Identification and Characterization of Novel Upstream Regulators of the Hippo Signalling Pathway

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Biochemistry
University of Toronto

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Abstract

The evolutionarily conserved Hippo signalling pathway is a major determinant of growth control and organ size. The co-transcription factors YAP/TAZ are downstream effectors of the Hippo pathway that regulate vital processes like cell proliferation, cell migration, apoptosis, stem cell self-renewal and differentiation. YAP/TAZ are regulated by the core Ser/Thr kinases of the Hippo pathway MST1/2 and LATS1/2. Upon activation, MST1/2 in association with the adaptor protein SAV, phosphorylate and activate the downstream kinases LATS1/2. In turn, activated LATS1/2 in association with the adaptor protein MOB, phosphorylate YAP/TAZ leading to cytoplasmic accumulation, degradation and transcriptional inactivation of YAP/TAZ. The Hippo pathway can be regulated by various intrinsic and extrinsic stimuli such as mechanotransduction, actin cytoskeleton dynamics, cell-cell contact and cell polarity, G-protein coupled receptor signalling and metabolic pathways. However, the mechanistic details and molecular mediators that connect the upstream stimuli to the core of the pathway are not fully understood. With the aim of identifying novel upstream regulators of the Hippo pathway we undertook a high-throughput LUMIER-based protein interaction screen complemented with a TEAD-luciferase reporter screen that led to the identification of βPIX and MARKs as positive and negative regulators of the Hippo pathway, respectively. Mechanistically, βPIX mediates the interaction between YAP/TAZ and the Hippo core kinase LATS to promote YAP/TAZ phosphorylation and transcriptional inhibition. Conversely, MARK4 enhances YAP/TAZ transcriptional activity in a kinase-dependent manner by phosphorylating MST and SAV, and disrupting complex formation with the downstream LATS kinase. Furthermore, abrogation of MARK4 or βPIX overexpression in breast cancer cells attenuates tumorigenic features such as proliferation and migration by activating the Hippo kinase.
cascade. Finally, βPIX and MARK binding partners GIT1 and DLG5 are respectively characterized as positive and negative regulators of YAP/TAZ transcriptional activity.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AMOT</td>
<td>Angiomotin</td>
</tr>
<tr>
<td>AMOTL</td>
<td>Angiomotin-like</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated kinase</td>
</tr>
<tr>
<td>ANKRD1</td>
<td>Ankyrin repeat domain 1</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical Protein kinase C</td>
</tr>
<tr>
<td>ARF</td>
<td>Adenosine diphosphate ribosylation factors</td>
</tr>
<tr>
<td>ARHGEF7</td>
<td>Rho Guanine Nucleotide Exchange Factor 7</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BRSK</td>
<td>Brain-specific serine/threonine protein kinase</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer associated fibroblast</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cbl</td>
<td>Casitas B-lineage lymphoma</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif chemokine ligand 2</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C motif chemokine receptor type 5</td>
</tr>
<tr>
<td>Cdc25</td>
<td>Cell division cycle 25</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDX2</td>
<td>Caudal type homeobox 2</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CRB3</td>
<td>Crumb 3</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CXCL5</td>
<td>C-X-C motif chemokine ligand 5</td>
</tr>
<tr>
<td>CXCR5</td>
<td>C-X-C motif chemokine receptor type 5</td>
</tr>
<tr>
<td>CYR61</td>
<td>Cysteine-rich angiogenic inducer 61</td>
</tr>
<tr>
<td>DAPI</td>
<td>6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Dbl</td>
<td>Diffuse B-lymphoma</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>DLG</td>
<td>Disc large homology</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Dvl</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAT</td>
<td>Focal adhesion targeting</td>
</tr>
<tr>
<td>FOXO3</td>
<td>Forkhead box O3</td>
</tr>
</tbody>
</table>
GAP: GTPase-activating protein
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GEF: Guanine-nucleotide exchange factor
GFP: Green fluorescent protein
GIT: GRK-interacting protein
GPCR: G-protein coupled receptor
GRK: G-protein coupled receptor kinase
GSK-3β: Glycogen synthase kinase-3β
GST: Glutathione S-transferase
GTP: Guanosine triphosphate

HA: Hemagglutinin
HDAC: Histone deacetylase
HEK: Human embryonic kidney
Hpo: Hippo
HPRT: Hypoxanthine-guanine phosphoribosyltransferase

ICM: Inner cell mass
IGF-1R: The insulin-like growth factor-1 receptor
IL-6: Interleukin-6
IP: Immunoprecipitation

KA: Kinase associated
KRa: Kirsten rat sarcoma
KSR: Kinase suppressor of Ras

LATS: Large tumor suppressor
LEF: Lymphoid enhancer factor
LGR5: Leucine-rich repeat-containing G-protein coupled receptor 5
LKB1: Liver kinase B1
LPA: Lysophosphatidic acid
LUMIER: Luminescence-based mammalian interactome
LZ: Leucine zipper

MAP: Microtubule associated protein
MAPK: Mitogen-activated protein kinase
MARK: Microtubule-affinity regulated kinase
MDSC: Myeloid-derived suppressive cell
MELK: Maternal embryonic leucine zipper kinase
MOB1: Mps one binder homolog1
mRNA: messenger Ribonucleic acid
MST: Macrophage stimulating 1

NDR: Nuclear Dbf-2-related
NF2: Neurofibromatosis 2
NMuMG: Normal murine mammary gland
NUAK: (Nua) kinase family
OCT4: Octamer-binding transcription factor 4

PAK: p21-activated kinase
PALS: Protein associated with Lin-7
Par-1: Partitioning defective protein 1
PATJ: Pals1-associated tight junction
PAX3: Paired box 3
PBS: Paxillin binding site
PCR: Polymerase chain reaction
PFK1: Phosphofructokinase-1
PH: Pleckstrin homology
PI(3)K: Phosphoinositide 3-kinase
PIX: PAK-interacting exchange factor
PKA: Protein kinase A
PTPN14: Protein tyrosine phosphatase non-receptor type 14

Rab: Ras-related in brain
Rac1: Ras-related C3 botulinum toxin substrate 1
Ran: Ras-related nuclear
Ras: Rat sarcoma
Rho: Ras homolog gene family, member A
RNA: Ribonucleic acid
ROCK: Rho kinase
RUNX: Runt-related transcription factor

S1P: Sphingosine-1-phosphate
SAV: Salvador
SCF: Skp, Cullin, F-box containing complex
SH3: Src homology 3
Shc: Src homology 2 domain containing transforming protein
SHD: Spa-homology domain
SIK: Salt inducible kinase
Smad: Sma and MAD-related
SOX: SRY (sex determining region Y)-box
SRC: Rous sarcoma oncogene
SRP: SH3 domain-containing proline-rich protein
STAT3: Signal transducer and activator of transcription 3
STE20: Sterile 20

TAO: Thousand-and-one amino acids
TAZ: Transcriptional coactivator with PDZ-binding motif
TBX5: T-box transcription factor 5
TCF: T-cell factor
TEAD: TEA domain family member 1
TEF: Transcriptional enhancer factor
TGF-β: Transforming growth factor-β

UBA: Ubiquitin associated
Wnt: Wingless and int-1
Wts: Warts
WWTR1: WW domain containing transcription regulator 1

YAP: Yes-associated protein
Yki: Yorki
Chapter 1
Introduction
1 Introduction

The size of an organ in multicellular organisms is determined by an intricate balance of cell proliferation, apoptosis, stem cell self-renewal and differentiation. Intrinsic and extrinsic factors tightly regulate these processes, which are essential for normal embryonic development and tissue homeostasis, and aberrations in their regulation lead to organ growth defects or tumorigenesis. The Hippo pathway has emerged as a major signalling pathway with evolutionarily conserved roles in regulating tissue growth and organ size.

1.1 Growth control by the evolutionarily conserved Hippo signalling pathway

One of the most fundamental questions in biology is how a single cell develops into a multicellular organism, giving rise to different tissues and organs. Although the major principles of tissue specification have been characterized, organ size determination is one aspect of animal development that has remained more mysterious. It is known that organ size is influenced by both intrinsic and extrinsic factors such as cell crowding and growth factor or nutrient availability, which regulate processes such as cell growth, proliferation and apoptosis. Nevertheless, how this information is integrated at tissue and organ levels to regulate organ size remains an outstanding question in biology.

A major step towards a better understanding of the mechanisms that control organ size, was the identification of the evolutionarily conserved Hippo signalling pathway (Pan 2007; Halder and Johnson 2011). Through a series of genetic screens in Drosophila, it was revealed that mutations in the Ste20 family Ser/Thr kinase Hippo (Hpo), which gives its name to the pathway, and the adaptor protein Salvador (Sav), results in the enlargement and overgrowth of various organs in the fly (Kango-Singh et al. 2002; Tapon et al. 2002; Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003). Mechanistic studies have revealed that Hpo, in association with Sav, phosphorylates and activates the NDR family Ser/Thr kinase, Warts (Wts) (Udan et al. 2003; Wu et al. 2003a), which had previously been identified as a tumor suppressor regulating cell proliferation and tissue size (Justice et al. 1995; Xu et al. 1995). The activated Wts in association with another adaptor protein Mob as tumor suppress (Mats), regulates the co-transcription factor Yorki (Yki) (Huang et al.
2005; Lai et al. 2005; Dong et al. 2007; Wei et al. 2007). When localized in the nucleus, Yki binds to the TEAD/TEF family transcription factor Scalloped (Figure 1.1) and promotes the expression of target genes such as DIAP1, Cyclin E and bantam that promote cell proliferation and resistance to apoptosis (Dong et al. 2007; Oh and Irvine 2008; Zhang et al. 2008; Halder and Johnson 2011). Wts can directly phosphorylate Yki at Ser168, which leads to transcriptional inactivation by cytoplasmic translocation and binding to 14-3-3 proteins (Dong et al. 2007; Oh and Irvine 2008; Zhang et al. 2008; Halder and Johnson 2011).

As an evolutionarily conserved pathway, the core components of the Hippo signalling have mammalian counterparts, which follow a similar order of activation. The Ser/Thr kinases MST1/2 are the mammalian orthologs of Hpo that in association with SAV, phosphorylate and activate the Wts orthologs, LATS1/2. In turn, LATS1/2, in association with the MOB1 adaptor protein, phosphorylate the related co-transcription factors YAP and TAZ, which are Yki orthologs. LATS1/2 can phosphorylate human YAP and TAZ on Ser127 and Ser89, respectively, that are the equivalents of Ser168 on Yki. Similar to Yki, phosphorylation of these sites results in cytoplasmic accumulation of YAP/TAZ and binding to 14-3-3. LATS can also phosphorylate YAP and TAZ on other Serine residues, a mechanism that is not observed in Drosophila. Phosphorylation by LATS1/2 primes YAP and TAZ for further phosphorylation by Casein Kinase 1 (CK1) which results in recognition by the SCFβ-TRCP E3 ubiquitin ligase and subsequent degradation by the proteosomal pathway (Liu et al. 2010; Zhao et al. 2010) (Figure 1.1). When unphosphorylated, YAP/TAZ are mainly localized in the nucleus and activate a gene expression program that regulates cell proliferation, differentiation, apoptosis and stem cell self-renewal (Mo et al. 2014; Varelas 2014).

YAP and TAZ do not have DNA binding activity, hence they rely on other transcription factors for regulating gene expression. TGF-β regulated SMADs, p53-related family of p73 proteins, TBX5, PAX3, RUNX and TEAD/TEF family of DNA binding proteins are some of the transcription factors that bind to and mediate the transcriptional activity of YAP/TAZ (Mauviel et al. 2012).
Figure 1.1: The Hippo pathway core components

The core of the Hippo pathway is composed of Ser/Thr kinases that phosphorylate the co-transcription factors YAP and TAZ. Upon activation, MST1/2 kinases, in association with the adaptor protein SAV, phosphorylate and activate the downstream kinases LATS1/2 and the adaptor protein MOB1. Activated LATS in association with MOB, phosphorylate the co-transcription factors YAP/TAZ which leads to transcriptional inactivation by promoting cytoplasmic retention by 14-3-3 binding or degradation by the proteosomal pathway. The kinase cascade is highly conserved between Drosophila and mammals, and the name of the fly orthologs for each pathway component are indicated in the figure (white letters). YAP/TAZ degradation upon phosphorylation seems to be a mammalian specific mechanism that has not been observed in Drosophila.
The importance of these transcription factors for YAP/TAZ biological functions are context and tissue-type dependent, however TEADs have been identified as the main transcription factors that mediate the growth promoting effects of YAP/TAZ such as cell proliferation, survival, anchorage-independent growth, epithelial-mesenchymal transition (EMT) and oncogenic transformation (Zhang et al. 2008; Zhao et al. 2008; Zanconato et al. 2015). Recently genome-wide mapping of YAP and TAZ binding sites revealed that YAP/TAZ regulate the expression of a repertoire of target genes that are important for cell proliferation including direct regulators of cell cycle progression such as cyclins and CDKs, factors that are required for DNA synthesis, replication and repair and genes that are involved in mitosis (Zanconato et al. 2015; Liu et al. 2016). TEADs were identified as the main transcription factors that mediate YAP/TAZ recruitment to DNA and gene expression. Interestingly, the majority of YAP/TAZ target genes also possess AP-1 binding sites and the cooperation between YAP/TAZ/TEADs with AP-1 transcription factors is critical to drive the YAP/TAZ oncogenic program (Zanconato et al. 2015; Liu et al. 2016).

1.2 Upstream regulators of the Hippo signalling pathway

Although the core kinase cascade is very well characterized both in Drosophila and in mammals, upstream regulators of the Hippo pathway are not as well-known and are currently the subject of intense investigation. Studies during the last few years have identified numerous factors such as cell density, cell polarity, actin cytoskeleton dynamics, G-protein coupled receptors and energy status of the cells as the regulators of the Hippo kinase cascade and YAP/TAZ transcriptional activity (Figure 1.2).

1.2.1 Cell polarity and junctional complexes

Contact inhibition is a known feature of epithelial tissues wherein cell density restricts proliferation and tissue overgrowth and loss of contact inhibition leads to oncogenic transformation. Cell-cell contact was among the first signals to be identified as an upstream regulator of the Hippo pathway (Zhao et al. 2007). Cell culture studies have shown that at low cell-density the Hippo pathway is inactive and YAP/TAZ are mainly localized in the nucleus. However, at high cell density the Hippo pathway becomes activated which leads to LATS-mediated phosphorylation of YAP/TAZ and subsequent cytoplasmic translocation (Zhao et al. 2007). Multiple components of junctional
Cell-Cell Contact

Cell morphology and actin cytoskeletal remodelling

G-protein coupled receptor signalling

Energy status (i.e. AMPK Pathway)
Figure 1.2: Upstream regulators of the Hippo signalling pathway.

Major upstream stimuli that regulate the Hippo pathway and YAP/TAZ localization. (A) Cell-cell contact and cell density, (B) Cell morphology and mechanotransduction through actin cytoskeletal reorganization, (C) G-protein coupled receptor (GPCR) signalling and (D) Energy status of the cell (i.e. AMPK pathway) are among the most important regulators of the Hippo pathway. Green color represents subcellular localization of YAP/TAZ.

complexes including adherens junctions, tight junctions and cell polarity complexes have since been identified to interact with and regulate the Hippo core components. In mammary epithelial cells, components of the Crumb polarity complex, PATJ, PALS, LIN7c and CRB3 become localized to tight junctions upon cell polarization and promote cytoplasmic localization of YAP/TAZ by activating the Hippo pathway (Varelas et al. 2010b). α-Catenin, a component of adherens junctions interacts with the phosphorylated form of YAP through 14-3-3 and acts as a cytoplasmic retention factor. Disruption of α-catenin in epidermal epithelial cells promotes nuclear accumulation of YAP, increased proliferation and expansion of progenitor cells that eventually causes tumor formation (Schlegelmilch et al. 2011; Silvis et al. 2011). Similarly, PTPN14 another member of the adherens junctions, has been shown to restrict YAP transcriptional activity by promoting phosphorylation and cytoplasmic translocation at high cell density, thereby suppressing transformation of mammary epithelial cells (Wang et al. 2012; Liu et al. 2013). Interestingly members of the Angiomotin (AMOT) family of tight junction proteins regulate YAP/TAZ activity either by binding and activating the Hippo core kinase LATS2 (Paramasivam et al. 2011), hence promoting YAP/TAZ phosphorylation, or by directly interacting with YAP/TAZ independently of Hippo core kinases (Chan et al. 2011; Wang et al. 2011; Zhao et al. 2011). Of note, AMOTs have been identified as direct substrates of LATS1/2 kinases and LATS-mediated phosphorylation has been shown to promote stabilization and abundance of AMOTs, thereby restricting YAP/TAZ activity in a feedforward mechanism (Adler et al. 2013).

1.2.2 Cell morphology, mechanotransduction and actin cytoskeleton reorganization

Cells are capable of sensing the physical aspects of their microenvironment such as changes in cell shape and geometry or the rigidity of the extracellular matrix and translating these into biochemical
signals. Mechanisms enabling cells to sense and respond to mechanical cues are known as mechanotransduction, which regulate multiple aspects of cell behavior such as proliferation, survival, migration and differentiation (Hoffman et al. 2011). Recent research has uncovered a central role for YAP/TAZ in mechanotransduction, revealing that YAP/TAZ can integrate mechanical cues into changes in cellular behavior by regulating gene expression (Halder et al. 2012; Low et al. 2014).

