells expressing Merlin or a control vector and YAP with mutated Lats target serines S127 and S381, n=4
d. Cell growth of Meso59 cells expressing Merlin or a control vector and YAP with mutated Lats target serines S127 and S381 and mutated WW domains, n=4
5. Merlin binding of YAP requires the WW domains of YAP

We next wanted to determine whether merlin regulation of YAP stemmed from a physical interaction between merlin and YAP. We expressed a FLAG-tagged version of either of the two major merlin isoforms in Meso59 cells (Figure 8a) and were able to immunoprecipitate endogenous YAP from the lysate of confluent cells using an anti-FLAG antibody. In a reciprocal experiment, endogenous merlin was immunoprecipitated from 293 cells expressing FLAG-tagged YAP or YAP mutants using an anti-FLAG antibody. However, FLAG-tagged YAP with mutated WW domains was unable to pull down endogenous merlin in 293 cells (Figure 8b). These results indicate the merlin is able to bind to YAP, and that this interaction requires the WW domain of YAP. However, this results do not prove that this interaction is necessarily a direct interaction, and whether other proteins associate with merlin and YAP and are required for this interaction to take place remains to be seen.
Figure 8: Merlin binds YAP and requires WW domains

a. Immunoprecipitation of FLAG-tagged merlin isoforms can pull down endogenous YAP in Meso59 cells expressing FLAG-tagged merlin.

b. Immunoprecipitation of FLAG-tagged YAP can pull down merlin, but YAP WW mutants are unable to pull down endogenous merlin in 293 cells expressing YAP or YAP mutants.
Discussion

Although many studies on YAP regulation have focused on the Hippo pathway, there has recently been research indicating that there are other factors that come into play. We initially focused on the role of merlin in regulating YAP due to its role in Drosophila, considering the high homology between members of the Hippo pathway in flies and mammals. Many mechanisms have been described to explain how merlin is able to suppress growth in multiple cell types; however, a mechanism for merlin regulation of YAP has not been shown. In these experiments we used a number of merlin-null mesothelioma cell lines. Since mesothelioma is one of the human diseases which shows a loss of merlin, and it has been previously implied that YAP may be regulated by merlin in mesothelioma, we decided that mesothelioma cell lines would be a biologically relevant context in which to investigate the possible functions of merlin and its role in YAP regulation.

Merlin expression in mesothelioma cells led to a decrease in YAP activity as evidenced proximally through reporter activity and target gene expression, and to a decrease YAP nuclear localization, as well as a decrease in cell proliferation. Surprisingly, merlin was able to suppress YAP activity even in the presence of a LATS-insensitive YAP. However, it is important to note that this LATS-insensitive YAP is only mutated on the two residues, S127 and S381, that have been most intensively investigated as targets of LATS, with S127 showing the most dramatic increase in YAP activity when mutated (3). It remains to be tested whether merlin can regulate a YAP in which all LATS target residues are mutated. Furthermore, in order to answer completely the question of whether merlin acts independently of the Hippo pathway when regulating YAP, these experiments should be done in the context of LATS knockdown. Due to difficulty in
completely eliminating both LATS1 and LATS2 these experiments were not done, but could possibly now be attempted following elimination of LATS1/2 expression through Crispr-Cas9 genome editing.

The role of angiomotin and its family members, angiomotin-like1 and angiomotin-like2, in YAP regulation is still unclear, with literature describing these proteins as YAP inhibitors (21, 22) or promoters (23) of YAP activity. However, one of the mechanisms described involves a Hippo-independent mechanism of YAP regulation, in which angiomotin PPxY motifs bind the WW domains of YAP and sequester it in the cytoplasm without requiring prior YAP phosphorylation by LATS (18). This hints at the ability of YAP to be regulated independently of the Hippo pathway. In Drosophila, Hippo-independent regulation of Yki, the YAP homolog, is mediated by the FERM-domain protein Expanded in a similar manner; PPxY motifs on Expanded can bind the Yki WW domain and sequester Yki in the cytoplasm (24). The mammalian homolog of Expanded, FRMD6, lacks these PPxY motifs, leaving open the question whether FERM-domain-mediated direct regulation of YAP through the WW domains can occur in mammals. We attempted to address this by showing that although merlin can suppress YAP activity, WW-mutated YAP is no longer regulated by merlin. In addition, merlin can bind YAP in immunoprecipitation experiments, but fails to bind a WW domain mutated YAP. An experiment that remains to be done is to determine whether merlin binds YAP in the biologically relevant mesothelioma cell lines and, furthermore, determine whether YAP can bind merlin directly. Merlin does contain a PPxY motif and mutating this motif would be an initial step in investigating the interaction between YAP and merlin. However, Yokoyama et al. (15) suggest through direct binding in-vitro experiments that merlin does not bind YAP directly, in which case the question remains which other proteins may be associated with the complex and, angiomotin, which has been shown separately to bind merlin (25) and
YAP (18) remains a candidate for this interaction. It remains to be seen whether angiometin is pulled down in a complex with YAP and merlin. Another possible candidate which has been shown to bind both merlin and YAP is α-catenin (26, 27) which might also be involved.