Cells cultured at low cell density on stiff matrices form extensive actin stress fibers, which promote nuclear localization of YAP/TAZ and higher transcriptional activity. Conversely, cells cultured on soft matrices or substrates with limited surface area lose their actin stress fibers which result in cytoplasmic localization of YAP/TAZ and transcriptional inactivation (Dupont et al. 2011; Wada et al. 2011). Mechanistically, active RhoA has been shown to promote actin stress fiber formation and increased YAP/TAZ transcriptional activity in response to mechanical cues. Therefore, disruption of actin stress fiber using small molecules that inhibit the activity of RhoA, ROCK or myosin type II result in cytoplasmic translocation and inactivation of YAP/TAZ. (Dupont et al. 2011; Wada et al. 2011; Zhao et al. 2012).

The role of the Hippo kinase cascade in this context is a matter of debate. A study by Dupont and colleagues proposes a model in which mechanotransduction regulates YAP/TAZ independent of the Hippo kinase cascade, as they noted that the loss of actin stress fibers promotes cytoplasmic YAP/TAZ without affecting phosphorylation. However, studies by other groups demonstrate that LATS-mediated phosphorylation downstream of actin cytoskeleton disruption is the main mechanism for regulating YAP/TAZ localization (Wada et al. 2011; Zhao et al. 2012).

1.2.3 G-Protein Coupled Receptors

Initially Hippo signaling was characterized to be regulated by intrinsic factors which unlike other major signalling pathways lacks a specific activating ligand-receptor. Recently, G-protein coupled receptors (GPCRs) have been identified as regulators of the Hippo pathway. Through biochemical analysis of the serum components in culture medium, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) were identified as potent activators of YAP/TAZ that signal through their cognate receptors belonging to the GPCR family (Miller et al. 2012; Yu et al. 2012b). Interestingly however, different GPCR family members have opposing effects on YAP/TAZ activity. Ligands that act through Gα12/13, Gq11 and Gαi/o families including LPA, S1P and thrombin
receptors, promote nuclear localization, hence transcriptional activation of YAP/TAZ, whereas the
$G_{\alpha_s}$ family including $\beta_2$ andrenergic receptors, inhibit YAP/TAZ upon activation by their cognate
ligands (i.e epinephrine and glucagon) (Yu et al. 2012b).

Mechanistic details of how GPCRs regulate YAP/TAZ activity are not fully understood, although
Rho GTPases have been implicated as downstream effectors of GPCRs (Miller et al. 2012; Yu et
al. 2012b). Activation of Protein Kinase A (PKA) upon cAMP production has also been shown to
inhibit YAP/TAZ activity by promoting LATS1/2 kinases in response to $G_{\alpha_s}$ activation (Kim et

G-protein coupled receptors are the largest family of cell surface receptors and are involved in
regulating a wide range of biological processes. Regulation of Hippo-YAP pathway downstream
of GPCRs, suggest that some of the biological effects of GPCRs might be mediated through Hippo
signalling. Indeed, YAP and TAZ have been shown to be important for regulation of cell
proliferation and migration downstream of LPA and S1P (Yu et al. 2012b). Furthermore, forskolin-
induced adipogenesis in mesenchymal stem cells (MSCs) is mediated by cAMP-PKA activation
downstream of GPCR signalling. It is known that YAP/TAZ transcriptional activity in cooperation
with RUNX transcription factor promotes osteogenic differentiation of mesenchymal stem cells at
the expense of adipogenic differentiation, therefore inhibition of YAP/TAZ activity is required for
adipogenesis (Hong et al. 2005; Dupont et al. 2011). Yu and colleagues have shown that activation
of cAMP-PKA in response to forskolin treatment promotes adipogenic differentiation of MSCs by
activating the Hippo kinase cascade and inhibition of YAP/TAZ activity (Yu et al. 2013).

1.2.4 Hippo pathway regulation by metabolic pathways

In multicellular organisms, organ growth relies on cell proliferation and cell growth, which are
intricately regulated by mitogens, growth factors and nutrient availability. Recently it has been
shown that the energy status of the cell regulates YAP transcriptional activity in both Hippo-
derpendent and Hippo-independent manners. Central to cell energy homeostasis is the Ser/Thr
kinase, AMP-activated kinase (AMPK) which in response to glucose deprivation and higher
AMP/ATP ratios, becomes activated and phosphorylates multiple downstream substrates (Hardie
et al. 2012). AMPK inhibits YAP/TAZ activity by both activating the Hippo kinases LATS1/2 or
by directly phosphorylating and restricting YAP transcriptional activity (Mo et al. 2015; Wang et
al. 2015).
Although glucose deprivation has been shown to promote activation of LATS kinases downstream of AMPK (Mo et al. 2015), loss of AMPK does not completely abolish LATS1/2 activation, suggesting the existence of other mechanisms that can activate Hippo pathway in response to metabolic stress independent of AMPK (DeRan et al. 2014; Wang et al. 2015). Although the mechanisms through which AMPK regulates LATS activity are not fully understood, a study by DeRan and colleagues identifies AMOTL1, a known upstream regulator of the Hippo kinase cascade, as a direct substrate of AMPK. Activation of AMPK in response to energy stress results in AMOTL1 phosphorylation and stabilization, hence promoting the kinase activity of LATS1/2 (DeRan et al. 2014). Furthermore, AMPK can directly phosphorylate YAP at multiple residues including Ser94, which is critical for interaction with TEAD, thereby disrupting YAP-TEAD interaction (Mo et al. 2015). Therefore, AMPK can regulate YAP transcriptional activity independent of or through the Hippo kinase cascade.

In addition to AMPK, glycolysis has been shown to be important for YAP/TAZ regulation in response to glucose deprivation. For example, Phosphofructokinase (PFK1), a central glycolytic enzyme that catalyzes fructose-6-phosphate conversion to fructose-1,6-biphosphate, is required to sustain YAP/TAZ transcriptional activity and inhibition of PFK1 in response to glucose deprivation results in inhibition of YAP/TAZ activity (Enzo et al. 2015). Beside glucose metabolism, the mevalonate pathway, which is crucial for cholesterol biosynthesis, has been shown to play a role in regulating YAP/TAZ activity. Accordingly, inhibition of the mevalonate pathway leads to YAP/TAZ cytoplasmic localization and a decrease in transcriptional activity (Sorrentino et al. 2014).
1.3 Biological implications of the Hippo pathway

1.3.1 Mouse genetic studies on Hippo pathway core components

Since the characterization of the Hippo pathway, genetic studies in mice have provided insights into the developmental and physiological roles of Hippo pathway core components. The major downstream effectors of the Hippo pathway are co-transcription factors YAP and TAZ that regulate the expression of target genes involved in cell survival and proliferation. Mice deficient in Yap die at embryonic stage E8.5 whereas Taz knockout mice are viable and fertile, however Taz deficiency results in polycystic kidney disease development and pulmonary emphysema (Hossain et al. 2007; Makita et al. 2008). Yap and Taz double knockout mice die prior to morula stage due to defects in trophoectoderm specification, indicating a redundant function for Yap and Taz in early embryonic development (Nishioka et al. 2009). The upstream kinases Lats1 and Lats2 restrict Yap/Taz transcriptional activity by phosphorylating and promoting cytoplasmic sequestration. Although Lats1 and Lats2 can redundantly inhibit Yap and Taz in cell culture, mice deficient for Lats1 or Lats2 exhibit distinct phenotypes suggesting either non-redundant physiological functions or differential expression patterns in different tissues. Lats1/- are viable and develop normally, however these mice are susceptible to soft tissue sarcoma formation and ovarian cancer (St John et al. 1999). Lats2 however, is crucial for normal development, thus germline deletion of Lats2 results in embryonic lethality at day 12.5. Lats2/- embryos demonstrate an overgrowth phenotype in a number of tissues of mesodermal lineage, however they exhibit major abnormalities in cell cycle progression in multiple tissues due to defective centrosomal duplication and cytokinesis that ultimately leads to embryonic lethality (McPherson et al. 2004; Yabuta et al. 2007). Concomitant deletion of Lats1 and Lats2, on the other hand results in early embryonic lethality at the morula stage due to failure to establish the Inner Cell Mass (ICM) lineage (Nishioka et al. 2009; Lorthongpanich et al. 2013).

Lats1/2 are regulated by the upstream kinases Mst1 and Mst2 that phosphorylate and activate Lats kinases. Mst1-null mice are viable and fertile, however they exhibit low numbers of T cells in peripheral lymphoid organs (Zhou et al. 2008; Katagiri et al. 2009), whereas Mst2/- mice do not exhibit any abnormalities (Oh et al. 2009; Zhou et al. 2009). Mst1/2 double knockout is embryonic lethal and Mst1/2-deficient mice die at embryonic day 8.5 to 9.5 due to severe growth retardation, failure in neural tube closure, placental development and impaired vascularization (Oh et al. 2009; Zhou et al. 2009). Interestingly, a single copy of either Mst1 or Mst2 is sufficient to rescue the
lethality associated with the Mst1/2 double knockout suggesting a redundant function for Mst1 and Mst2 during development, however these mice have a significant risk of developing hepatocellular carcinoma (Zhou et al. 2009).

The adaptor proteins Sav1 and Mob1 are the other core components of the Hippo pathway that associate with and activate Mst1/2 and Lats1/2 kinases, respectively. Germline deletion of Sav1 results in embryonic lethality at day 17.5 due to defective and immature placental development that display poor vascularization (Lee et al. 2008). Interestingly epithelial tissues such as epidermis and intestine in Sav1−/− mice are hyperproliferative and fail to undergo terminal differentiation (Lee et al. 2008). Among seven MOB homologs encoded in the human genome, only MOB1a and MOB1b, which share more than 95% sequence homology, can bind to and activate LATS kinases (Chow et al. 2010). Single deletion of either Mob1a or Mob1b does not interfere with embryonic development and Mob1a−/− or Mob1b−/− mice are viable, fertile and without any developmental and physiological abnormalities (Nishio et al. 2012). However Mob1a and Mob1b double knockout mice die before completing gastrulation at E6.5 stage of embryonic development mainly due to failure to form primitive endoderm (Nishio et al. 2012). Phenotypes associated with genetic disruption of the Hippo pathway core components have been summarized in Table 1.
Table 1: Phenotypes associated with germline deletion of Hippo pathway core components

<table>
<thead>
<tr>
<th>Genotype</th>
<th>-/-</th>
<th>+/-</th>
<th>-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yap</strong></td>
<td>Embryonic lethal (E8.5)</td>
<td>Viable</td>
<td>Embryonic lethal (Morula Stage)</td>
</tr>
<tr>
<td><strong>Taz</strong></td>
<td>Viable</td>
<td>Polycystic Kidney Disease</td>
<td>Pulmonary emphysema</td>
</tr>
<tr>
<td><strong>Mst1</strong></td>
<td>Viable</td>
<td>Viable</td>
<td>Embryonic lethal (E9.5)</td>
</tr>
<tr>
<td><strong>Mst2</strong></td>
<td>T cell development deficiency</td>
<td>No abnormalities</td>
<td></td>
</tr>
<tr>
<td><strong>Lats1</strong></td>
<td>Viable</td>
<td>Embryonic lethal (E12.5)</td>
<td>Embryonic lethal (Morula Stage)</td>
</tr>
<tr>
<td><strong>Lats2</strong></td>
<td>Soft tissue sarcoma</td>
<td>Widespread cell division defects</td>
<td></td>
</tr>
<tr>
<td><strong>Mob1a</strong></td>
<td>Viable</td>
<td>Viable</td>
<td>Embryonic lethal (E6.5)</td>
</tr>
<tr>
<td><strong>Mob1b</strong></td>
<td>No abnormalities</td>
<td>No abnormalities</td>
<td>Failure to form Primitive Endoderm</td>
</tr>
<tr>
<td><strong>Sav1</strong></td>
<td>N/A</td>
<td>N/A</td>
<td>Embryonic lethal (E17.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Placental defects</td>
</tr>
</tbody>
</table>
1.3.2 The Hippo pathway in early embryonic development

One of the first and crucial lineage specification events during mammalian development is the formation of trophoectoderm cells and their segregation from the inner cell mass. This specification is mainly determined by the mutual antagonism between transcription factors Pou5f (Oct3/4) and Cdx2, which are responsible for inner cell mass (ICM) or trophoectoderm formation respectively (Niwa et al. 2005). It has been shown that TEAD family transcription factors have an important role in trophoectoderm lineage determination by regulating Cdx2 expression (Nishioka et al. 2009). Specifically, TEAD4 transcriptional activity is differentially regulated by the Hippo pathway effectors YAP and TAZ in the trophoectoderm and inner cell mass. After the 8-cell stage, YAP is mainly nuclear in the outer cells of the blastula while the inner cells show a predominantly cytoplasmic localization, which is correlated with Cdx2 expression. Interestingly the differential localization of YAP in the blastula is Hippo pathway dependent and greater cell-cell contact in the inner cells has been shown to promote YAP phosphorylation and cytoplasmic localization through LATS1/2 kinases (Nishioka et al. 2009). Furthermore, NF2/Merlin the upstream regulator of the Hippo pathway has been identified as the critical regulator of LATS1/2 kinases during ICM/trophoectoderm specification in the preimplantation embryo (Cockburn et al. 2013).

1.3.3 The Hippo pathway in heart development

The mammalian heart is an organ with limited regenerative capacity, however from embryonic development to adulthood the heart size shows a considerable increase. During embryonic development, cardiac growth relies on cardiomyocyte progenitor proliferation and cardiomyocyte fusion, while postnatally an increase in cell size is the main mechanism of heart growth (Vincent and Buckingham 2010; Laflamme and Murry 2011).

Specific ablation of YAP in cardiomyocytes of early embryos interferes with normal heart development and results in embryonic lethality (Xin et al. 2011; von Gise et al. 2012). Although loss of YAP does not impair myocardial specification, YAP-deficient cardiomyocytes fail to grow and show a marked decrease in proliferation. Thus, YAP deficiency does not disrupt overall cardiac patterning but results in the formation of a markedly smaller heart. On the other hand, expression of an active version of YAP that cannot be inhibited by the Hippo pathway, induces proliferation and cell cycle progression both in vitro and in vivo when expressed in cardiomyocyte progenitors and this results in cardiac hypertrophy and heart overgrowth (Xin et al. 2011; von Gise
et al. 2012). Consistently, increased YAP activity upon deletion of the Hippo pathway core components SAV and MST, also promotes cardiomyocyte proliferation and heart overgrowth (Heallen et al. 2011). Gene expression analysis shows that YAP induces the expression of genes important for cell cycle progression, mitosis and cytokinesis including cyclins and CDKs (Cyclin A, Cyclin b, Cdc25 and Cyclin-dependent kinase (Cdc2)) (von Gise et al. 2012). Interestingly, YAP cooperates with the Wnt signalling transducer β-catenin to induce the expression of at least a subset of target genes including Sox2, Snai2, Cdc2 and Survivin that are important for tissue regeneration, cell cycle regulation, stem cell self-renewal and survival of cardiomyocytes (Heallen et al. 2011; Xin et al. 2011). Moreover, YAP promotes the activation of β-catenin in cardiomyocytes by inducing IGF-1R expression and activation of the PI(3)K-Akt pathway which results in phosphorylation of GSK-3β by Akt. This phosphorylation inhibits GSK-3β kinase activity, which reduces β-catenin phosphorylation and subsequent degradation by proteosomal pathway and ultimately results in β-catenin stabilization (Xin et al. 2011).

1.3.4 The Hippo pathway in skin development and regeneration

Skin is another organ that shows a great dependence on Hippo transducer YAP for normal development. An epithelial tissue that protects the body from environmental stress, dehydration, and infection, the skin epidermis is highly proliferative and is composed of various appendages such as hair follicles and sweat and oil glands that are maintained by a variety of stem/progenitor cells. An intricate balance of self-renewal, proliferation and differentiation of these stem/progenitor cells is important for skin homeostasis and regeneration in response to injury (Blanpain and Fuchs 2009).

During embryonic development, the epidermis forms shortly after gastrulation as a single layer of epithelial cells which start to stratify at the E12.5 stage. Conditional ablation of YAP interferes with normal skin epidermis development, characterized by deficient stem/progenitor cell self-renewal, proliferation and premature keratinocyte differentiation, suggesting that YAP is required for stem cell maintenance and suppression of differentiation (Zhang et al. 2011). Conversely, increased YAP activity in SAV-deficient mice promotes the expansion of basal progenitor cells and suppresses terminal differentiation by inhibiting cell-cycle exit, which is required for terminal differentiation (Lee et al. 2008). Consistently, overexpression of an active version of YAP also results in expansion of stem cells, increased proliferation, suppression of terminal differentiation
and ultimately squamous cell carcinomas (Schlegelmilch et al. 2011). YAP inhibits the expression of the Notch signalling effector, Hes1 which has been shown to promote terminal differentiation of basal progenitor cells to suprabasal spinous cells (Blanpain et al. 2006). Thus, the effect of YAP on stem cell expansion may be mediated, at least partially by inhibiting Notch signalling (Zhang et al. 2011).

1.3.5 The Hippo pathway in reprogramming of intestinal stem cells

The mammalian intestine is a single-layered epithelial tissue that is organized into crypt and villus structures and can be completely renewed within 4-5 days, making it one of the fastest proliferating tissues in the body. The constant turnover of cells in the intestine is guaranteed by a specialized population of stem cells at the bottom of the crypt that give rise to differentiated cells including absorptive enterocytes and secretory goblet and Paneth cells (van der Flier and Clevers 2009).

Unlike the epidermal epithelium, which relies on YAP for the formation and maintenance of progenitor cells, intestinal epithelium does not show such dependence, and YAP/TAZ are dispensable for normal development or homeostasis of intestinal tissue. Nevertheless, YAP has crucial and rather complex roles in repair and regeneration of intestinal epithelium upon damage and inflammation. During homeostatic conditions YAP is mainly dispersed throughout the cytoplasm and excluded from the nucleus of intestinal epithelial cells, suggestive of active Hippo signalling in this tissue (Cai et al. 2010; Barry et al. 2013). Indeed, core components of the Hippo pathway, MST1/2 and SAV are abundantly expressed in the intestine and their specific ablation causes a dramatic increase in cell proliferation and expansion of stem and undifferentiated progenitor cells by promoting YAP activity (Lee et al. 2008; Cai et al. 2010; Zhou et al. 2011). Increased transcriptional activity of YAP has been shown to be crucial for intestinal tissue regeneration upon injury by protecting intestinal stem cells (Cai et al. 2010; Gregorieff et al. 2015; Taniguchi et al. 2015). Damage incurred by irradiation or colitis-associated inflammation, promotes a regenerative response mediated by inflammatory cytokines such as IL-6, that activate multiple downstream pathways to stimulate proliferation and expansion of stem cells in order to restore homeostasis. In particular, activation of STAT3 transcription factor in response to IL-6 has been shown to be crucial for mucosal regeneration (Bollrath et al. 2009; Grivennikov et al. 2009). Interestingly, YAP was identified as the downstream effector of IL-6 that is required for the homeostatic reconstitution of the intestinal epithelium, in parallel to and independent of STAT3
signalling (Taniguchi et al. 2015). Notably, activation of YAP in this context occurs independent of the Hippo kinase cascade and is mediated by the Src-family kinases (Taniguchi et al. 2015). A recent study by Gregorieff and colleagues, has also identified a YAP-driven regenerative gene expression program that protects and prevents loss of intestinal stem cells in response to injury and tissue damage (Gregorieff et al. 2015). By inhibiting Wnt signalling, YAP reprograms LGR5+ stem cells to suppress their differentiation into Paneth cells. At the same time, YAP protects stem cells from cell death and promotes their proliferation through EGFR signalling by inducing EGFR ligands such as epiregulin and amphiregulin.

1.3.6 The Hippo pathway in the Liver

Liver is an organ with very high regenerative capacities, capable of proliferating and restoring normal size even after a large portion of it has been removed. Depending on the type and extent of the injury, hepatocyte duplication or activation of oval like progenitor cells have been shown to contribute to liver regeneration and repair (Stanger 2015). Genetic manipulation of Hippo pathway components in mice has dramatic effects on liver size, suggesting a paramount role for Hippo signalling in liver homeostasis and regeneration. Hepatic-specific ablation of the Hippo core components MST1/2, SAV1 and NF2 or transgenic activation of YAP promotes cell proliferation, loss of hepatocyte quiescence, and activation and expansion of biliary progenitor cells that leads to a dramatic overgrowth phenotype and ultimately tumor formation (Camargo et al. 2007; Dong et al. 2007; Zhou et al. 2009; Lee et al. 2010; Lu et al. 2010; Zhang et al. 2010).

Using a combination of lineage tracing and clonal analysis, Yimalmalie and colleagues investigated the consequences of YAP activation in hepatocytes or biliary epithelial cells (Yimlamai et al. 2014). Specific expression of YAP in the biliary cells results in expansion of biliary progenitor cells and ductal hyperplasia, however hepatocytes expressing activated-YAP adopt a smaller, oval morphology and form ductal structures. These findings indicate that YAP induction is capable of reprogramming differentiated hepatocytes to progenitor like biliary cells and suggest that dedifferentiation is the primary mechanism for appearance and proliferation of progenitor cells in the liver of Hippo deficient mouse models (Yimlamai et al. 2014). Consistently, more recent studies show that ablation of the Hippo core components LATS1/2 inhibits terminal differentiation of hepatoblasts to hepatocytes during embryonic development, and also promotes the dedifferentiation of mature hepatocytes to biliary progenitor cells (Yi et al. 2016).
Gene expression profiling in the aforementioned studies identified multiple cell cycle regulators, cell death inhibitors and stem cell markers as the targets of Hippo-YAP signalling in the liver. Notch and TGF-β pathways have also been identified as potential effectors for hepatocyte reprogramming downstream of YAP activation (Nishio et al. 2016; Yi et al. 2016). Indeed, previous studies have shown that Notch signalling is required for biliary epithelial cell specification during embryonic development and activation of Notch signalling in mature hepatocytes is sufficient for reprogramming of hepatocyte into biliary cells (Zong et al. 2009; Yanger et al. 2013).

1.3.7 Roles of the Hippo pathway in tumorigenesis

Since the characterization of the Hippo signalling pathway, it was speculated that defects in Hippo pathway regulation would be associated with various types of cancers given the prominent role in growth control. Interestingly, LATS (large tumor suppressor) was originally identified as a tumor suppressor in *Drosophila* through genetic screens before being recognized as the core component of the Hippo pathway. Subsequent genetic studies in mice showed that LATS1-deficiency results in soft tissue sarcoma formation and ovarian cancer (Justice et al. 1995; Xu et al. 1995; St John et al. 1999). Genomic analysis identified YAP as a major oncogene on the 11q22 locus that becomes amplified in various cancers, and increased expression of YAP has been shown to be able to transform normal mammary and hepatic epithelial cells (Overholtzer et al. 2006; Zender et al. 2006). Although recurrent genetic mutations in the core components of the Hippo pathway are not usually observed in human cancers, altered expression and regulation of these components, especially YAP and TAZ is a frequent event in most types of malignancies (Harvey et al. 2013). Furthermore, various mouse models have shown that targeted perturbation of the Hippo pathway core components including MST1/2, SAV, MOB and LATS1/2 promotes tumorigenesis (Harvey et al. 2013; Zanconato et al. 2016).

Gene expression profiling in different tissues have identified multiple YAP/TAZ target genes that are important for cell proliferation and resistance to apoptosis (Harvey et al. 2013; Zanconato et al. 2016). Beyond regulating cell proliferation and cell death, YAP/TAZ also contribute to tumor progression by regulating various aspects of tumorigenesis such as cell migration, EMT, cancer stem cell content and tumor microenvironment (Zanconato et al. 2016).
1.3.7.1 YAP/TAZ in cancer stem cells, metastasis and drug resistance

Metastasis is a multistep process through which cancer cells lose their contact with neighboring cells, enter the circulation and finally grow in the host organ after colonizing a secondary site. To be able to form tumors in other organs, metastatic cells must acquire a variety of adaptive features such as increased motility, extracellular matrix invasion, ability to enter the blood vessels (intravasation), survival in the blood stream and leaving the circulation to colonize the host organ (extravasation) (Nguyen et al. 2009; Valastyan and Weinberg 2011). Epithelial mesenchymal transition (EMT) is a developmental program that is believed to confer cancer cells with many of the adaptive traits necessary for successful metastasis, including increased motility, invasiveness, cellular plasticity and stem-cell like properties (Yang and Weinberg 2008).

Ectopic expression of YAP and TAZ increases cell migration and induces EMT, thus higher YAP/TAZ activity has been associated with cancer invasiveness and metastasis (Lei et al. 2008a; Lamar et al. 2012). Moreover, YAP/TAZ enable cancer cells to bypass contact inhibition and anchorage independent growth, which are essential for intravasation and survival in the bloodstream. In breast cancer, increased TAZ levels are associated with high grade tumors and elevated cancer stem cell content (Cordenonsi et al. 2011). TAZ is functionally important for the stemness of cancer stem cells, promoting self-renewal, tumor initiating capacity and chemoresistance of malignant cells (Cordenonsi et al. 2011). In esophageal cancer, YAP promotes the acquisition of cancer stem cell properties by inducing the expression of the well-known stem cell marker SOX9 (Song et al. 2014).

1.3.7.2 YAP/TAZ bypass oncogene addiction

Although cancer cells usually bear multiple mutations in oncogenes or tumor suppressors that are important for tumor formation, they tend to heavily rely on continued activation or overexpression of specific oncogenes for tumor maintenance and progression, a phenomenon known as “oncogene addiction” (Weinstein 2002). Activating mutations or increased expression of the KRAS oncogene has been shown to be essential for tumor maintenance in a variety of cancers including pancreatic, lung and colon cancer (Karnoub and Weinberg 2008). Although efforts to target KRAS have been futile so far, multiple upstream activators or downstream effectors of RAS-MAPK pathway have been targeted for cancer therapy (Montagut and Settleman 2009). Targeting these components
provokes striking initial therapeutic responses, however acquired resistance and tumor recurrence is almost inevitable (Holohan et al. 2013). Therefore, it’s crucial to understand mechanisms that are involved in therapeutic resistance and identify oncogenes that can compensate for loss of KRAS and promote tumor progression.

Recently YAP was identified as an essential oncogene in KRAS-mutated colon and pancreatic cancers and it has been shown that cancer cells with increased expression of YAP are able to bypass KRAS oncogene addiction. In KRAS-addicted pancreatic cancer cells, the YAP/TEAD complex in association with the E2F transcription factor can compensate for loss of KRAS by inducing the expression of multiple targets involved in cell cycle progression, proliferation, DNA synthesis and replication thereby suppressing tumor regression upon KRAS inactivation (Kapoor et al. 2014). In colon cancer cells, YAP can compensate for KRAS deficiency by inhibiting cell death and promoting epithelial-mesenchymal transition (EMT) in cooperation with the FOS transcription factor, rather than regulating cell cycle (Shao et al. 2014). Of note, EMT had previously been identified as an important mechanism to bypass KRAS-addiction in multiple cancer cell lines (Singh et al. 2009).

1.3.7.3 YAP/TAZ in tumor microenvironment

Malignant cells within a tumor are associated with a variety of non-cancerous stromal cells such as fibroblasts, endothelial and immune cells collectively referred to as the tumor microenvironment (Quail and Joyce 2013). One of the major components of the tumor microenvironment are cancer associated fibroblasts (CAFs), that have crucial roles in tumor growth and progression by producing extracellular matrix components, chemokines and growth factors. Interestingly, in invasive breast cancer YAP has been shown to be predominately nuclear in CAFs, whereas fibroblasts associated with normal mammary epithelial cells demonstrate a cytoplasmic YAP distribution. In cancer associated fibroblasts, YAP induces the expression of extracellular matrix components and actin cytoskeleton regulators that increase the stiffness of tumor microenvironment to support higher YAP activity both in CAFs and in cancer cells. Thus, YAP activity in CAFs creates a favorable microenvironment for tumor growth and invasion (Calvo et al. 2013).

Tumor cells usually generate an immune suppressive environment by recruiting myeloid-derived suppressive cells (MDSC) or macrophages that suppress antitumor T-cell immunity. The
recruitment of these cell populations is mediated by a variety of cytokines and chemokines secreted by tumor cells and other components of the tumor microenvironment (Quail and Joyce 2013). Interestingly, YAP has recently been identified as a critical mediator of immune cell response in prostate and liver cancers. In prostate cancer, YAP has been shown to recruit myeloid-derived suppressive cells by inducing the expression and secretion of CXCL5 that engages the CXCR5 and recruits MDSCs to tumor microenvironment (Wang et al. 2016). In the liver, hyperactive YAP promotes the expression of the CCL2 cytokine that attracts CCR2 expressing macrophages to the tumor parenchyma even at very early stages of tumor formation (Guo et al. 2017). Both MDSCs and macrophages recruited to the tumor microenvironment promote tumor progression by suppressing anti-tumor immunity mediated by T cells. (Wang et al. 2016; Guo et al. 2017).

1.4 Pix/Git proteins in cell shape regulation and cell signalling

1.4.1 Pix as a GEF for Rac1/Cdc42

Small molecule GTPases are a large family of GTP-binding proteins that act as molecular switches to regulate a plethora of biological and cellular processes. They can be categorized into five major families, including Ras, Rab, Rho, Ran and Arf, each with multiple members (Wennerberg et al. 2005). The Rho family of GTPases is comprised of twenty different members which regulate a variety of biological processes such as cell polarity, cell cycle progression, vesicular transport, actin cytoskeleton and focal adhesion dynamics (Etienne-Manneville and Hall 2002; Sahai and Marshall 2002). RhoA, Rac1 and Cdc42 are the best-known Rho family members that have been extensively studied for their roles in regulating actin cytoskeleton reorganization. Activation of RhoA results in the formation of contractile and actin stress fibers, whereas Rac1 and Cdc42 promote lamellipodia or filopodia formation, respectively. The activity of each of these Rho GTPases can be activated by specific Guanine Nucleotide Exchange Factors (GEFs) or inhibited by GTPase Activating Proteins (GAPs) (Etienne-Manneville and Hall 2002).

α and βPix (for PAK-interacting exchange factor α and β) belong to the DBL family of guanine nucleotide exchange factors (GEF) with specific activity toward Rac1 and Cdc42. As depicted in Figure 1.3, βPix is composed of an N-terminal SH3 domain, followed by a tandem Dbl homology (DH) and Pleckstrin homology (PH) domains, which comprises the GEF activity. The C-terminal
region contains a leucine-rich coiled-coil domain that is responsible for homo- and hetero-multimerization (Rosenberger and Kutsche 2006).

Overexpression of βPix induces membrane ruffling and filopodia formation which is reminiscent of Rac1/Cdc42 activation (Koh et al. 2001; ten Klooster et al. 2006) (Figure 1.3). This can be blocked by DH domain deletion indicating that βPix overexpression can activate Rac1 in a GEF dependent manner and places βPix upstream of Rac1. Rac1 and Cdc42 exert their biological effects through multiple downstream effectors such as p21-activated kinases (PAKs), a family of Ser/Thr kinases with important roles in actin cytoskeleton dynamics, migration and cell survival (Bokoch 2003). In response to Rac1/Cdc42 activation, Pix is recruited to focal adhesions and in association with PAK, induces membrane ruffling and cell migration (Koh et al. 2001; Filipenko et al. 2005; Rosenberger and Kutsche 2006). Moreover, βPix can be phosphorylated by the focal-adhesion kinase (FAK), an event that enhances βPix interaction with Rac1 and promotes βPix recruitment to focal adhesions (Chang et al. 2007). Altogether, these studies demonstrate that βPix has crucial roles in actin cytoskeleton reorganization and focal adhesion dynamics by serving both as an upstream activator and a downstream effector of Rac1/Cdc42.

1.4.2 βPix functions as an adaptor protein

βPix, is not only involved in focal adhesion dynamics but also functions as a scaffolding protein that mediates multiprotein complex formation and organization of signalling events. βPix was first cloned and characterized as p85SRP (SH3 domain-containing proline-rich protein) based on its N-terminal SH3 domain, which tightly binds to a proline-rich region on PAK (Oh et al. 1997; Bagrodia et al. 1998; Manser et al. 1998). While PAK is the best-known binding partner of βPix, the SH3 domain also mediates a specific interaction with a proline-arginine rich domain in the ubiquitin ligase Cbl (Flanders et al. 2003; Wu et al. 2003b; Schmidt et al. 2006). Cbl is responsible for ubiquitin-mediated degradation of epidermal growth factor receptor (EGFR) and by blocking Cbl-EGFR binding, βPix inhibits EGFR downregulation (Schmidt and Dikic 2005). The tandem DH/PH domain not only contains the GEF activity, but also mediates interaction with 14-3-3. The association between βPix and 14-3-3 inhibits βPix-GEF activity through a conformational change in the DH domain (Chahdi and Sorokin 2008b).
Figure 1.3: The structure and function of βPIX.

(A) βPIX is composed of an N-terminal SH3 domain followed by a tandem Dbl-homology (DH) and Pleckstrin homology (PH) domains that contain the GEF activity. The C-terminal region is comprised of a domain enriched in charged amino acids (KER) and a Leucine zipper (LZ) region that is responsible for homo- and heterodimerization. (B) βPIX functions as a Guanine Nucleotide Exchange Factor (GEF) for Rac1 and Cdc42 GTPases that cycle between an active GTP-bound and inactive GDP bound states.
The C-terminal region of βPix contains a glutamate rich region and a leucine-rich coiled-coil domain or leucine zipper, which are necessary for dimerization and complex formation with other binding partners such as p66shc, FoxO3 (Chahdi and Sorokin 2008a). This region is also responsible for interaction with Git1, one of the well-known binding partners of βPix, which together form multimeric complexes involved in various cell signalling events.