In summary, merlin is able to regulate YAP in a manner which appears to be largely Hippo-pathway independent. This regulation is dependent on a functional WW domain of YAP. Merlin can bind YAP but requires the WW domain to do so. These results indicate that merlin might be able to regulate YAP through binding the WW domains, whether directly or with an intermediate, and might be a mechanistic link between FERM domains and YAP in mammals.

References


Chapter 5. Discussion

The role of FERM domain proteins in tumorigenesis and metastasis

Although our study did not find any additional roles for ERM proteins in tumorigenesis and metastasis using a murine mammary carcinoma model, this does not rule out a role for these proteins in other tumor models. Indeed, the ERM proteins specifically and the 4.1 family of proteins in general have been shown to play roles in both metastasis and tumorigenesis (reviewed in (1), and in (2)). The question still remains as to whether other 4.1 family proteins, which were not screened in these experiments, contribute to tumor formation or metastasis. Using a more efficient system such as CRISPR-Cas9 to knock out the proteins in question, while using an in vivo barcoded vector system such as the one we developed to analyze the contribution of the knockdown or overexpressing cells to the tumor or metastases in the tumor model could serve to address this question.

Choosing the appropriate model in which to screen these proteins remains difficult, since expression levels vary in different tissues and it appears that, depending on the model used, there can be redundancies. For example, although in vivo expression of the ERM proteins is dependent on cell type, cells in culture express ezrin, radixin and moesin simultaneously and redundantly, such that these proteins compensate for one another. This could be the case for other 4.1 proteins as well.

Despite these difficulties, the 4.1 family of proteins remain interesting candidates for future study of metastasis due to their roles in maintaining cell-cell contacts, determining cell polarity, and organizing the cell cortex, all necessary in preventing metastasis.
Suppression of cell growth by both major merlin isoforms

While investigating the role of ERM proteins in tumorigenesis and metastasis, I became interested in merlin, a FERM-domain-containing tumor suppressor that has been extensively studied and well characterized. Merlin was originally considered to be regulated in a manner similar to its close homologs, the ERM proteins, through intramolecular associations that determine whether it is in a closed or open conformation, and this was thought to govern merlin's activity. Since isoform II had been shown to be unable to achieve the closed conformation (3) and the closed conformation had been shown to regulate growth (reviewed by (4)) it was assumed that isoform II was an inactive form of merlin. As recent literature suggested that the conformational switch was more fluid and that the closed form of merlin was not required for merlin activity (5), I questioned whether isoform II was able to suppress cell growth, and whether it was regulated similarly to isoform I. I have shown conclusively that isoform II indeed does suppress cell growth. Furthermore, isoform II experiences differential phosphorylation when compared with isoform I in a number of cell lines. The inhibitory phosphorylation on Ser518 is decreased in isoform II when compared to isoform I under similar culture conditions, and isoform II is more stable in culture than isoform I. This indicates that the isoforms experience different regulatory control and, rather than being a non-active isoform, isoform II can persist in culture. This might be a consequence of the Ser518 site in isoform II being protected by the alternate C-terminal tail, which is the only difference between the two major isoforms. In the unprotected isoform I, phosphorylation of Ser518 leads to additional phosphorylation events and ultimately to ubiquitination and degradation of merlin (6). Hypothetically, if the Ser518 residue on isoform II were to be shielded by the C-terminal tail, that might protect it from further phosphorylation events and consequentially, from degradation, leading to improved stability of isoform II in culture. It remains to be seen whether these additional events of phosphorylation,
ubiquitination and degradation indeed differ in isoform II, and what would be the in vivo contexts of such differential regulation.

One experiment to address the differences between the isoforms would be phosphopeptide mapping. Although the major phosphorylation sites are shared between the two isoforms, and the short C-terminal tail that distinguishes between the isoforms does not contain known phosphorylation sites, the fact that isoform I shows hyperphosphorylation under some circumstances when isoform II does not shows that hyperphosphorylation could be a way to investigate the differences between the isoforms. Determining where the additional phosphorylation occurs would be the first step in elucidating the differences in regulation between the isoforms.

Another way to address the difference between the two major isoforms would involve determining the localization of the two isoforms. Merlin has been shown to localize to the cell cortex, to the cytoplasm, and to the nucleus. Is there a preference for either of the isoforms for any single compartment? Since the localization could determine the mechanism by which merlin inhibits cell growth, a preference of an isoform for a certain location within the cell could determine the way by which merlin inhibits proliferation in that cell type, and differential regulation between the isoforms might explain how merlin can sustain multiple modes of regulation. An experiment to determine isoform localization would best be done using isoform-specific antibodies or tagged versions of each form, maintaining expression at endogenous levels, preferably in cells which are otherwise merlin-null.