1.4.3 Versatile GIT proteins regulate multiple cellular processes

G-protein coupled receptor kinase interacting protein 1 and 2 (GITs) are versatile multidomain containing proteins that are involved in diverse biological processes including focal adhesion dynamics, cell migration, feedback regulation of GPCR signalling and vesicular trafficking. GIT1 was initially identified as an interacting partner of G-protein coupled receptor kinases (GRKs) which are crucial regulators of GPCR signalling (Premont et al. 1998). GRKs are a family of Ser/Thr kinases that phosphorylate activated GPCR after receptor binding and this phosphorylation facilitates recruitment of multiple binding partners such as arrestin and clathrin that are involved in termination of GPCR signalling by receptor internalization (Pitcher et al. 1998). Thus, GIT proteins are involved in negative feedback regulation of GPCR signalling. Shortly after their characterization as GRK binding partners, GITs were shown to be localized in focal adhesions and interact with Paxillin and Pix proteins (Turner et al. 1999). As depicted in Figure 1.4, GIT proteins have a complex multidomain structure composed of an N-terminal ARF-GAP domain followed by three Ankyrin repeats (ANK), a Spa2-homolgy domain (SHD), a coiled-coil and a C-terminal paxillin-binding domain (PBD) also known at focal adhesion targeting (FAT) domain. Compared to GIT1, GIT2 has multiple isoforms and truncated versions that lack the C-terminal paxillin binding domain.

ARF proteins are small GTPases that are involved in vesicular trafficking and endosomal recycling (D’Souza-Schorey and Chavrier 2006). The N-terminal domain of GITs contain GAP (GTPase activation protein) activity towards ARF proteins that lack inherent GTPase activity (Hoefen and Berk 2006), therefore GITs might be involved in endosomal pathway regulation. Indeed, mutations in the GAP domain of GITs result in abnormal localization to vesicular structures, confirming the importance of GIT ARF-GAP activity in regulating membrane and vesicular trafficking pathways (Di Cesare et al. 2000; Matafora et al. 2001).
Figure 1.4: The structure of GIT1, interacting partners and cellular functions.

The GIT1 domain structure is composed of an N-terminal ARF-GAP domain followed by three Ankyrin repeats (ANK), Spa-homology domain (SHD), Coiled-coil domain (CC) and a Paxillin-binding site (PBS). Through interactions with ARF-GTPases, PIX, PAK and Paxillin, GIT1 regulates different cellular processes such as membrane trafficking, focal adhesion dynamics and cell morphology.
The Spa2-homolgy domain (SHD) mediates GIT interaction with its most prominent binding partners α- and βPix (Brown et al. 2002). The coiled-coil domain mediates GIT dimerization that in association with oligomeric Pix proteins, form a large multimeric complex, involved in focal adhesion dynamics (Brown et al. 2002; Manabe et al. 2002; Paris et al. 2003). The GIT paxillin-binding domain mediates the recruitment of GIT-PIX complex to focal adhesions during cell-ECM contact, which further facilitates downstream signalling such as Rac1/Cdc42 activation, actin cytoskeleton reorganization and cell migration (Brown et al. 2002; Manabe et al. 2002; Paris et al. 2003; Hoefen and Berk 2006; Frank and Hansen 2008).

1.5 Par-1/MARK kinase family

Microtubule associated regulating kinases (MARKs) are a family of Ser/Thr kinases and the mammalian orthologs of the evolutionarily conserved Par-1 polarity proteins. MARKs are comprised of four family members, MARK1/Par-1c, MARK2/Par-1b, MARK3/Par-1a and MARK4/Par-1d and as the name implies, they were identified based on their ability to phosphorylate microtubule associated proteins, Tau and MAPs (Drewes et al. 1997; Hurov and Piwnica-Worms 2007). Phosphorylation by MARKs, was reported to disrupt Tau and MAP binding to microtubules and results in microtubule instability (Drewes et al. 1997). MARK family members share a similar domain structure which is composed of a kinase domain, a ubiquitin-associated domain (UBA) and a C-terminal Kinase-associated domain (KA) (Figure 1.5). The UBA domain has been suggested to regulate the kinase activity of the MARKs through intramolecular interaction with the kinase domain and by conformational changes in response to post-translational modification (Jaleel et al. 2006). The C-terminal region of the MARKs which contains the kinase-associated (KA) domain has been implicated in the reversible autoinhibition of kinase activity (Elbert et al. 2005). The kinase-associated domain mainly contains positively charged amino acids and recently has been identified as a membrane association domain that binds to negatively charged phospholipids thereby mediating membrane localization of MARKs (Moravcevic et al. 2010). The kinase domain of the Par-1/MARKs shows a high degree of similarity to AMP-activated kinase (AMPK) and along with 8 other kinases including NUAK1, NUAK2, BRSK1, BRSK2, SIK1-3, and MELK comprise the AMPK related subfamily of Ser/Thr kinases. Although AMPK is the best-known member of this family and has been extensively studied for its role in organismal metabolic regulation, recent genetic studies in mice have revealed metabolic roles for MARK family members too.
Figure 1.5: The structure of MARKs, upstream regulators and downstream substrates.

Microtubule affinity regulated kinases (MARKs) have a simple domain structure composed of a Ser/Thr Kinase domain, a Ubiquitin-associated domain (UBA) and a C-terminal Kinase-associated domain (KA). The kinase activity of MARKs can be positively or negatively regulated by upstream kinases such as LKB1 and TAO1 that activate, or GSK3 and aPKC that inhibit MARKs by phosphorylating different residues. In turn, by phosphorylating different substrates such as PAR3, DLG1, Tau, MAPs, CDC25 and Dishevelled (DVL), MARKs regulate a number of cellular processes including cell polarity, microtubule dynamics, cell cycle progression and Wnt signalling.
MARK2/Par-1b was the first member to be genetically targeted in mice and MARK2-deficient mice display metabolic alterations including decreased adiposity, resistance to weight gain, insulin hypersensitivity and increased glucose uptake in adipose tissue (Hurov et al. 2007). MARK3/Par-1a knockout mice also show most of the phenotypes observed in MARK2-deficient mice such as increased metabolism, decreased adipose tissue mass and resistance to obesity (Lennerz et al. 2010). Despite great redundancy, MARK3 deficient mice demonstrate specific phenotypes that are not observed in MARK2-/− mice. Specific phenotypes associated with MARK3 deficiency include resistance to high-fat diet induced steatohepatitis, increased hepatic autophagy and glycogen depletion in response to starvation (Lennerz et al. 2010). Interestingly, although mice deficient for either MARK2 or MARK3 are viable, the double knockout mice die during embryonic development suggesting that MARK2 and MARK3 function redundantly (Lennerz et al. 2010). Phenotypes associated with MARK4 disruption are also very similar to MARK2 and MARK3 deletion, including hypermetabolism, resistance to obesity and insulin hypersensitivity (Sun et al. 2012).

MARK4 is located on the human chromosome 19q13.2, encoding two alternatively spliced Large (L) and Short (S) isoforms that differ in the C-terminal region. The MARK4 cDNA was initially isolated from hepatocellular carcinomas cell lines where it is highly expressed. It was also shown to be downregulated in response to TCF/LEF expression, suggesting that MARK4 might be regulated by Wnt signalling (Kato et al. 2001). MARK4 is ubiquitously expressed with the highest expression in testis and the nervous system, especially in undifferentiated neuronal progenitor or glial precursor cells (Moroni et al. 2006). It has been shown that MARK4 is downregulated during glial differentiation and MARK4 overexpression is frequently observed in glioma cells, thereby maintaining the progenitor state (Beghini et al. 2003). Similar to other Par-1/MARK family members, MARK4 is capable of phosphorylating Tau and MAPs and overexpression of MARK4 results in microtubule reorganization (Trinczek et al. 2004).

Par-1/MARK proteins have been extensively studied for the roles they play in regulating cell polarity from *C.elegans* to mammals (Guo and Kemphues 1995; Bohm et al. 1997). Par-1 can be phosphorylated and negatively regulated by aPKC, a component of the PAR complex which is composed of Par3, Par6 and aPKC. The PAR complex is localized at the apical surface of epithelial cells and has crucial roles in regulating apico-basal polarity and tight junction formation (Hurov et al. 2004; Suzuki et al. 2004). Phosphorylation by aPKC excludes Par-1 from the apical surface,
thereby creating non-overlapping domains between the PAR complex at the apical surface and Par-1 at the basolateral domain of epithelial cells (Hurov et al. 2004; Suzuki et al. 2004). The antagonism between Par-3/Par-6/aPKC complex and Par-1/MARKs is not only important for establishment of epithelial cell polarity but is also required for proper neuronal polarization. During neurogenesis, inhibition of the MARK/Par-1 proteins by the PAR complex promotes axon formation by microtubule stabilization (Chen et al. 2006). Interestingly, a recent study identifies that dystrophin regulates the asymmetric distribution of PAR complexes in satellite muscle stem cells by interacting with and stabilizing MARK2/Par-1. Thus, loss of function mutations in dystrophin results in MARK2 downregulation, disruption of cell polarity, defective mitotic spindle orientation and loss of asymmetric cell division which leads to stem cell dysfunction and impaired regenerative capacity, the underlying cause of Duchenne muscular dystrophy (Dumont et al. 2015).

The kinase activity of MARKs/Par-1 proteins can be regulated by a number of other upstream kinases (Figure 1.5). LKB1 is a master upstream kinase that phosphorylates AMPK family kinases, including MARKs on a Threonine residue in the T-loop region, leading to activation of kinase activity (Lizcano et al. 2004). MARKs can also be phosphorylated by TAO-1 and GSK3-β that results in activation or inhibition of MARK kinase activity, respectively (Timm et al. 2003; Timm et al. 2008). Multiple proteins have also been identified as MARK substrates in addition to MAPs and Tau, including Dishevelled (DVL), CDC25, HDAC, KSR, RAB11 and DLG1, suggesting that MARKs are involved in regulating a variety of cellular processes from microtubule dynamics and cell polarity to Wnt signalling and cell cycle regulation (Hurov and Piwnica-Worms 2007) (Figure 1.5).
1.6 High-throughput screenings to identify novel regulators of the Hippo pathway

Cells rely on signal transduction pathways to integrate the information received from extracellular environment into biological responses. Dynamic and highly modulated protein-protein interactions are essential for signal transduction, therefore in order to understand how signalling pathways work, it is crucial to identify the components of each pathway and to characterize the spatial and temporal pattern of protein interactions (Scott and Pawson 2009). Since the advent of high throughput technologies, a variety of approaches including genetic or physical mapping-based methods such as affinity purification-mass spectrometry and LUMIER have been applied to systematically characterize protein interaction networks in different model organisms. These interaction studies combined with functional assays such as gain or loss of function or luciferase-reporter assay screens have been very informative in identification and characterization of novel components of various signalling pathways. LUMIER is a cell-based high throughput screening method to map protein interactions in mammalian cells first developed by Wrana and colleagues (Barrios-Rodiles et al. 2005). In this method, a protein of interest tagged with luciferase is used to test for interactions with a library of co-expressed Flag-tagged proteins, in mammalian cells (Figure 1.6). The degree of interaction is measured by anti-Flag immunoprecipitation followed by a luciferase assay (Barrios-Rodiles et al. 2005). This method was originally applied to identify novel TGF-β signalling regulators (Barrios-Rodiles et al. 2005) and has since been successfully applied to other signalling pathways including Wnt (Miller et al. 2009; Varelas et al. 2010a).
Figure 1.6: Luminescence-based mammalian interactome mapping (LUMIER) method to detect protein interactions.

LUMIER uses Firefly luciferase enzyme fused with the protein of interest (bait) which is co-expressed with a Flag-tagged protein (prey) in mammalian cells. The cell lysates are then subject to anti-Flag immunoprecipitation and the interaction between bait and prey is determined by performing a luciferase enzymatic assay on immunoprecipitates.
1.7 Thesis Overview

The Hippo pathway is a signalling pathway that responds to and integrates a variety of intrinsic and extrinsic stimuli such as cell-cell contacts, cell morphology, actin cytoskeleton dynamics, nutrient availability and G-protein coupled receptor activation to regulate vital processes like cell proliferation, apoptosis and differentiation. Nevertheless, the molecular mechanisms and protein components that connect these upstream regulators to the core of the Hippo pathway are not fully understood. In this thesis, results from a LUMIER-based protein interaction screen combined with a functional cDNA overexpression screen using a TEAD-luciferase reporter have been utilized to identify novel modulators of the Hippo pathway.

ARHGEF7 also known as βPIX was identified by LUMIER to interact with the Hippo core components YAP/TAZ and LATS kinases. Through functional studies, I demonstrated that βPIX acts as a scaffolding protein that promotes complex formation between LATS and YAP/TAZ, thereby restricting YAP/TAZ transcriptional activity. Furthermore, I showed that overexpression of βPIX in aggressive breast cancer cells with constitutively active YAP/TAZ, re-engages the Hippo kinase cascade and diminishes cell proliferation and migration, hence the tumorigenic properties of these cells.

In chapter 3, I identified the microtubule affinity regulated kinases (MARKs) as negative regulators of the Hippo pathway from a TEAD-luciferase reporter functional screening. I showed that MARK4, by phosphorylating MST and SAV, disrupts complex formation between MST, SAV and downstream LATS kinases thereby inducing YAP/TAZ activity. Furthermore, I demonstrated that MARK4 loss of function attenuates YAP/TAZ mediated oncogenic features such as cell proliferation and cell migration in breast cancer cells.

In chapter 4, I investigated the potential role of βPIX and MARK4 binding partners, GIT1 and DLG5, respectively, in regulating YAP/TAZ activity. Through loss of function experiments, I showed that GIT1 deletion increases YAP/TAZ transcriptional activity and also promotes cell proliferation in mammary epithelial cells. Conversely, disruption of DLG5 expression results in inhibition of YAP/TAZ activity, concomitant with increased phosphorylation and loss of nuclear localization.
Altogether the work presented in this thesis identifies multiple upstream regulators of the Hippo signalling pathway that control YAP/TAZ transcriptional activity in response to various stimuli and provide insights into the mechanisms by which they modulate the Hippo kinase cascade.
Chapter 2
Arhgef7 promotes activation of the Hippo core kinase Lats

The work included in this chapter was published in:


Data attribution:
I initiated the project and designed and performed experiments to investigate the effect of βPix loss of function on Yap/Taz subcellular localization, phosphorylation and transcriptional activity in response to actin dynamics and cell density. I also designed and performed experiments to elucidate βPix mechanisms of action including mapping the interaction between βPix and the Hippo core components Yap and Lats, and functional studies with βPix mutant constructs. Furthermore, I generated MDA-MB-231 cells stably expressing βPix to evaluate the tumorigenic properties of these cells. I generated the data presented in:
Figure 2.1C and D; 2.2; 2.4; 2.5C and E; 2.6 and 2.8C

Sharfee Shiban performed the LUMIER screen and produced the data presented in:
Figure 1.1A

Ki Song performed experiment related to αPix-Yap interactions and examined the effect of Rac1/Cdc42 depletion on Yap/Taz and produced the data presented in:
Figure 1.1B; 2.2 and 2.5A

Siyuan Song performed the experiments related to cell proliferation/migration assays and I assisted in analyzing the data presented in:
Figure 2.7; 2.8A, B, D and E
2 Arhgef7 promotes activation of the Hippo core kinase Lats

2.1 Abstract

The Hippo pathway regulates tissue growth and organ size and inactivation contributes to cancer. Signals flow through Mst/Lats kinases, which phosphorylate and promote cytoplasmic localization of the transcriptional regulators, Yap and Taz to inhibit transcription. Here, we identify the multidomain-containing guanine nucleotide exchange factor (GEF), Arhgef7, or βPix, as a positive Hippo pathway regulator. We show that βPix, which localizes to the cytoplasm, binds both Lats and Yap/Taz and thereby promotes Lats-mediated phosphorylation of Yap/Taz in a GEF independent manner. βPix is required downstream of both cell density sensing and actin cytoskeletal rearrangements and we demonstrate that loss of βPix expression in normal mammary epithelial cells strongly reduces Yap/Taz phosphorylation, promotes nuclear localization and increases target gene expression. Conversely, increased expression of βPIX in breast cancer cell lines, recouples the Hippo kinase cassette to Yap/Taz, promoting localization of Yap/Taz to the cytoplasm and inhibiting cell migration and proliferation. These studies thus define βPix as a key component that links the Hippo kinase cassette to Yap/Taz in response to multiple upstream Hippo pathway activators.
2.2 Introduction

The Hippo signalling pathway is a major regulator of cell proliferation and tissue growth control. First uncovered using genetic screens in *Drosophila*, the conservation of this pathway in mammals has been firmly established (Halder and Johnson 2011; Irvine 2012; Ramos and Camargo 2012; Harvey et al. 2013; Yu and Guan 2013). At the core of the mammalian Hippo pathway is a kinase cassette comprised of the *Drosophila* Hippo homologs, Mammalian STE20-like protein kinase 1/2 (Mst1/2, gene name STK4/3) and Large tumor suppressors 1 and 2 (Lats1/2). Upon activation, Mst1/2 in association with the adaptor protein, Salvador (Sav1), phosphorylates and activates Mob1A/B-bound Lats1/2 kinases that in turn phosphorylate the related transcriptional regulators, Yes-associated protein (Yap) and Transcriptional co-activator with PDZ-binding motif (Taz).

When Hippo is inactive, Yap/Taz are primarily localized in the nucleus and in association with diverse transcription factors such as Teads, Runx and Smads, regulate the expression of target genes including connective tissue growth factor (Ctgf), Ankrd1 and Cyr61. However, phosphorylation of Yap/Taz by Lats1/2 kinases leads to their cytoplasmic accumulation and enhanced ubiquitin-dependent degradation that thereby prevents transcriptional activity (Halder and Johnson 2011; Irvine 2012; Harvey et al. 2013; Yu and Guan 2013).

Disruption of Hippo signalling in mouse models promotes tumor formation and overexpression and constitutive nuclear localization of Yap/Taz occurs in many human cancers (Harvey et al. 2013). Yap/Taz regulated transcriptional programs are associated with tumor initiation, progression and metastasis by promoting cell proliferation, migration, survival and epithelial-mesenchymal transition (Harvey et al. 2013). Functional interactions of Yap/Taz with many cancer-associated signalling networks also contributes to their tumor promoting activities and to related physiological processes such as the regulation of stem cell maintenance and differentiation (Irvine 2012; Ramos and Camargo 2012; Attisano and Wrana 2013).

Cell-cell contact was one of the first identified regulators of Hippo signalling (Zhao et al. 2007; Ota and Sasaki 2008) and is sensed and transmitted to the pathway by proteins that are involved in maintenance of cell architecture, such as polarity complexes and junctional proteins (Genevet and Tapon 2011; Boggiano and Fehon 2012; Schroeder and Halder 2012). The apically-localized Crumbs complex, that includes Angiomotin, activates the Hippo kinase cassette in flies and mammals, possibly in connection with the sub-apically-localized Kibra/NF2/Willin complex.
(Chen et al. 2010; Grzeschik et al. 2010; Ling et al. 2010; Robinson et al. 2010; Varelas et al. 2010b; Genevet and Tapon 2011; Zhao et al. 2011; Boggiano and Fehon 2012; Schroeder and Halder 2012). Other components of polarity complexes including Scribble and adherens junction proteins, such as α-Catenin and Ajuba, and protocadherins, such as Fat, also promote Hippo pathway activity (Sopko and McNeill 2009; Das Thakur et al. 2010; Kim et al. 2011; Schlegelmilch et al. 2011; Boggiano and Fehon 2012; Reddy and Irvine 2013). Yap/Taz activity is also modulated by rearrangements of the actin cytoskeleton that can occur with changes in cell morphology, attachment to the extracellular matrix and in response to mechanical forces (Dupont et al. 2011; Wada et al. 2011; Halder et al. 2012; Zhao et al. 2012). Pathways emanating from G-protein coupled receptors can also positively or negatively regulate the Hippo pathway (Yu et al. 2012b).

Despite extensive interest in understanding how the Hippo pathway is regulated, mechanistic details and the molecular mediators that connect upstream signals to the Hippo kinase cassette remain elusive. Using LUMIER, a high throughput protein-protein interaction screen (Barrios-Rodiles et al. 2005; Miller et al. 2009; Varelas et al. 2010a), we identified Arhgef7, more commonly known as βPix (for PAK-interacting exchange factor beta) as a Taz interacting protein. βPix, is a member of Dbl family of Guanine nucleotide Exchange Factors (GEF) for the small GTPases, Rac1 and Cdc42 (Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012). βPix contains several protein-protein interaction domains, including Src Homology 3 (SH3), a tandem Dbl homology (DH) and Pleckstrin homology (PH) domain that mediates GEF activity, and a carboxy-terminal leucine zipper (LZ) domain (Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012). As a multidomain-containing protein, βPix is thought to act as a signal organizer by scaffolding the formation of multiprotein complexes. In this study, we show that βPix is required for Hippo pathway activity in response to multiple upstream stimuli. Mechanistically, we demonstrate that βPix scaffolds Lats to Yap/Taz, thereby promoting Yap/Taz phosphorylation and cytoplasmic sequestration. Thus, we delineate βPix, as a novel regulator of the Hippo core kinase cassette.
2.3 Materials and Methods

2.3.1 Cell Culture and Transfection

For cell culturing, NMuMG cells were grown in DMEM supplemented with 10% FBS and 10 μg/ml insulin, EpH4 and HEK293T cells in DMEM with 10% FBS and MDA-MB-231 cells in RPMI with 5% FBS. Cells were transfected with Dharmacon siGENOME pools of four individual siRNAs (Thermo Scientific) using Lipofectamine RNAiMAX (Life Technologies) or with cDNAs, using Lipofectamine LTX or Lipofectamine 3000 (Life Technologies) according to the manufacturer’s instructions.

2.3.2 Plasmids and Chemicals

The βPIX construct was generated by PCR using isoform a of human βPIX (NM_001113513.1) and was N-terminally tagged with Flag or HA in a pCMV5 vector. βPIX and βPIX deletion constructs were generated by PCR-mediated site directed mutagenesis. Flag- or HA- tagged constructs for LATS1, TAZ and YAP in pCMV5 were previously described (Varelas et al. 2010a). For MDA-MB-231 cells stably expressing βPIX, Flag-tagged βPIX was subcloned into pBABE-puro vector (addgene #1764, (Mani et al. 2007)). pBABE-puro empty vector was used as a control. Chemicals used in this study were: Latruncin A (Tocris Bioscience #3973), C3 (Cytoskeleton Inc #CT04), Y-27632 (Sigma-Aldrich #Y0503), and Blebbistatin (Sigma-Aldrich #B0506).

2.3.3 Immunoblotting, Immunoprecipitation and Subcellular Fractionation

Cells were lysed in lysis buffer [50 mM tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X100, 1 mM DTT containing phosphatase and protease inhibitors]. Lysates were separated on SDS-PAGE gels and immunoblotting was performed using standard protocols as previously described (Labbe et al. 2000). Phos-Tag gels, using reagents purchased from Waco Chemicals, were prepared according to manufacturer’s instructions. Nuclear and cytoplasmic fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific #78833). For immunoprecipitations, cell lysates were subject to anti-Flag or anti-Lats1 immunoprecipitation and proteins collected using protein G-Sepharose prior to analyses by immunoblotting. The antibodies used were: pYAP (D9W2I; Cell Signalling #13008); YAP (Cell Signalling #4912); TAZ (Cell
Signalling #2149); Lats1 (C66B5; Cell Signalling #3477); Cool1/βPix (Cell Signalling #4515); rat anti-HA (Roche #1867423) and anti-Flag M2 (Sigma-Aldrich #F1804).

2.3.4 Immunofluorescence Microscopy

Cell were plated in 4-well Lab-Tek chambers (#154526) and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were permeabilized with 0.5% Triton-X100 in PBS for 10 min at room temperature. Samples were washed three times with 0.01% PBS-Tween, and then blocked in 2% BSA-PBS for 30 min before treatment with primary antibody. Samples were then incubated with primary antibodies (mouse anti-YAP 1:300; Santa Cruz sc-101199; or rabbit anti-Flag 1:500, Sigma F7425) in 2% BSA-PBS overnight at 4°C. After washing three times with 0.01% PBS-Tween, slides were incubated with the secondary antibodies, goat anti-rabbit Alexa-fluor 488 (Life Technologies #A11305, 1:1000 in 2% BSA-PBS) or goat-anti-mouse Alexa Fluor 546 (Invitrogen #A11029, 1:1000 in 2% BSA-PBS) for 1-2 h at room temperature. Slides were washed three times with 0.01%PBS-Tween and once with PBS and mounted with ProLong Gold Antifade Reagent (Life Technologies #P36035). Cell nuclei were visualized by DAPI staining and Alexa Fluor 568-Phalloidin (Life technologies #12380) was used for actin cytoskeleton staining. Images were captured using a spinning disk confocal scanner (CSU10, Yokogawa) on Leica DMI6000B microscope and Volocity software was used for image acquisition and processing. For quantification of Yap localization transfected with different Flag-tagged βPIX cDNA constructs, a minimum of 30 transfected cells were counted and nuclear/cytoplasmic localization of Yap was evaluated in transfected cells compared to the surrounding non-transfected cells.

2.3.5 Quantitative Real-Time PCR

Total RNA was purified using PureLink RNA Mini Kit (Life Technologies. cDNA was synthesized using 1 μg of purified RNA using Oligo-dT primers and M-MLV Reverse Transcriptase (Invitrogen #28025-013). Real-Time PCR was performed using the SYBR Green master mix (Applied Biosystems) on the ABI Prism 7900 HT system (Applied Biosystems). Relative gene expression was quantified by ΔΔCt method and normalized to Gapdh. The sequence of the primers used for qPCR is this study are listed below:
<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
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<td>Arhgef7</td>
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<tr>
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<td>ATAGGCCAGATTCAGTTGGT</td>
</tr>
<tr>
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<tr>
<td>Lats2</td>
<td>TTTATCCAGGACATCAAGCCT</td>
<td>AGTTGGAACATCGTCCCAGAGGT</td>
</tr>
</tbody>
</table>

2.3.6 Wound Healing and Cell Growth Assays

For wound healing migration assay, cells were seeded in a 6-well plate and were grown to confluency. The wound was introduced by scraping with a sterile 200 μl pipette tip and the unfilled area was quantified by ImageJ at the 8 h time point. Cell growth determined using the Sulforhodamine B (SRB) assay. Cells were plated overnight in 96-well dishes and after 48 h, were fixed with 10% (w/v) trichloroacetic acid and stained as previously described (Bao et al. 2012). The amount of SRB present in each well was determined by optical density reading at 490 nm.
2.4 Results

2.4.1 βPix interacts with Yap and TAZ

Yap/Taz are the key effectors of the Hippo pathway. Thus, to identify putative pathway modulators, we undertook LUMIER, a mammalian cell-based protein-protein interaction screen (Barrios-Rodiles et al. 2005; Miller et al. 2009; Varelas et al. 2010a), to uncover novel TAZ binding partners. For this, TAZ, fused to Firefly luciferase, was used as a bait to screen a library of Flag-tagged proteins and interactions were detected by conducting a luciferase assay on anti-Flag immunoprecipitates (Figure 2.1A). Identified binding proteins included known partners, such as DVL1/2, 14-3-3 proteins, βTrCP and TAZ, which forms a dimer (Kanai et al. 2000; Tian et al. 2007; Varelas et al. 2010a), and also revealed a novel interaction with ARHGEF7, commonly known as βPIX. βPIX and the closely related αPIX are proteins comprised of diverse binding domains including an SH3, a LZ and centrally localized tandem DH and PH domains, characteristic of GEFs (Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012). To confirm the association of TAZ with βPIX we performed immunoprecipitations of either Flag-tagged TAZ or βPIX, followed by anti-HA immunoblotting, and observed an interaction between the two proteins regardless of whether TAZ or βPIX was immunoprecipitated (Figure 2.1B). Similarly, YAP interacted with βPIX (Figure 2.1B).
2.4.2 βPix is required for cytoplasmic localization of Yap/Taz in response to diverse cues

High cell density and the formation of cell-cell junctions activates the Hippo pathway and results in cytoplasmic retention of the transcriptional regulators, Yap/Taz (Genevet and Tapon 2011; Boggiano and Fehon 2012; Schroeder and Halder 2012). Thus, to understand the role of βPix in regulating Yap/Taz activity, we sought to determine the effect of loss of βPix expression on Yap/Taz localization in response to cell density. For this, we first used mouse mammary EpH4 cells in which cytoplasmic localization of Yap/Taz occurs upon assembly of the Crumbs complex during epithelial cell polarization (Varelas et al. 2010b). Consistent with previous findings, Yap/Taz was primarily nuclear in sparse cultures, but was predominantly found in the cytoplasm at high density (Figure 2.1C). Notably, abrogation of βPix expression using a pool of four siRNAs, markedly attenuated the cytoplasmic sequestration of Yap/Taz (Figure 2.1C). Analysis of expression of the well-characterized Yap/Taz target genes, Ctgf and Ankrd1, revealed a concomitant upregulation of expression of both genes upon loss of βPix (Figure 2.1D). In mouse mammary NMuMG cells, abrogation of βPix expression also increased expression of the Yap/Taz
target genes, Ctgf, Ankrd1 and Cyr61 (Figure 2.2A). Deconvolving of the βPix siRNAs confirmed that all four individual siRNAs, efficiently reduced βPix expression and concomitantly activated Yap/Taz target gene expression (data not shown). While, βPix is widely expressed, βPix displays a more limited distribution pattern (Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012) and consistent with this, αPix is not expressed in NMuMG cells nor did siRNA-mediated targeting of αPix alter Yap/Taz target gene expression (Figure 2.2A). These results demonstrate that βPIX is important for cell density-dependent activation of the Hippo pathway in mammary epithelial cells.

Changes in the organization of the actin cytoskeleton can regulate the subcellular distribution of Yap/Taz though little is known of the mechanisms that connect dynamic actin cytoskeletal rearrangements to Yap/Taz (Genevet and Tapon 2011; Boggiano and Fehon 2012; Schroeder and Halder 2012). Thus, to examine if βPix is required for regulation of Yap/Taz in response to actin cytoskeleton dynamics, we abrogated the expression of βPix in NMuMG cells and treated them with various actin disrupting agents, including Latrunculin A, which disrupts F-actin, Blebbistatin, which inhibits myosin-II-ATPase and C3, an inhibitor of Rho GTPase. As previously reported, cells plated at low density displayed primarily nuclear Yap/Taz that relocalized to the cytoplasm upon disruption of the actin cytoskeleton (Figure 2.2B). However, in the absence of βPix, Yap/Taz cytoplasmic accumulation was markedly attenuated and concordantly, Yap/Taz target gene expression was enhanced (Figure 2.2B and 2C). Cell detachment/attachment can also regulate the subcellular distribution of Yap/Taz (Zhao et al. 2012). Accordingly, detachment of EpH4 cells obtained from low density cultures followed by a brief re-plating, caused pronounced cytoplasmic sequestration of Yap/Taz, which re-accumulated in the nucleus after 80 min of cell attachment (Figure 2.2D). Abrogation of βPIX expression attenuated the cytosolic sequestration and promoted nuclear retention of Yap/Taz upon cell detachment (Figure 2.2D). These results suggest that βPix plays a key role in mediating the cytoplasmic localization of Yap/Taz during cytoskeletal remodelling and high cell density. We therefore, next asked whether overexpression of βPIX might enhance cytoplasmic localization of Yap/Taz under low density conditions, where Yap is predominantly nuclear. Indeed, transient overexpression of βPIX in low density NMuMG cells led to redistribution of Yap/Taz such that levels of Yap/Taz in nucleus and cytoplasmic were roughly equivalent (Figure 2.2E and quantitated in Figure 2.3A and Figure 2.6A).
**Figure 2.2:** βPix regulates Yap/Taz localization and transcriptional activity during actin cytoskeleton reorganization.

NMuMG (A-C) or EpH4 cells (D) were transfected with control siRNA or siRNA targeting βPix or αPix, as indicated. (A) A representative experiment showing the relative expression of the Yap/Taz target genes Ctgf, Cyr61 and Ankrd1 in NMuMG cells was measured by qPCR and is plotted as the mean +/- the range. (B) NMuMG cells cultured at low density for 48 h were treated with the F-actin inhibitor Latrunculin A (LatA, 0.5 μM), the Rho inhibitor C3 (3 μg/ml) or the non-muscle myosin inhibitor Blebbistatin (Blebb, 50 μM) for 4 h. Cells were fixed and Yap/Taz localization was analyzed by immunofluorescence confocal microscopy. Scale bar, 25 μm (C) A representative experiment showing the relative expression of Yap/Taz target genes in LatA treated NMuMG cells was determined by qPCR and is plotted as the mean +/- the range. (D) EpH4 cells cultured at low cell density for 48 h were trypsinized, kept in suspension for 1 h and then re-plated on fibronectin coated chambers for either 10 or 80 min. Samples were fixed and Yap/Taz localization was analyzed by immunofluorescence confocal microscopy. Scale bar, 15 μm (E, F) βPix is localized in the cytoplasm and promotes Yap/Taz cytoplasmic translocation. (E) NMuMG cells were transfected with Flag-tagged βPIX and endogenous Yap and βPIX localization was analyzed by immunofluorescence microscopy using anti-YAP and anti-Flag antibodies respectively. Scale bar, 15 μm (F) NMuMG cells were treated with DMSO or LatA, nuclear (N) and cytoplasmic (C) fractions were isolated and samples analyzed by immunoblotting. Gapdh and Histone H1 were used as cytoplasmic and nuclear markers, respectively.

Collectively, these results show that βPix promotes increased cytoplasmic localization of Yap/Taz and concomitantly inhibits target gene activation in diverse contexts, including epithelial cell polarization, high cell density and disruption of the actin cytoskeleton.

### 2.4.3 βPix regulates Yap/Taz localization in a GEF-independent manner

To better understand how βPix might modulate Yap/Taz, we first examined the subcellular localization of endogenous βPix in NMuMG cells by subcellular fractionation. βPix was predominantly localized in the cytoplasm both in control cells and cells treated with the actin disrupting agent, Latrunculin A (Figure 2.2F). This is consistent with the strong cytoplasmic localization of transiently overexpressed Flag-βPIX observed by immunofluorescence microscopy.
These results suggest βPix acts in the cytoplasm rather than the nucleus to prevent Yap/Taz nuclear accumulation. Since βPix has GEF activity towards the monomeric RhoGTPases, Cdc42 and Rac1 (Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012), both of which are known modulators of the actin cytoskeleton, we considered whether βPix GEF activity was important for regulating Yap localization. However, overexpression of the GEF-domain double-point mutant (L238R/L239S) that abrogates GEF activity (Manser et al. 1998), drove cytoplasmic localization of Yap/Taz in low density NMuMG cells, similar to overexpressed WT βPIX (Figure 2.3A). We also examined whether abrogation of Cdc42 and Rac1 expression affected Yap/Taz localization and activity. While loss of βPix promoted nuclear Yap/Taz localization and increased target gene expression as above, loss of Cdc42 or Rac1, either individually or together, had no effect (Figure 2.3B and C). These findings are consistent with previous observations indicating that Cdc42/Rac1 are not involved in attachment-mediated control of Yap localization (Zhao et al. 2012) and together indicate that GEF activity is not the means through which βPix controls Yap/Taz.