Merlin has been shown to be more fluid in conformation than other FERM-domain proteins (5), which utilize a phosphorylation-driven conformational change to switch between an active and inactive form (7). Although merlin can experience regulatory phosphorylation, that does not necessarily coincide with a change in conformation. I have shown that isoform II, which does not readily achieve a closed conformation, can
suppress growth and even do so more efficiently than isoform I under certain conditions - as it can persist in culture longer. Similarly to localization, the conformation of merlin might dictate which mechanism merlin uses to suppress growth, and again, this might explain the multiple mechanisms described for merlin regulation.

**Hippo-independent regulation of YAP by merlin**

Merlin can suppress cell growth through a number of intertwined mechanisms. Merlin can inhibit Rac-PAK (8, 9), PI3K-Akt (10) and FAK (11) signaling, as well as negatively regulate EGFR signaling through control of EGFR localization (12). Merlin has also been shown to localize to the nucleus, where it may act as an inhibitor of the ubiquitin ligase, CRL4DCAF1 (13). In addition to modulating signal transduction and gene expression, merlin has also been shown act as a scaffold, stabilizing adherens junctions through binding alpha-catenin (14), and potentiating Hippo pathway activity by recruiting Lats to the membrane (15). Merlin’s role as a scaffold protein explains its ability to orchestrate growth suppression through multiple pathways and players, but its regulation of YAP might be a clue to a common upstream input for the many subsequent pathways used to regulate growth suppression. YAP regulation has been shown to correlate with cell shape and stiffness, with YAP activity being inhibited in cells plated on a soft matrix, or cells experiencing contact inhibition (16). Merlin, as a FERM-domain protein which acts as a scaffold linking the actin cytoskeleton to the plasma membrane, could be a link between the cytoskeleton and YAP, allowing the cell to sense and respond to changes in its environment. In *Drosophila*, merlin was placed upstream or parallel to the growth-suppressing Hippo pathway through epistasis experiments. Further biochemical experiments demonstrated multiple interactions between merlin, expanded, a FERM-domain protein, and kibra, a WW-domain-containing protein, which together provided upstream input into the Hippo pathway (reviewed by (17)).
I used Lats-insensitive YAP mutants to show that merlin is able to regulate YAP independently of the Hippo pathway. Merlin has been shown to stabilize Lats at the cell membrane and allow potentiation of Hippo signaling (15), however this is the first time that Hippo-independent regulation of YAP through merlin has been shown. Furthermore, I showed that merlin regulation of YAP is dependent on the WW domains of YAP. This is a similar mode of regulation to that seen in *Drosophila*, where expanded mediates Yki cytoplasmic retention and inhibition through binding the Yki WW domain. The mammalian ortholog of expanded, FRMD6, does not contain a PPxY motif necessary for binding the WW domains of YAP, however, a similar Hippo-independent regulation has been shown in mammals and is mediated through the PPxY-containing protein angiominotin (18, 19). We show that the WW domains of YAP are necessary for merlin-mediated regulation of YAP. Merlin can bind YAP and requires the WW domains of YAP to do so efficiently. It remains to be seen whether this binding is direct, for example, through an interaction between merlin proline-proline motifs and YAP WW domains. Although merlin lacks traditional PPxY motifs, some evidence has shown that other proline-proline motifs (20) or proline-serine/proline-threonine motifs (21) can bind WW domains. Alternatively, a direct binding interaction could occur through an interaction between the merlin coiled-coil domain and the YAP coiled coil domain. If merlin is binding YAP indirectly, possible proteins that might be involved or required in a YAP-merlin interaction are α-catenin and angiominotin, as well as angiominotin family members AMOTL1 and AMOTL2. Both have previously been shown to bind merlin (14, 22) and YAP and regulate YAP in a Hippo-pathway independent manner (19, 23). α-catenin has been described as a binding partner for YAP in keratinocytes, and promotes cytoplasmic sequestration through forming a complex with YAP and 14-3-3. Although phosphorylated YAP is preferentially bound to 14-3-3 (24), the α-catenin-YAP interaction does not
require prior phosphorylation of YAP by the Hippo pathway (23). α-catenin also binds merlin (14), and this interaction has been shown to stabilize adherens junctions in keratinocytes, coordinating cell polarity and adhesion by creating a complex with Par3 proteins. Merlin might be playing a dual, linked role in binding α-catenin, by maintaining cell junctions and contact inhibition while inhibiting downstream YAP transcriptional activity. The FERM domain of merlin is required for its interaction with α-catenin, and it would be interesting to see if the FERM domain of merlin is also responsible for its interaction with YAP.