2.4.4 βPix promotes Hippo-dependent Yap/Taz phosphorylation and interaction with Lats

The Hippo pathway signals through a core kinase cassette comprised of Mst1/2 and Lats1/2 kinases that promote phosphorylation of Yap/Taz, thereby driving Yap/Taz localization to the cytoplasm (Halder and Johnson 2011; Yu and Guan 2013). Therefore, we explored the involvement of the Hippo pathway in βPix-mediated regulation of Yap localization by examining the phosphorylation status of Yap on Ser127, the Lats target site that mediates cytoplasmic sequestration. In EpH4 cells, immunoblotting with a phospho-Ser127 Yap antibody revealed that abrogation of βPix expression reduced the levels of phospho-Yap in cells cultured at either low or high density (Figure 2.4A). In NMuMG cells treated with the actin-disrupting compound, LatA, we observed the expected robust increase in phosphorylation of Yap, as determined by Yap mobility shift in a Phos-Tag gel, and by immunoblotting in a standard gel using the phospho-Ser127 Yap antibody (Figure 2.4B and C). Of note, abrogation of βPix expression decreased phosphorylated Yap both in basal conditions and in the context of LatA. Similar results were obtained with C3 and the ROCK inhibitor, Y27632, for both Yap and Taz (Figure 2.4D). Thus, βPix is required for efficient phosphorylation of Yap/Taz.
**Figure 2.3: βPix functions independent of Rac1 and Cdc42 to regulate Yap/Taz localization.**

(A) βPix GEF activity is dispensable for regulation of Yap/Taz. NMuMG cells were transfected with Flag-tagged wild type or L238R/L239S double mutant version of βPIX (GEFm), which lacks GEF activity. βPix and Yap localization was analyzed by immunofluorescence microscopy. A representative experiment showing Yap localization quantitated from n > 40 cells per condition is plotted. Scale bar, 20 μm. (B, C) NMuMG cells transfected with control siRNA or siRNA targeting βPix, Rac1 or Cdc42 were plated at high cell density. (B) A representative experiment showing the relative expression of Yap/Taz target genes, Ctgf and Cyr61 and the knockdown efficiency of βPix, Rac1 and Cdc42 were determined by qPCR and is plotted as the mean +/- the range. (C) Yap/Taz localization was visualized by immunofluorescence confocal microscopy. Scale bar, 25 μm.
Figure 2.4: βPix regulates Yap phosphorylation.

EpH4 (A) and NMuMG cells (B-D) were transfected with control siRNA or siRNA targeting βPix as indicated. (A) EpH4 cells were cultured at low and high cell densities for 48 h. Cells lysates were subject to immunoblotting to assess Yap phosphorylation using anti-phospho YAP antibodies. (B-D) NMuMG cells were treated with Latrunculin A (B,C), C3 or Y27632 (D) for 4 h. Cell lysates, separated on Phos-Tag or regular gels, were analyzed by immunoblotting using the indicated antibodies. Total levels of YAP, βPix and Gapdh or actin as loading controls, was determined as indicated. (C) The ratio of P-Yap to total Yap in the Phos-Tag gel (B) was quantitated by measuring the intensity of the upper band (P-Yap) over all bands (total Yap). (E) βPix acts upstream of Lats1/2 kinases to regulate Yap/Taz activity. NMuMg cells were transfected with control siRNA or siRNAs targeting Lats1 and Lats2 (siLats1/2) and 24 hours later with Flag-tagged βPIX. Localization of βPIX and Yap/Taz was analyzed by immunofluorescence microscopy. A representative experiment showing Yap localization, quantitated from n > 20 cells per condition is plotted. Scale bar, 20 μm (F) βPIX interacts with LATS1. Lysates from HEK293T cells co-transfected with Flag-tagged βPIX and HA-tagged LATS1 were subjected to anti-Flag immunoprecipitation (α-Flag IP) and the presence of LATS1 was detected by anti-HA immunoblotting. Equivalent protein expression levels were confirmed (Totals).

Our results thus far show that βPix promotes phosphorylation of Yap/Taz on the Lats kinase target site (Halder and Johnson 2011; Yu and Guan 2013). We therefore explored whether Lats is required for βPIX function. Overexpression of βPIX induces cytoplasmic sequestration of Yap/Taz (Figure 2.2E and 2.3A) and as expected, abrogation of Lats1/2 expression using siRNAs enhanced the nuclear accumulation of Yap/Taz (Figure 2.4E). Importantly, loss of Lats1/2 expression prevented the βPIX-induced cytoplasmic accumulation of Yap/Taz, suggesting that βPix functions upstream of Lats kinases to regulate Yap/Taz activity (Figure 2.4E). We next examined whether βPix might directly function at the level of Lats by first testing for physical interaction. Immunoprecipitation of Flag-βPIX revealed that LATS1 interacted with βPIX (Figure 2.4F). Thus, Lats1 interacts with and is required for βPix function towards Yap/Taz.
2.4.5 βPIX binds Lats1 and Yap via an internal domain in the carboxy-terminus

To map the determinants of the interactions between βPIX and LATS1 or YAP, we constructed a series of βPIX deletion mutants and assessed interactions by immunoprecipitation and immunoblotting (Figure 2.5). Deletion of the amino-terminus comprising the SH3 and DH domains (βPIX 272-646), which bind PAK1 or are required for GEF activity, respectively, was dispensable for interaction with YAP or LATS1. This is consistent with our observation that GEF activity is not required to promote cytoplasmic Yap localization (Figure 2.3A). We next explored the C-terminal region and observed that a truncation mutant (construct 1-495) lacking the last 151 amino acids, displayed reduced interaction with both YAP and LATS1. The C-terminal deletion encompasses two regions, a lysine (K)- and glutamate (E)-rich region (KER, amino acids 496-555, that has been reported to mediate interactions with other βPix partners such as Git1 (Flanders et al. 2003; Audebert et al. 2004; Jin et al. 2004; Hoefen and Berk 2006; Chahdi and Sorokin 2008a) or Naa10p (Hua et al. 2011) and is also referred to as the GB (Git-binding) domain, as well as a C-terminal leucine zipper motif (LZ; amino acids 586-646) that is required for βPix dimerization. An internal deletion of the KER that retains an intact LZ (ΔKER; lacking amino acid 586-555) also failed to interact with YAP and LATS1. Thus, the KER is required for interaction between βPIX and both YAP and LATS1. Analysis of the requirement for the LZ was more complex, since this region mediates βPix dimerization and is essential to maintain βPix in the cytoplasm (Kim et al. 2001). Interestingly, while the LZ deletion mutant (1-585) retained interaction with YAP, both of which are localized to the nucleus (see Figure 2.6A), the interaction with LATS1 was disrupted. Thus, for YAP interaction, neither the LZ or βPIX dimerization per se is required. In the case of LATS1, the βPIX KER-domain only deletion restored cytoplasmic βPIX, but still failed to interact with LATS1. These findings suggest that loss of interaction between the LZ mutant and LATS1 is secondary to their nuclear versus cytoplasmic compartmentalization. Thus, a region (KER) in α/βPIX, rich in charged residues including Lys and Glu, is required for interaction with both YAP and LATS. As βPix typically exists as either a dimer, or even a trimer in vivo (Schlenker and Rütinger 2009), our findings are compatible with the notion that βPix can simultaneously recruit both Yap and Lats into a multimeric complex.
Figure 2.5: C-terminal region of βPIX is required for binding to YAP and LATS.

(A) A schematic depicting the different βPIX cDNA constructs used for mapping interactions is shown. Positive or negative interactions with YAP and LATS1 are indicated on the right. (B, C) HEK293T cells were co-transfected with wild-type or mutant constructs of Flag-βPIX along with HA-YAP (B) or HA-LATS1 (C). Cell lysates were subject to anti-Flag IP and the presence of YAP or LATS1 was determined by anti-HA immunoblotting. A dashed line on blots indicates removal of a sample lane. (D, E) Quantitation of βPIX interaction mapping from replicate experiments YAP (D), n = 3, and LATS1 (E), n = 2 is plotted.

2.4.6 Disruption of Lats/Yap binding to βPix prevents cytoplasmic sequestration of Yap

We next examined the ability of βPIX mutants to regulate Taz/Yap localization. For this, NMuMG cells were transiently transfected with the Flag-tagged βPIX constructs and Yap/Taz localization was examined by immunofluorescence microscopy. As noted above, while the majority of control cells not expressing WT βPIX had nuclear Yap/Taz, cells overexpressing either WT βPIX, or the amino terminal deletion mutant lacking the PAK binding (SH3) and GEF (DH) domains, displayed marked enrichment in cytoplasmic Yap/Taz (Figure 2.6A). As previously reported (Kim et al. 2001), the LZ mutant is primarily localized to the nucleus and did not alter the nuclear localization of YAP. In contrast, the βPIX mutant lacking the KER (ΔKER, amino acids 496-555) was localized in the cytoplasm like the WT βPIX, but failed to promote Yap/Taz cytoplasmic sequestration. Since the KER deletion mutant fails to associate with either Yap or Lats, and the LZ mutant, which binds only Yap, both fail to drive cytoplasmic Yap sequestration, these results indicate that the interaction of βPIX with both Yap and Lats is required for control of Taz/Yap localization.

The ability of βPix to bind both Yap and Lats1, suggests that βPix may act to scaffold to promote the association of Lats1 with its substrate, Yap. To test this possibility, we examined the effect of βPIX on the interaction between YAP and LATS1. We observed that overexpression of WT βPIX, but not a βPIX mutant lacking the KER domain, enhanced the interaction of LATS1 with YAP as determined by YAP immunoprecipitation followed by LATS1 immunoblotting (Figure 2.6B).
Figure 2.6: C-terminal region of βPIX is important for regulating Yap localization.

(A) NMuMG cells were transfected with the indicated Flag-βPIX WT or mutant constructs. Localization of endogenous Yap along with expressed Flag-βPIX constructs was determined by immunofluorescence confocal microscopy using anti-Yap and anti-Flag antibodies, respectively. Quantification of the percentage of cells with the indicated patterns of Yap localization from a representative experiment with n > 30 per condition is shown. Scale bar, 20 μm (B) βPIX enhances YAP and LATS1 interaction. HEK293T cells co-transfected with Flag-tagged YAP and HA-tagged LATS1 in the presence or absence of HA-tagged WT or ΔKER mutant βPIX were lysed and subject to α-Flag IP. The interaction between YAP and LATS1 was determined by immunoblotting using an anti-HA antibody. (C) βPix knockdown reduces the strength of interaction between Yap and Lats1. EpH4 cells were transfected with either siCTRL or a pool and a single siβPix. After 48 h, cells were lysed and subject to immunoprecipitation using either α-IgG or α-Yap antibodies. The strength of interaction between Yap and Lats1 was determined by immunoblotting using anti-Lats1 antibody. Equivalent protein expression levels and βPix knockdown efficiency was confirmed by immunoblotting (Totals).

In line with a scaffolding function for βPix, abrogation of the expression of βPix using either a pool or a single siRNA, dramatically reduced the interaction of endogenous Yap with Lats1 in EpH4 cells (Figure 2.6C). Altogether, these results indicate that βPix can act as a scaffold to promote the interaction of Lats with its substrate, Yap/Taz.

2.4.7 βPIX attenuates the tumorigenic properties of MDA-MB-231 breast cancer cells

Studies in cells, mice and human tumor samples indicate that Yap/Taz display oncogenic activities, whereas the Hippo pathway, which restrains nuclear Yap/Taz, is tumor suppressive (Harvey et al. 2013). Tumor cells have thus acquired the ability to bypass the Hippo pathway thereby permitting the emergence of the pro-tumorigenic Yap/Taz-mediated transcriptional program.
Figure 2.7: YAP and TAZ promote cell proliferation and migration in MDA-MB-231 cells.

Cells were transfected with control siRNA or siRNA targeting YAP or TAZ (WWTR1) and the effect on cell proliferation or migration was determined by a SRB or wound healing assay, respectively. Knockdown efficiency was confirmed by qPCR. A representative experiment is shown.
Indeed, it has been shown that overexpression of Yap/Taz promotes proliferation, migration and tumor initiation in breast cancer cells, while loss of Taz inhibits tumor formation (Chan et al. 2008; Lei et al. 2008b; Zhao et al. 2008; Cordenonsi et al. 2011; Lamar et al. 2012; Harvey et al. 2013; Serrano et al. 2013; Hiemer et al. 2014; Mi et al. 2014; Sorrentino et al. 2014). Consistent with this, we confirmed that loss of YAP or TAZ in the triple-negative breast cancer cell line, MDA-MB-231 decreased the rate of cell migration and proliferation (Figure 2.7). Thus we used these cells to generate clones stably overexpressing βPIX to determine whether βPix might re-couple the Hippo pathway to YAP/TAZ in a cancer context. Analysis of YAP/TAZ localization showed that in control MDA-MB-231 cell clones, YAP/TAZ were predominantly nuclear, while in two independently-derived βPIX over-expressing clones, abundant cytoplasmic YAP/TAZ was detected, with some cells displaying nuclear exclusion (Figure 2.8A). A concomitant reduction in YAP/TAZ target gene expression was also observed in the βPIX-expressing clones (Figure 2.8B). Thus, increased expression of βPIX inhibits the nuclear localization and transcriptional activity of YAP/TAZ in these breast cancer cells.

We next determined how ectopic βPIX expression affected properties typically associated with tumorigenesis. Clones overexpressing βPIX, displayed a marked attenuation of cell migration in an in vitro wound healing scratch assay (Figure 2.8C) as well as a decreased rate of cell proliferation (Figure 2.8D). Thus, expression of βPIX, which enhances the cytoplasmic localization of YAP/TAZ results responses characteristic of tumor suppressive activity, in a manner that parallels that of loss of YAP/TAZ.

Although YAP/TAZ are predominantly nuclear, MDA-MB-231 cells nevertheless, retain some LATS activity as siRNA-mediated depletion of LATS1/2 resulted in a more pronounced nuclear accumulation of YAP/TAZ and enhanced YAP/TAZ target gene expression (Figure 2.8E and F). Thus, while upstream signals are disconnected from the core Hippo kinases, LATS1/2 are expressed and are able to limit YAP/TAZ activity. We thus examined the effect of loss of LATS1/2 on YAP/TAZ localization in the βPIX-expressing clones and observed that siLATS1/2 overcame the effects of βPIX and resulted in strong nuclear localization of YAP/TAZ (Figure 2.8E and F) and enhanced expression of ANKRD1 (Figure 2.8F).
Figure 2.8: Effect of βPix expression on tumorigenic properties of MDA-MB-231 breast cancer cells.

(A) YAP is mainly cytoplasmic in MDA-MB-231 cells stably expressing βPIX. Parental MDA-MB-231 cells or MDA-MB-231 cells stably expressing Flag-βPIX or empty vector were fixed and YAP localization was visualized by immunofluorescence microscopy. Scale bar, 25 μm (B) A representative experiment showing the expression levels of βPIX and YAP/TAZ target genes, CTGF and ANKRD1, were determined by qPCR and is plotted as the mean +/- the range. (C) βPIX regulates cell migration. A wound was introduced in a confluent monolayer of MDA-MB-231 cells stably expressing either control vector or βPIX and the migration of the cells into the wound was assessed by live-cell phase contrast microscopy. Quantitation of cell migration, determined by measuring the cell free area within the wound, of three independent experiments, n=4, is plotted as the mean +/- S.E.M. Scale bar, 120 μm (D) βPIX attenuates cell proliferation. An SRB assay was used to measure cell proliferation. Data is plotted as the mean +/- S.E.M. of 6 independent experiments, n = 5. (E, F) LATS1/2 kinases are required for βPIX mediated YAP/TAZ inactivation. (E) MDA-MB-231 cells stably expressing control vector or βPIX were transfected with siCTL or siLATS1/2 and YAP localization was analyzed by immunofluorescence microscopy. Scale bar, 25 μm (F) Expression level of YAP target gene, ANKRD1, was determined by qPCR and is plotted as the relative expression in siLATS1/2 over siControl (CTL).