Angiomotin and its close family members AMOTL1 and AMOTL2 have also been shown to bind merlin and YAP. Angiomotin binds Rich, a GAP for Rac. Merlin competes with Rich for angiomotin binding, releasing Rich to inhibit Rac. Angiomotin has also been shown to bind YAP and sequester it in the cytoplasm. As an inhibitor of Rich, leading to cell proliferation and migration, yet also an inhibitor of YAP, angiomotin seems to have conflicting roles in cell growth, which are also reflected in the literature regarding its role in YAP regulation [(25-27) for inhibition, (28-31) for activation]. Merlin might provide a mechanism to explain how angiomotin can act as either an inhibitor or an enhancer of cell growth. This might depend on the cell type, the expression levels of angiomotin, angiomotin isoforms or angiomotin family members, and the expression and activity of merlin. Merlin binds angiomotin through an interaction between the merlin coiled-coil domain and the angiomotin coiled-coil domain. Since angiomotin has already been shown to bind YAP through the YAP WW domains, it seems a likely candidate as an interacting protein in a merlin/YAP complex. Considering the complexity of the multiple isoforms and closely related family members in the angiomotin family, careful consideration of the cell line used for these experiments would be necessary, utilizing knock down or knockout to establish a framework of players while eliminating redundancies or competition. Finally, mass spectrometry might be useful in determining
any other proteins involved in the merlin-YAP interaction, and especially in elucidating the possible differences in different cell lines.

Our lab has shown that YAP is a promoter of metastasis (32), and it will be interesting to see in what contexts merlin can suppress tumorigenesis or metastasis through regulation of YAP. I have shown that merlin can regulate YAP in mesothelioma cell lines which have lost endogenous merlin. Mesothelioma is an aggressive tumor of the lung mesothelium that has been directly linked to asbestos exposure. Inhalation of asbestos fibers results in localized fibrosis and inflammation, followed by lung cancer and mesothelioma. Studies have proposed that the fibrosis and repeated inflammation in the lung pleura could cause the development of cancer at the site (reviewed in (33, 34)). Interestingly, inhalation of another hazardous environmental particulate, silica, causes fibrosis as well, but it does not progress into lung cancer or mesothelioma. A study which exposed human lung epithelial cells to silica dust versus asbestos fibers showed distinct changes in gene expression depending on the substance to which the cells were exposed (34). Although both the silica-exposed samples and the asbestos-exposed samples showed a common response with an upregulation of genes involved in inflammation, fibrotic response, and apoptosis, gene profiling was able to distinguish between the cells exposed to silica and those exposed to asbestos. In the cells exposed to asbestos, there was a change in expression in genes associated with tumorigenesis, tumor remodeling, and cell proliferation (34). A number of these genes have also been implicated as targets of YAP/TEAD through Chip-Seq experiments (35). It would be interesting to determine whether exposure to asbestos fibers results in an increase in YAP activity, and the mechanism that might be involved. The fact that silica dust doesn’t promote mesothelioma or the associated changes in gene expression despite causing similar inflammation and fibrosis could be related to the structure of the asbestos fibers,
and insertion of these fibers into the cells and surrounding matrix might affect mechanotransduction and the cytoskeleton, leading to an increase in YAP activity. If YAP activity is a driver of tumorigenesis in mesothelioma, and merlin regulates YAP activity in mesothelial cells, it could explain why cells that have lost merlin would be sensitized to an increase in YAP activity following dysregulation by asbestos fibers. In addition to its role in tumorigenesis, YAP also plays a role in organ size regulation, and consequently, in organ regeneration. The liver has been an amenable model in which to study organ regeneration, since it has the ability to regenerate following hepatocyte loss, as seen clinically following liver resection or transplant. Interestingly, the liver is also extremely responsive to alterations in YAP expression or the Hippo pathway. Overexpression of YAP or conditional knockout of Hippo pathway components in the liver results in an increase in liver size and ultimately in the development of hepatocellular carcinoma (36-40). In rats that have undergone a partial hepatectomy, YAP activity is increased as the liver regenerates, with activity decreasing as the liver approaches normal ratios of body weight to liver size (41). Interestingly, during the first three days post-hepatectomy, when there is a large increase in liver size, along with nuclear localization of YAP and an increase in YAP target gene expression, phosphorylation of YAP does not decrease concomitantly. The greatest decrease in YAP phosphorylation only occurs later, after day three, with YAP phosphorylation being at its lowest on day seven post-hepatectomy (41). This could indicate that the YAP-inhibitory mechanism being targeted at the earliest stages of liver regeneration is a Hippo-pathway-independent mechanism, which precedes the downregulation of the canonical Hippo pathway at later stages in regeneration. An interesting experiment to do using this model of rat hepatectomy would be to examine the expression levels and phosphorylation status of merlin at early points in regeneration, and see if conditional expression of a constitutively active merlin could decrease the regenerative ability of the
liver post-hepatectomy. Another possible regeneration model is the heart. Adult hearts have lost the ability to regenerate, leading to loss of cardiomyocytes and decreased contractility after injury. However, the hearts of neonatal mice have the ability to regenerate following partial resection or injury within the first week after birth (42). This regenerative ability is lost after one week, and subsequently injury will result in a fibrotic response that leads to decreased contractility and heart function. Similarly to studies in the liver, conditional knockout of Hippo pathway components (43) or overexpression of YAP (44, 45) lead to increased cardiomyocyte proliferation in the mouse embryo.