Thus, βPIX-mediated regulation of YAP/TAZ is dependent on the Hippo pathway kinase, LATS. Taken together, our findings show that enhanced expression of βPIX can re-engage the Hippo core kinases, LATS1/2 and that βPIX thereby functions as a tumor suppressor to restrain the pro-oncogenic properties of YAP/TAZ in metastatic breast cancer cells.
2.5 Discussion

The Hippo pathway has been established as a key regulator of tissue growth and cell fate and disruption of the pathway promotes tumorigenic processes such as cell migration and proliferation (Halder and Johnson 2011; Ramos and Camargo 2012; Harvey et al. 2013; Yu and Guan 2013). Several upstream mediators, including determinants of apical-basal polarity, mechanical forces acting through the actin cytoskeleton and G-protein coupled receptors have emerged as pathway activators (Genevet and Tapon 2011; Boggiano and Fehon 2012; Schroeder and Halder 2012). How these signals are transduced to the core Mst/Lats kinase cassette is the subject of intense investigation. Ultimately, however, activation of the Hippo pathway results in Lats kinase-mediated phosphorylation of Yap/Taz, which then drives Yap/Taz localization to the cytoplasm. Here, we identify βPix as a key positive regulator of the Hippo kinase. Mechanistically, we demonstrate that βPix binds both Lats and its substrate target, Yap and that loss of βPIX impairs the interaction between Lats and Yap/Taz, resulting in decreased Yap/Taz phosphorylation. Moreover, we show that increased expression of βPIX in cancer cells can restore cytoplasmic localization of Yap/Taz in a Lats-dependent manner. Altogether our findings suggest a model in which βPix promotes Hippo pathway activity by scaffolding Lats to its Yap/Taz substrates to stimulate phosphorylation and localization to the cytoplasm (Figure 2.9).

Signals emanating from polarity determinants, such as the Crumbs/Amot complex, processes such as mechanotransduction that act through the actin cytoskeleton and mediators of cell-density sensing, all can control Yap localization (Genevet and Tapon 2011; Boggiano and Fehon 2012; Schroeder and Halder 2012). In general, these signals flow to the Mst/Lats kinase cassette, and while Mst1/2-independent Yap phosphorylation has been reported (Yu et al. 2012b; Zhao et al. 2012; Kim et al. 2013; Yu et al. 2013), there appears to be a more ubiquitous requirement for Lats. Our data demonstrates that βPix functions at the level of Lats, consistent with the notion that βPix acts in the Hippo pathway downstream of multiple cues. Accordingly, we observed a requirement for βPix in regulating Yap activity in response to cell-cell contact and cell density, actin cytoskeleton disruption and in attachment/detachment events. In all the cases, loss of βPix expression, resulted in retention of Yap/Taz in the nucleus and continued activation of a Yap/Taz transcriptional program. In solid tumors, YAP and/or TAZ are frequently overexpressed and unlike normal cells, most cancer cells have acquired the means to bypass Hippo-dependent regulation.
Figure 2.9: Model of the mechanism for βPix function in Hippo signalling.

The Hippo pathway is activated by upstream signals such as high cell density and actin cytoskeleton remodeling. βPix, which exists as a dimer or a trimer in the cytoplasm, forms a multiprotein complex with both Lats and Yap, acting as a scaffold to promote phosphorylation Yap/Taz by Lats and resulting in cytoplasmic accumulation of Yap/Taz.
YAP/TAZ become constitutively nuclear and act to promote the tumorigenic phenotype (Harvey et al. 2013). In breast cancer cells for example, overexpression of TAZ and YAP promotes proliferation, migration, invasion, epithelial–mesenchymal transition (EMT), acquisition of cancer stem cell (CSC) properties and sustains CSC self-renewal (Chan et al. 2008; Lei et al. 2008b; Zhao et al. 2008; Cordenonsi et al. 2011; Lamar et al. 2012; Harvey et al. 2013; Hiemer et al. 2014). In MDA-MB-231 cells, the hippo pathway is inactive, possibly through loss of the upstream component NF2 (Dupont et al. 2011) resulting in a pronounced nuclear accumulation of YAP/TAZ. Remarkably, we found that ectopic expression of βPIX alone in MDA-MB-231 cells was sufficient to restrain YAP/TAZ activity and yielded a concomitant suppression of cell proliferation and cell migration. This activity of βPIX was dependent on the presence of LATS1/2. Thus, we uncovered a tumor suppressor function for βPix that acts via recoupling of the Hippo kinase cassette to its Yap/Taz substrates. Given that these cells lack NF2 (Dupont et al. 2011), these findings also suggest the intriguing possibility that βPix might function to link NF2 to the hippo kinase cassette.

Our interaction mapping studies revealed that Yap and Lats both bind to an approximately 50 amino acid region located just upstream of the carboxy-terminus that we termed the KER and which is highly conserved in both αPix and βPix. This region does not contain any recognizable protein-protein interaction motifs, but rather is rich in charged amino acids including Lys and Glu. Nevertheless, this region also mediates interactions with other proteins such as Git1 (Hoefen and Berk 2006; Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012). We showed that loss of βPix attenuated the interaction of Lats1 with Yap while overexpression of βPIX enhanced association in a KER domain-dependent manner. βPix was first characterized as a dimer, although a more recent study suggests that trimers may be the more typical state (Schlenker and Rittinger 2009). Taken together, we speculate that simultaneous binding of Yap and Lats to individual βPix proteins within the context of a multimerized complex provides the scaffolding function and allows for enhanced Yap phosphorylation (Figure 2.9). In this study, we focused on βPix, since abrogation of βPix expression alone was sufficient to inhibit Hippo pathway activity in our cell models. This is consistent with a more ubiquitous expression pattern for βPix, as compared to βPix (Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012). However, the KER region is highly conserved and also mediates βPix binding to Yap/Taz and Lats. Interestingly, mutations in αPIX (ARHGEF6) have been associated with X-linked intellectual disability in humans.
and αPix/Arhge6-deficient mice display alterations in synaptic and immune system function (Missy et al. 2008; Ramakers et al. 2012) whereas βPix mutants display early embryonic lethality (Missy et al. 2008). Thus, it will be important to determine whether αPix might also modulate Hippo signalling in these distinct contexts.

αPix and βPix have been most studied for their function as Guanine nucleotide Exchange Factors (GEFs) for the RhoGTPases, Cdc42 and Rac1, although the mechanisms whereby GEF activity is controlled remain unclear (Rosenberger and Kutsche 2006; Staruschenko and Sorokin 2012). We showed that loss of Cdc42, Rac1 or both, has no effect on Yap/Taz subcellular localization or target gene activation, suggesting that GEF activity is not required for βPix function in the Hippo pathway. Consistent with this, a βPIX construct harboring point mutations in the GEF domain that prevent guanine nucleotide exchange (Manser et al. 1998) still functioned to sequester Yap/Taz in the cytoplasm. βPix has also been studied as a binding partner for p21-activating kinase 1, PAK1 (Chan and Manser 2012), and can stimulate PAK, via the GEF activity. Although we did not directly test whether PAK activity alters Yap/Taz function, deletion of the SH3 domain of βPIX, which mediates binding to PAK1, was not required to promote Yap localization to the cytoplasm. Thus, our findings suggest that the well-characterized role of βPix in complex with Cdc42/Rac1 and PAK1 is molecularly distinct from its promotion of Hippo pathway activity. Instead, our studies demonstrate that βPix functions in the Hippo pathway by scaffolding Yap and Lats. A general role for βPix as a scaffold is suggested by the ability of βPix to bind a diverse array of proteins, some of which form large macromolecular assemblies, to control cellular processes such as focal adhesion formation and function, cell migration or G-protein coupled receptor signalling. For instance, βPix can bind to 14-3-3 proteins, p66Shc, Scribble, Cbl and the multi-domain containing Git1 (Flanders et al. 2003; Audebert et al. 2004; Jin et al. 2004; Hoefen and Berk 2006; Chahdi and Sorokin 2008a). Although mechanistic understanding of how βPix functions in so many processes is not well understood, the ability of βPix to engage in diverse protein complexes indicates that scaffolding functions for βPix are likely to be widespread. Whether any of the known βPix interactors might co-operate in regulating the Hippo pathway is an interesting area for future investigations.
Chapter 3
MARK4 inhibits Hippo signalling to promote proliferation and cell migration of breast cancer cells

The work presented in this chapter was published in:


Data attribution:
I designed and performed experiments related to the mechanistic investigation of MARKs in the Hippo signalling, including the effect of MARK4/MARK3 deletion on YAP/TAZ subcellular localization, phosphorylation and target gene expression in addition to biochemical studies examining MST-SAV phosphorylation by MARK4 and protein-protein interaction experiments. I also generated MDA-MB-231 cells stably expressing wild-type or S89A-mutant versions of TAZ. I produced the data presented in:
Figure 3.1E; 3.2C; 3.3A, B, D, E; 3.4A, D, 3.5; 3.7B, F; 3.8; 3.9 and 3.10

Sharfee Shiban performed the TEAD-reporter screen and produced the data presented in:
Figure 3.1A, B, D; 3.2A, B; 3.3C and 3.7A

Siyuan Song generated MARK4 CRISPR-knockout clones, performed rescue experiment and examined the effect of MARK4 deletion on proliferation and migration of MDA-MB-231 cells. I assisted in analyzing the data. Siyuan produced the data presented in:
Figure 3.1C; 3.4B and C; 3.6 and 3.11
3 MARK4 inhibits Hippo signalling to promote proliferation and cell migration of breast cancer cells

3.1 Abstract

The Hippo pathway is a critical regulator of tissue size and aberrations in pathway regulation leads to cancer. MST1/2 and LATS1/2 kinases comprise the core of the pathway that in association with adaptor proteins SAV and MOB, function in a sequential manner to phosphorylate and inhibit the transcription factors, YAP and TAZ. Here we identify mammalian MARK family members as activators of YAP/TAZ. We show that depletion of MARK4 in MDA-MB-231 breast cancer cells results in loss of nuclear YAP/TAZ and decreases expression of YAP/TAZ target genes. Mechanistically, we demonstrate that MARK4 can bind to and phosphorylate MST and SAV and that MARK4 expression attenuates formation of a complex between MST/SAV and LATS in a manner that is dependent on MARK4 kinase activity. Abrogation of MARK4 expression using siRNAs and CRISPR/Cas9 gene editing attenuates proliferation and migration of MDA-MB-231 cells. Our results thus demonstrate that MARK4 acts as negative regulator of the Hippo kinase cassette to promote YAP/TAZ activity and that loss of MARK4 restrains the tumorigenic properties of breast cancer cells.
3.2 Introduction

The size of an organ in multicellular organisms is determined by an intricate balance of cell proliferation, apoptosis, stem cell self-renewal and differentiation. Intrinsic and extrinsic factors tightly regulate these processes, which are essential for normal embryonic development and tissue homeostasis, and aberrations in their regulation lead to organ growth defects or tumorigenesis. The Hippo pathway has emerged as a major signalling pathway with evolutionarily conserved roles in regulating tissue growth and organ size (Pan 2010; Halder and Johnson 2011; Ramos and Camargo 2012; Piccolo et al. 2014; Varelas 2014; Meng et al. 2016). The major effectors of the pathway are the related transcriptional co-activators, YAP and TAZ, which in association with various transcription factors such as TEADs, induce a growth promoting gene expression program to regulate cell proliferation, apoptosis, self-renewal and differentiation (Pan 2010; Halder and Johnson 2011; Ramos and Camargo 2012; Piccolo et al. 2014; Varelas 2014; Meng et al. 2016).

The core of the Hippo pathway in vertebrates is composed of the conserved Ser/Thr kinases, MST1/2 and LATS1/2 that associate with the adaptor proteins, SAV and MOB, to restrict YAP/TAZ transcriptional activity. Specifically, MST1/2 phosphorylates and activates LATS1/2, which in turn directly phosphorylates YAP/TAZ to promote cytoplasmic sequestration and subsequent degradation through the proteasomal pathway (Pan 2010; Halder and Johnson 2011; Ramos and Camargo 2012; Piccolo et al. 2014; Varelas 2014; Meng et al. 2016). Although this kinase cascade is considered as the core of the Hippo pathway, multiple upstream regulators can act at many levels to modulate YAP/TAZ activity (Irvine 2012; Piccolo et al. 2014; Meng et al. 2016). Initial studies on Hippo pathway regulation mainly focused on the role of intrinsic factors such as cell polarity, junctional complexes and changes in cell shape and size as major determinants of Hippo-YAP activity (Polesello et al. 2006; Harvey and Tapon 2007; Matallanas et al. 2007; Sopko and McNeill 2009; Dupont et al. 2011; Genevet and Tapon 2011; Wada et al. 2011; Schroeder and Halder 2012). However, it is now clear that the Hippo pathway also responds to environmental cues such as G-protein coupled receptors, nutrient availability particularly glucose, and metabolic pathways (Yu et al. 2012a; DeRan et al. 2014; Sorrentino et al. 2014; Enzo et al. 2015; Mo et al. 2015; Wang et al. 2015; Santinon et al. 2016).

Given the growth promoting effects of YAP/TAZ, disruptions of YAP/TAZ activity are commonly associated with pathological conditions, especially cancer (Harvey and Tapon 2007; Pan 2010; Harvey et al. 2013; Yu et al. 2015). Diverse genetic mouse models have shown that loss of Hippo
core components such as MST1/2 or SAV that restrict YAP/TAZ activity, lead to overgrowth phenotypes and ultimately cancer (Zhou et al. 2009; Lee et al. 2010; Lu et al. 2010; Zhang et al. 2010; Zhou et al. 2011). Furthermore in vitro and in vivo models of tumorigenesis have shown that increased expression of YAP and TAZ is sufficient to transform normal epithelial cells, induce epithelial-mesenchymal transition, cooperate with other proto-oncogenes to bypass oncogene addiction and increase cancer stem cell content of tumors (Harvey and Tapon 2007; Pan 2010; Harvey et al. 2013; Yu et al. 2015). Hence, a better understanding of the modulators of YAP/TAZ activity is crucial for understanding tumorigenesis.

Previously, we used a LUMIER-based protein interaction screen (Barrios-Rodiles et al. 2005; Miller et al. 2009; Varelas et al. 2010a), and identified βPIX, as a novel upstream regulator of the Hippo pathway (Heidary Arash et al. 2014). Here, we complemented this physical map, with a functional cDNA overexpression screen using a TEAD-luciferase reporter to identify genes that modulate YAP/TAZ transcriptional activity. We identified MAP/microtubule affinity regulated kinase (MARK) family members as potent activators of YAP/TAZ activity. MARKs were originally identified based on their ability to phosphorylate microtubule regulating proteins Tau and MAPs (Drewes et al. 1997). They belong to the larger AMPK family that includes AMPK, the master regulator of cellular energy balance (Tassan and Goff 2004; Hurov and Piwnica-Worms 2007; Shackelford and Shaw 2009; Mihaylova and Shaw 2011). Several AMPK family members have recently been shown to be important regulators of Hippo pathway (Wehr et al. 2013; DeRan et al. 2014; Mo et al. 2015; Wang et al. 2015). MARK1-4 are the mammalian orthologs of the Drosophila Par-1 kinase, and have evolutionarily conserved roles in embryonic development, asymmetric cell division, and cell polarity regulation (Tassan and Goff 2004; Hurov and Piwnica-Worms 2007; Naz et al. 2013; Dumont et al. 2015).

Here we show that MARK family members activate a YAP/TAZ responsive luciferase reporter, and concordantly, that MARK4 deletion in breast cancer cells leads to loss of nuclear YAP/TAZ and inhibits activation of YAP/TAZ target genes. Furthermore, we show that abrogation of MARK4 expression either by siRNAs or CRISPR/Cas9 mediated knockout attenuates the tumorigenic properties of breast cancer cells including cell proliferation and cell migration. Mechanistically, we show that MARK4 binds to the Hippo core components MST1/2 and SAV and subsequently phosphorylates both. Phosphorylation of MST1/2 and SAV by MARK4 leads to
disruption of complex formation between MST/SAV and their downstream targets, LATS kinases, hence blocking YAP/TAZ inactivation by the Hippo kinase cassette.
3.3 Materials and Methods

3.3.1 TEAD-luciferase Reporter Screen

The TEAD-reporter construct is comprised of tandem TEAD binding sites fused to Firefly luciferase as previously described (Couzens et al. 2013). HEK293T cells were seeded on poly-L-lysine coated 96-well dishes at 70% confluency. Cells were transfected with 50 ng TEAD-reporter, 25 ng of β-galactosidase and 100 ng of Flag-tagged cDNA constructs using Lipofectamine 2000 according to the manufacturer’s instructions. Cells were lysed 48 h after transfection in 100 μl of lysis buffer (25 mM Tris, 2 mM DTT, 2 mM DCTA, 10% glycerol, 1% Triton X-100). Aliquots of the cell lysates were used to read luciferase and β-galactosidase activity using a PerkinElmer Envision Xcite multilabel reader. Luciferase activities were normalized to β-galactosidase activity and fold over run median was determined.

3.3.2 Cell Culture and Transfection

For cell culturing, HEK293T and 293 T-REx cells were grown in DMEM with 10% FBS and MDA-MB-231 cells in RPMI with 5% FBS. Cells were transfected with Dharmacon siGENOME pools of four individual siRNAs (Thermo Scientific) using Lipofectamine RNAiMAX (Life Technologies) or with cDNAs, using Lipofectamine 2000, Lipofectamine LTX or Lipofectamine 3000.