Similarly, conditional expression of activated YAP in the neonatal heart led to an extension of the window of regeneration in neonates, with improved heart function and less fibrotic scarring following injury (46). The question remains how upstream regulation of YAP affects its ability to be active in the case of regeneration, and how this upstream regulation changes during development to prevent regeneration in the adult. Of course, ultimately the question would be how this regulation could be reversed when the need arises to induce regeneration in an adult heart following injury. As regeneration is a process that must be closely regulated through cell-contact inhibition and the dynamics of the actin cytoskeleton, merlin, as a link between these processes and YAP regulation, could hold the key to understanding YAP-driven organ regeneration.

In summary, there are many exciting questions that remain unanswered when considering the upstream regulation of YAP and its clinical implications. In this thesis I present one mechanism by which merlin can regulate YAP, but it remains to be seen what the physiological implications of this regulation are, and in which contexts this regulation is relevant.
References


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Materials and Methods Chapter 2

Cell culture

All cells were cultured in a 5% CO₂ humidified incubator at 37°C.

The 4T1, 66Cl4, 168FARN, 4T07 and 67NR murine mammary carcinoma cell lines (a gift from Fred Miller, Wayne State University, Detroit, MI) were described previously (1) and were cultured in DMEM with 5% fetal bovine serum, 5% newborn calf serum, 2mM L-glutamine, 100U/ml penicillin and streptomycin, and 1mM of non-essential amino acids.

The murine mammary carcinoma cell lines D2, D2.1 and D2A1 (a gift from Robert Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA) were described previously (2) and were cultured in DMEM with 10% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and streptomycin.

Retroviral packaging and production was performed using 293FT cells (ATCC), cultured in DMEM with 10% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and streptomycin.

Vectors

All knockdowns were done using the MSCV-ZSGreen-2A-Puro-mir30 vector or the MSCV-Puro-2A-GFP-mir30 vector (3). The following mir30-based shRNA sequences were used for targeting (2 sequences per gene):

Ezrin shRNA1: TGCTGGTTGACAGTGAGCGACTCCAGTATGAGACAAATAATAGTA
AGCCACAGATGTATTTTATGTCTACATGTGAGGTGCCTACTGCCTCGGA

Ezrin shRNA2: TGCTGGTTGACAGTGAGCGCAGCGATAATATGGGTTTGTAATAGTA
AGCCACAGATGTATTACAAACCCATATTATCGCTTTGCCTACTGCCTCGGA
Radixin shRNA 1: TGCTGTTGACAGTGAGCGCAAGGATATTCTACATGGCTTATAG
TGAAGCCACAGATGTATAAGCCATATCATCCTTTTGCTACTGCTACTCGGA
Radixin shRNA 2: TGCTGTTGACAGTGAGCGACTGCAATATGTAGACAGCAAATAGT
GAAGCCACAGATGTATAAGCCATGTAGAATATCCTTTTGCCTACTGCCTCGGA
Moesin shRNA 1: TGCTGTTGACAGTGAGCGAGTTGGCTGAAACTCAATAAGAA
TAGTGAAGCCACAGATGTATTTGAGTTTCAGCCAAGTGACTGCTACTGCCTCGGA
Moesin shRNA 2: TGCTGTTGACAGTGAGCGATAGGATTTAGCCTCTTAATTAGT
AGTGAAGCCACAGATGTATAATTAAGAGGCTAAATCTAGTGCCTACTGCTCGGA
4.1B shRNA 1: TGCTGTTGACAGTGAGCGCAACCAACTATATTAGTGAGCTGAAATAGTGAA
GCCACAGATGTATTTACGCTCAATAATATTGAGTTTCAGCTACTGCTCGGA
4.1B shRNA 2: TGCTGTTGACAGTGAGCGCAACTCTCAATATGTAAATGAATAGTGAA
GCCACAGATGTATTTACGCTCAATAATATTGAGTTTCAGCTACTGCTCGGA
For retroviral production the MLV gag-pol and VSVg expression vectors were used as
described previously (3).

Retroviral production and infection
For production of retrovirus, 293FT cells were seeded in 6-well plates and transfected
the following day with 2µg of the MSCV vector along with 1µg each of MLV gag/pol and
VSVg, using Fugene6 (Roche) per manufacturer protocol. Media was changed the
following day, and collected at 48 hours. Collected media was filtered with a 0.45µm
filter, and added to target cells with 4µg/ml Polybrene (Sigma). On day 2, media was
changed on the target cells. On day 4, 2µg/ml puromycin was added for selection of
transduced cells.
Western blotting and quantitative PCR

For immunoblotting, cells were lysed in RIPA buffer (10mM Tris, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% Na-DOC, 0.1% SDS, 140mM NaCl) with protease and phosphatase inhibitors (Roche) and 20μg of protein was used for SDS/PAGE using 4-20% Tris-Glycine gels (Invitrogen), transferred to nitrocellulose membranes and assayed by immunoblotting. Primary antibodies were diluted in Tris-buffered saline with 3% milk at the following concentrations: rabbit anti-ezrin (#3145, Cell Signaling Technology) 1:5000; rabbit anti-radixin (C4G7, #2636, Cell Signaling Technology) 1:5000; rabbit anti-moesin (#3146, Cell Signaling Technology) 1:5000; mouse anti-GAPDH (Millipore) 1:10000, and rabbit anti-4.1B (McCarty et al. 2005). HRP-conjugated secondary antibodies were used at the following concentrations: goat anti-rabbit IgG (Jackson Labs) 1:10000, goat anti-mouse IgG (Jackson Labs) 1:10000.

For quantitative PCR (qPCR) cells were lysed in TRizol reagent (Invitrogen) and RNA was isolated according to manufacturer protocol. 1μg of RNA was reverse transcribed to produce cDNA using the Promega Reverse Transcription System (Promega). qPCR was carried out using 2μl of cDNA, with 12.5μl of SYBR Green Supermix (Bio-Rad) and 0.4μmol of each primer, following manufacturer protocol. Analysis was performed using the MyiQ real-time PCR detection system (Bio-Rad). PCR conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30sec, 60°C for 30sec, and 72°C for 30sec.

Primers for qPCR:

4.1G fwd CCCGCACAGAGTTAATGGAGAGGT, rev GTACGGCGTTAAGACTTTGGATGG
4.1N fwd GACCGACCACGCCCTTTCTTTG, rev CGTGCGCGTGGTGTTTGAACC
4.1RI fwd CCCTTCGGACTCTTTACATCAAC rev GCTGCAGGCTGGCTTCTTTATCAC
4.1RII/III fwd TGAGACCCCGATCGAGAAGAAT rev CAACCACCCCATAAAAACATCAAA
Invasion and migration assays

To assay invasive or migratory potential, 4x10^4 4T1 or 67NR cells were seeded into the wells of a transwell plate (Costar #3422 for migration assay, BD BioCoat Matrigel Invasion Chamber #354480 for the invasion assay). After 24 hours the cells on top of the well were removed with a cotton swab, and the cells were fixed in 3.7% formalin for 15 minutes. Cells were then stained with Hoechst nuclear staining (10µg/ml) for 10 minutes, and the cells that had invaded onto the bottom of the well filter were counted. For the Luminex assay of migration the cells at the bottom of the filter were washed off with PBS and collected for analysis.

In-vivo tumor growth and metastasis assays

All animal experiments and husbandry were approved by the MIT Department of Comparative Medicine. For tail vein experimental metastasis assays 5x10^5 cells were injected into the lateral tail vein of 6-10 week old female syngeneic Balb/C mice (Taconic) in 100µl of HBSS. After 20 days the mice were sacrificed and lungs were inflated and fixed with 3.7% formalin for 24 hours, followed by 75% ethanol for 24 hours. Metastasis were counted in whole lungs. For Luminex analysis, genomic DNA was isolated from fresh lungs.

Orthotopic transplants were performed to assay tumor growth and spontaneous metastasis to the lung. For these assays 6-10 week old female Balb/C mice (Taconic) were anesthetized by intraperitoneal injection of 125-250 mg/kg body weight of Avertin, followed by 100µl of 12µg/ml of buprenorphine for analgesia. A small incision was made on the right flank, and 2x10^5 of cells were injected in 25µl of HBSS into the right #4 fat pad using a Hamilton syringe. Mice received three additional injections of buprenorphine at 24-hour intervals following the surgery. After the indicated number of days tumors
were removed and weighed, and lungs were fixed and assayed for metastasis as described above. For Luminex experiments genomic DNA was isolated from the lungs and tumors.

**Luminex-based assays**

Unique oligo barcodes were cloned into MSCV retroviral vectors containing mir30-based shRNA targeting the genes of interest as described above. Equal numbers of cells that had been validated for knockdown were mixed and used in the Luminex-based assays. For tumor growth and metastasis, cells were injected orthotopically or into the tail vein as described above, for migration cells were plated in transwell plates as described above and for in vitro proliferation cells were plated in 6 well plates and cultured for the indicated number of days, with cells being collected at intervals for analysis. For all experiments, a starting population was collected when the cell populations were mixed and used for normalization of subsequent samples. Genomic DNA was collected from all samples and the barcodes were amplified using biotinylated primers that bound to common regions flanking the barcodes. The PCR output was then quantified at the Genetic Analysis Platform at the Broad Institute (Cambridge, MA) using Luminex technology as described previously (4).
Materials and Methods Chapter 3

Cell culture

All cells were cultured in a 5% CO₂ humidified incubator at 37°C.

The human mesothelioma cell lines Meso59 and Meso428 (a gift from Jonathan Fletcher, Brigham and Women’s Hospital and Harvard Cancer Center, Boston, MA) were cultured in RPMI or in DMEM with 10% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and streptomycin. The human mammary carcinoma cell line MDA-MB-231 (ATCC) was cultured in DMEM with 10% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and streptomycin. The murine mammary epithelial cell line NMuMG (ATCC) was cultured in DMEM with 10% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and streptomycin, and 10μg/ml insulin.

Retroviral packaging and production was performed using 293FT cells (ATCC), cultured in DMEM with 10% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and streptomycin as described in chapter 2.

Vectors

Merlin (pBABE NF2/WT, isoform II, Addgene) was cloned into the MSCV-IRES-GFP and the MSCV-IRES-Puro expression vectors (Stern et al. 2008). Using standard molecular biology techniques and the following oligos, the sequence corresponding with isoform II was replaced with a sequence corresponding with isoform I:

5'ggagcagctcaacgagctcaagacggagatcgaggccttgaaactcaaagagcggagacggcctggacgtcctacacagcgagagctcagacagaggcggccccagcagcaagtataataaaaagctcactctgcagagcgccaagtcccgagtggccttcttgaagaactctgaagtctcgtgtagctgctgtagctgctcctgca 3'
5'aatctagagttcttcaaagaaggccactcgggacttggcgctctgcagagtgagctttaatggtattatgcttgctgctggggccgcctctgtctgagctctcgctgtgtagctgctgtagctgctcctgca 3'
Mutant forms of merlin isoform I or isoform II were generated via PCR-mediated site-directed mutagenesis using a pcDNA shuttle vector, and cloned into the MSCV-IRES-Puro or MSCV-IRES-GFP vectors.

**Western blotting**

For immunoblotting, cells were lysed in CSK buffer (100mM NaCl, 300mM sucrose, 3mM MgCl$_2$$\cdot$6H$_2$O, 10mM PIPES, 0.5% IPEGAL, 1mM EDTA) with protease and phosphotase inhibitors (Roche). 20μg of protein was used for SDS/PAGE using 4-20% Tris-Glycine gels (Invitrogen), transferred to nitrocellulose membranes and assayed by immunoblotting. For phosphotase treatment of lysates no phosphotase inhibitors or EDTA was used in the lysis buffers. Calf Intestinal Phosphotase (CIP) was added at 20U CIP for 20μg of protein and incubated at 37°C for one hour.

Primary antibodies were diluted in Tris-buffered saline with 5% BSA at the following concentrations: rabbit anti-merlin monoclonal D1D8,(#6995, Cell Signaling Technology) 1:5000; rabbit anti-phospo S518 merlin (#9163, Cell Signaling Technology) 1:5000; and mouse anti-GAPDH (Millipore) 1:10000. HRP-conjugated secondary antibodies were used at the following concentrations: goat anti-rabbit IgG (Jackson Labs) 1:10000, goat anti-mouse IgG (Jackson Labs) 1:10000.

**Proliferation assays**

For standard proliferation assays cells were seeded at starting numbers as indicated, collected at days post seeding as indicated, and counted manually with a hemacytometer.

For flow-cytometry based proliferation assays, GFP- and GFP+ merlin-expressing cells were mixed at equal numbers and plated and cultured over time. After the indicated number of days, culture cells were collected, trypsinized, and resuspended in PBS with
5% fetal bovine serum, and stained with propidium iodide. The percentages of green vs.
non-green cells were quantified using a FACSCalibur flow cytometer (BD). In all flow-
cytometry based proliferation assays samples were normalized to a starting population.
Raw flow data was analyzed using Flow Jo version 8.8.
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Cell culture

All cells were cultured in a 5% CO₂ humidified incubator at 37°C.

The human mesothelioma cell lines Meso59 and Meso428 (a gift from Jonathan Fletcher, Brigham and Women’s Hospital and Harvard Cancer Center, Boston, MA) used were described in chapter 3. The human mammary epithelial cells lines HMLE and HMLER (a gift from Robert Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA) were grown in a 1:1 mixture of Mammary Epithelial Cell Growth Media (C-21110, PromoCell) and DMEM/F12 (1:1) with 100U/ml penicillin and streptomycin, 10μg/ml insulin, 1μg/ml hydrocortisone, and 10ng/ml EGF.

Vectors

Merlin-GFP and Merlin-Puro vectors, for both isoforms, were described in chapter 3.

For knockdown of merlin, mir30-based shRNA targeting sequences were:

NF2 shRNA1: TGCTGTGACAGTGAGCGCCAGCTTGTCATTTGACTTCAATAGTGAAGCACAGATGTATTGAAGTCAAATGACAAGCTGTTGCCTACTGCCTCGGA

NF2 shRNA2: TGCTGTGACAGTGAGCGCAAGGGTGATAAATCTCTATCATAGTGAAGCCACAGATGTATGAGATTTATCACCCTTTTGCTACTGCCTCGGA

Knockdown was performed and validated as described in chapter 2.

Generation of the YAP and YAP mutant vectors used was described previously (4). The TEAD-dependent luciferase reporter construct, pGL3-5xMCAT(SV)-49 (a gift from Ian Farrance, Virginia Technological Institute and State University, Blacksburg, VA) was described previously (6).
Western blotting and immunoprecipitation

For immunoprecipitation, cells were lysed in CSK buffer (as described in chapter 3) with protease and phosphotase inhibitors (Roche) and 0.1% SDS, 0.5% NP-40, 0.1% Na-DOC. 1mg of total cell lysate was used for immunoprecipitation. 1:2 CSK-diluted lysate was precleared with 1µg of mouse IgG (Santa Cruz Biotechnology) along with 30µl of either magnetic beads or protein A/G agarose and incubated at 4°C for 30 minutes, then samples were spun down or beads removed with a magnet. Following preclear, 3µl of anti-mouse FLAG primary antibody (Sigma) was added to the cleared supernatant and incubated at 4°C for one hour. 30µl of either magnetic beads or protein A/G agarose was added and incubated overnight at 4°C on a rotating device. Immunoprecipitate was collected by magnet or gentle spin, and pellet washed 4 times with CSK buffer before resuspension in Laemmeli sample buffer with β-mercaptoethanol (Biorad).

For immunoblotting, immunoprecipitate or 20µg of total cell lysate was used for SDS/PAGE using 4-20% Tris-Glycine gels (Invitrogen), followed by transfer to nitrocellulose membranes and assayed by immunoblotting. Primary antibodies were diluted in Tris-buffered saline with 5% BSA at the following concentrations: rabbit anti-merlin monoclonal D1D8, (#6995, Cell Signaling Technology) 1:5000; rabbit anti-YAP (#4912, Cell Signaling Technology) 1:5000; rabbit anti-phospho YAP S127 (#4911, Cell Signaling Technology) 1:5000; and mouse anti-GAPDH (Millipore) 1:10000. HRP-conjugated secondary antibodies were used at the following concentrations: goat anti-rabbit IgG (Jackson Labs) 1:10000, goat anti-mouse IgG (Jackson Labs) 1:10000.

Immunofluorescence

Confluent cells on coverslips were fixed for 5 minutes in 4% paraformaldehyde, then permeabilized for 5 minutes with 0.5% NP-40, 0.2% Triton in PBS. Coverslips were then
blocked in 2% BSA in PBS for one hour, and incubated overnight at 4°C with rabbit monoclonal anti-YAP (YAP [D8H1X] XP, #14074 Cell Signaling Technology) primary antibody in 2% BSA in PBS. Coverslips were rinsed four times in PBS and then incubated for 45 minutes at 37°C in Alexa 488-conjugated anti-rabbit secondary antibodies (Invitrogen) diluted with 2% BSA with PBS and DAPI (Invitrogen). Coverslips were mounted on slides using Fluoromount-G (Southern Biotech).

**Dual luciferase reporter assay**

For TEAD transcriptional activity assays, cells stably expressing the vectors indicated were plated on 12-well plates in duplicate or triplicate and co-transfected with 500ng of a 20:1 mixture of pGL3-5xMCAT(SV)-49 and PRL-TK (Promega) using Lipofectamine Plus (Invitrogen) as per manufacturer instructions. Alternatively cells were co-transfected with the reporter mixture and with YAP or merlin vectors described above. After 48-72 hours, luciferase activity was assayed in duplicate for each sample using the dual luciferase reporter assay system (Promega) and a Tecan Infinite 200Pro plate reader. For each well the signal of firefly luciferase (pGL3-5xMCAT(SV)-49) was normalized to the signal for Renilla luciferase (PRL-TK).

**Proliferation assay**

Proliferation experiments were done as described in chapter 3.

**References**