3.3.3 Plasmids and Stable Cell Lines

The MARK construct was generated by PCR using MARK4 isoform 2 (NM_031417) as template and tagged with Flag or HA in either pCMV5 or Gateway destination vectors as specified below. Flag- or HA- tagged constructs for LATS1, MST1, MST2, SAV1, TAZ (WT) and TAZ (S89A) were previously described (Varelas et al. 2010a). For MDA-MB-231 cells stably expressing TAZ (WT or S89A), HA-TAZ was subcloned into pCAG-ires-puro vector. The Dox-inducible Flag-MARK4 expressing stable cell line was generated using Flp-In 293 T-REx cells as previously described (Couzens et al. 2013). Briefly, human MARK4 was subcloned into Triple FLAG Destination vector using Gateway Technology (Thermo Fisher Scientific) and 48 h after transfection, Flp-In 293 T-REx cells were cultured in selection media (DMEM with 10% FBS...
supplemented with 200 μg/ml hygromycin) to generate stable cell lines. Expression of Flag-MARK4 was induced by overnight Doxycycline (1 μg/ml) treatment prior to analysis by immunoblotting or immunofluorescence confocal microscopy.

The CRISPR design tool (Hsu et al. 2013) [54] was used to design MARK4 targeting guide RNAs that were cloned into the px459 plasmid (Addgene #48139 (Ran et al. 2013)). Individual MDA-MB-231 cell clones were selected using media containing 1 μg/ml puromycin and loss of MARK4 protein expression was confirmed by immunoblotting.

3.3.4 Immunoblotting, Immunoprecipitation and in vitro Kinase Assay

Cells were lysed in lysis buffer (50 mM tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X100, 1 mM DTT containing phosphatase and protease inhibitors). Lysates were separated on SDS-PAGE gels and immunoblotting was performed using standard protocols as previously described (Heidary Arash et al. 2014). For subcellular fractionation experiments, cells were scraped prior to lysis using NE-PER Nuclear and Cytoplasmic Reagents (ThermoFisher Scientific #78833). Phos-Tag gels, using reagents purchased from Waco Chemicals, were prepared according to manufacturer’s instructions. For immunoprecipitations, cell lysates were subject to immunoprecipitation with anti-Flag or target specific antibodies as indicated and proteins collected using protein G-Sepharose prior to analyses by immunoblotting. LUMIER experiments were performed as previously described (Barrios-Rodiles et al. 2005; Miller et al. 2009; Varelas et al. 2010a). Briefly, HEK293T cells were transfected with firefly luciferase tagged bait and Flag-tagged prey constructs and were lysed and lysates subjected to anti-Flag immunoprecipitation. Luciferase activity from the immunoprecipitates and in aliquots of total cell lysates was measured and normalized to β-galactosidase readings. For in vitro kinase assays, immunoprecipitates were washed three times with kinase assay buffer (100 mM HEBES, 100 mM NaCl, 5 mM MgCl₂) and then incubated with 0.25 mM DTT, 250 μM ATP and 200 ng GST-tagged MARK4 protein purified from Sf9 cells (Sigma #SRP5046) for 30 min in 30°C. The antibodies used were: MST1 (Cell Signaling #3682); MST1/2 (Bethyl Laboratories #A300-468A); pMST1/2(Thr10/183) (Cell Signaling #3681); MARK4 (Cell Signaling #4834); MARK3 (Cell Signaling #9311); YAP (Cell Signaling #4912); pYAP (Ser127) (D9W2I; Cell Signaling #13008); pYAP (Ser397) (D1E7Y; Cell Signaling #13619); pMOB1 (Thr35) (D2F10; Cell Signaling #8699); TAZ (BD Pharmingen #560235); LATS1 (C66B5; Cell Signaling #3477); pLATS1 (Thr1079) (D57D3; Cell Signaling
#8654); rat anti-HA (Roche #1867423); GST (91G1, Cell Signaling #2625); and anti-Flag M2 (Sigma-Aldrich #F1804).

3.3.5 Immunofluorescence Confocal Microscopy

Cells were plated in 4-well Lab-Tek chambers (#154526), fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X100 in PBS and blocked in 2% BSA-PBS prior to addition of antibodies. Primary antibodies used were mouse anti-YAP (Santa Cruz #sc-101199, 1:300); mouse anti-TAZ (BD Pharmingen #560235, 1:300); rat anti-HA (Roche #1867423); or rabbit anti-Flag (Sigma #F7425, 1:500) and secondary antibodies were goat anti-rabbit Alexa-fluor 488 (Life Technologies #A11035, 1:1000); goat anti-rat Alexa-Fluor 488 (Life Technologies #A11006, 1:1000); or goat-anti-mouse Alexa Fluor 546 (Life Technologies #A11029, 1:1000) in 2% BSA-PBS. Cell nuclei were visualized by DAPI staining. Images were captured using a spinning disk confocal scanner (CSU10, Yokogawa) on Leica DMI6000B microscope and Volocity software was used for image acquisition and processing. For quantification of YAP localization, a minimum of 30 transfected cells were counted and nuclear/cytoplasmic localization of YAP was evaluated in transfected cells compared to the surrounding non-transfected cells.

3.3.6 Quantitative Real-Time PCR

Total RNA was purified using PureLink RNA Mini Kit (Life Technologies) and cDNA was synthesized using 1 μg of purified RNA using Oligo-dT primers and M-MLV Reverse Transcriptase (Invitrogen #28025-013). Real-Time PCR was performed using the SYBR Green master mix (Applied Biosystems) on the ABI Prism 7900 HT system (Applied Biosystems). Relative gene expression was quantified by ΔΔCt method and normalized to HPRT. The sequence of the primers used for qPCR is this study are listed below:

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER NAME</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANKRD1</td>
<td>ANKRD1 FOR</td>
<td>AGTAGAGGAACTGGTGACTGG</td>
</tr>
<tr>
<td></td>
<td>ANKRD1 REV</td>
<td>TGGGCTAGAAGTGCTCTAGAT</td>
</tr>
<tr>
<td>CTGF</td>
<td>CTGF FOR</td>
<td>AGGAGTGGGTTGGTGACGGA</td>
</tr>
<tr>
<td></td>
<td>CTGF REV</td>
<td>CCAGGCAGTTGGCTCTAATCC</td>
</tr>
<tr>
<td>CYR61</td>
<td>CYR61 FOR</td>
<td>CGAGGTGGAGTTGACGAGAAA</td>
</tr>
<tr>
<td></td>
<td>CYR61 REV</td>
<td>CTTTGAGCAGCGTGAGCAGAT</td>
</tr>
</tbody>
</table>
3.3.7 Wound Healing and Cell Growth Assays

For the wound healing migration assay, cells were seeded in a 6-well plate and were grown overnight to confluency. The wound was introduced by scraping with a sterile 200 μl pipette tip and the progress of wound closure was monitored by microphotographs at 10x magnification taken with Zeiss inverted microscope equipped with a CCD camera (Hamamatsu Photonic Systems). The unfilled area was quantified by Volocity at different time points. Cell growth was determined using the Sulforhodamine B (SRB) assay. For this, cells transfected with siRNAs were plated in 96-well dishes, fixed at varying times with 10% (w/v) trichloroacetic acid and then stained as previously described (Bao et al. 2012).

3.3.8 Statistical Analysis

For statistical tests performed, \( p \)-values were calculated using Student’s two-tailed \( t \)-test. The number of replicates and independent experiments are indicated in figure legends. Sample size was not predetermined using a statistical method.
3.4 Results

3.4.1 Identification of MARK4 as a regulator of YAP/TAZ activity

To identify novel Hippo pathway modulators, we undertook a functional screen that examined the effect of cDNA overexpression on transcriptional outcome using a YAP/TAZ-dependent transcriptional reporter, TEAD-luciferase, which harbors multiple TEAD binding sites located upstream of Firefly luciferase (Couzens et al. 2013). HEK293T cells were transfected with cDNAs from an augmented version of the previously described libraries (Miller et al. 2009; Varelas et al. 2010a), that encode Flag-tagged mouse and/or human proteins comprised of diverse signalling-associated domains (Figure 3.1A). TEAD reporter activity in cells transfected with each cDNA was determined by measuring luciferase activity, normalized for transfection efficiency with a coexpressed β-galactosidase reporter gene. Comparisons of duplicate runs revealed excellent correlation (Figure 3.1B) and identified both known positive regulators, such as YAP and TAZ as well as negative regulators, such as LATS2. Among the top hits that enhanced TEAD-luciferase transcriptional activity, were three members of the Microtubule-affinity regulating kinases (MARK) family, MARK2, 3 and 4. We confirmed that transient overexpression of MARK2, 3 and 4 potently activated YAP/TAZ transcriptional reporter activity (Figure 3.1C). YAP/TAZ regulate the expression of diverse target genes, thus as a complement to overexpression using a transcriptional reporter, we next examined the effect of the loss of expression of MARKs on endogenous target genes. For this, we used the triple negative breast cancer cell line, MDA-MB-231, which displays constitutive TAZ/YAP activity (Cordenonsi et al. 2011; Heidary Arash et al. 2014). Abrogation of expression of MARK4 using a pool of siRNAs (Figure 3.1D) or 3 out of 4 individual siRNAs (Figure 3.2A), markedly attenuated the expression of the well-characterized TAZ/YAP target genes, ANKRD1 and CTGF while loss of expression of MARK2 or MARK3 had little or no effect on gene expression in these cells (Figure 3.2B). A similar reduction of ANKRD1 expression by siMARK4 was also observed in MCF10A breast cancer cells and in two colorectal cancer cell lines, DLD-1 and SW480 (Figure 3.1E). Of note, in MDA-MB-468 cells, loss of MARK3 attenuated expression of ANKRD1 and CTGF suggesting the existence of redundant, context-dependent activities for distinct MARK family members (Figure 3.2C). Here, we focused on MARK4 for further study.
**Figure 3.1: Identification of MARK family members as activators of YAP/TAZ.**

(A) A TEAD-luciferase reporter assay was designed to identify modulators of YAP/TAZ transcriptional activity upon overexpression. (B) Luminescence intensities from the TEAD-luciferase reporter screen were normalized to β-gal. Comparison of fold over median values from two runs is shown as a scatterplot. (C) MARK family members activate the TEAD-luciferase reporter. Flag-tagged wild-type MARK1, 2, 3 and 4 and kinase-dead (KD) MARK4 were co-transfected with wild-type and mutant TEAD-luciferase reporter constructs. Transcriptional activity was measured by luciferase assay and data are shown as the mean +/-SD of triplicate samples from a representative experiment performed four times. (D-E) MARK4 loss of function decreases YAP/TAZ target gene expression. Cell lines, as indicated, were transfected with control siRNA or siRNA targeting MARK4. The levels of the YAP/TAZ target genes ANKRD1 or CTGF and the knockdown efficiency for MARK4 was determined by real-time qPCR. (D) Gene expression in MDA-MB-231 cells is plotted as the mean +/- SD of three independent experiments each performed with three technical replicates, (*p=0.001 and **p=0.0002 calculated using Student’s two-tailed t-test). (E) Gene expression from a representative experiment performed twice in MCF10A, SW480 and DLD-1 cells is plotted as the mean +/- range of three technical replicates.
**Figure 3.2: Loss of MARK expression decreases YAP/TAZ target genes.**

(A) Deconvolution of MARK4 siRNA. MDA-MB-231 cells were transfected with single siMARK4 oligonucleotides that comprise the pool. The expression of YAP/TAZ target gene, ANKRD1, and MARK4 knockdown efficiency was determined by real-time qPCR and is plotted as the mean +/-SD of three independent experiments each performed with three technical replicates. (B) MDA-MB-231 cells were transfected with siRNAs targeting MARK2, MARK3 and MARK4. The expression of YAP/TAZ target gene, ANKRD1, and MARK4 knockdown efficiency was determined by real-time qPCR and is plotted as the mean +/- SD of three independent experiments each performed with three technical replicates. (C) MDA-MB-468 cells were transfected with siControl or siMARK3. The expression of YAP/TAZ target genes, ANKRD1, CTGF, and MARK3 knockdown efficiency was determined by real-time qPCR and plotted as the mean +/- range of a representative of two independent experiments each with three technical replicates.

**3.4.2 MARK4 inhibits YAP/TAZ phosphorylation**

The Hippo pathway signals through a core kinase cassette comprised of MST1/2 and LATS1/2 in a complex with the adapter/scaffolding proteins, SAV and MOB that function to induce phosphorylation of YAP/TAZ (Pan 2010; Kim et al. 2011; Ramos and Camargo 2012; Piccolo et al. 2014; Varelas 2014; Meng et al. 2016). Phosphorylated YAP/TAZ is localized to the cytoplasm where it is subsequently degraded thus preventing YAP/TAZ-induced transcriptional activity. To explore whether MARK4 alters YAP/TAZ activity by regulating the Hippo pathway, YAP/TAZ subcellular localization and phosphorylation. For this, we examined the effect of abrogating MARK4 expression using siRNAs in MDA-MB-231 cells and observed a loss of the prominent YAP/TAZ nuclear localization found in controls when analyzed by immunofluorescence microscopy or by subcellular fractionation (Figure 3.3A and B). A parallel increase in the relative levels of phosphorylated to unphosphorylated YAP and TAZ was also observed using either PhosTag gels or by immunoblotting regular gels with phospho-YAP Ser127 or Ser397 antibodies which recognize two distinct LATS-mediated phosphorylation sites in YAP (Figure 3.3C and D). Degradation of YAP and TAZ that occurs subsequent to phosphorylation was also evident (Figure 3.3D).
A. Immunofluorescence images showing the distribution of YAP and TAZ in siCtrl and siMARK4 cells. The bar graph on the right indicates the percentage of cells with cytoplasmic, even, and nuclear distribution for YAP and TAZ.

B. Western blot analysis of YAP, TAZ, MARK4, β-Tubulin, and Lamin B1 under control (CTL) and siMARK4 conditions. The ratio of cytoplasmic to nuclear proteins is plotted for YAP and TAZ.

C. Phospho-Tag and SDS-PAGE analysis showing the levels of P-TAZ, TAZ, P-YAP, YAP, YAP(S127), YAP(S397), pLATS1(T1079), and LATS1 under control and siMARK4 conditions.

D. Western blot analysis showing the ratio of pYAP(S127)/YAP under control and siMARK4 conditions.

E. Western blot analysis showing the expression of pYAP(S127), YAP, TAZ, MARK3, and GAPDH under control and siMARK3 conditions.
Figure 3.3: MARK regulates YAP/TAZ phosphorylation and localization.

(A-D) MDA-MB-231 cells were transfected with control siRNA or siRNAs targeting MARK4, YAP or TAZ as indicated. (A-B) Subcellular localization of YAP/TAZ was analyzed by (A) immunofluorescence confocal microscopy and subcellular localization in at least 50 cells for each condition was quantified and is plotted as a percentage of cells displaying the indicated distribution in a representative experiment of two independent experiments. Scale bar 15 μm or by (B) immunoblotting after biochemical fractionation. β-Tubulin and Lamin B1 were used as cytoplasmic and nuclear markers, respectively. The ratio of cytoplasmic to nuclear YAP and TAZ in blots was quantified. C, Cytoplasmic; N, Nuclear. The data are representative of two independent experiments. (C-D) Cell lysates, separated on PhosTag or regular SDS-PAGE gels, were analyzed by immunoblotting using the indicated antibodies. (C) YAP/TAZ phosphorylation was assessed by mobility shift on PhosTag gels using YAP and TAZ antibodies. The data are representative of at least three independent experiments. (D) YAP phosphorylation on Ser127 and Ser397 and LATS phosphorylation on Thr1079 were analyzed by immunoblotting using phospho-specific antibodies. The data are representative of three independent experiments. The ratio of phospho-YAP at Ser127 and Ser397 to total YAP from the blots was quantified. Total levels of YAP, TAZ, LAT51, MARK4 and GAPDH as the loading control were determined as indicated. (E) MDA-MB-468 cells were transfected with siControl or siMARK3. YAP phosphorylation and YAP/TAZ total levels were analyzed by immunoblotting using anti-phospho-YAP (S127), YAP and TAZ antibodies respectively. Knockdown efficiency was determined using MARK3 antibody and GAPDH was used and the loading control. YAP phosphorylation in blots was quantified by measuring the ratio of phospho-YAP (S127) to total YAP. The data are representative of two independent experiments.

In MDA-MB-468 cells, loss of MARK3 similarly induced an increase in the relative level of phosphorylated YAP (Figure 3.3E). Of note, in cells expressing a variant of TAZ harboring a mutation in key LATS-targeted phosphorylation site (TAZ S89A), loss of MARK4 did not promote cytoplasmic accumulation of TAZ (Figure 3.4A). As MST/LATS are key mediators of YAP/TAZ phosphorylation, these results are consistent with a role for MARK4 in directly modulating the Hippo pathway.
3.4.3 MARK4 knockout promotes cytoplasmic YAP/TAZ in MDA-MB-231 cells

To further investigate the role of MARK4 in regulating the Hippo pathway, we used CRISPR/Cas9 genome editing technology (Ran et al. 2013) to knockout MARK4 in MDA-MB-231 breast cancer cells. We designed several guideRNAs targeting human MARK4 and obtained multiple clones that showed complete knockout as assessed by immunoblotting (Figure 3.4B). MARK4 knockout resulted in an increase in YAP and TAZ phosphorylation levels as assessed by immunoblotting of regular gels using anti-phospho-YAP (Ser127) antibody or by examining migration on PhosTag gels (Figure 3.4B). Consistent with the increased phosphorylation, MARK4 knockout clones showed a marked loss of nuclear YAP/TAZ and an increase in cytoplasmic localization compared to control clones as assessed by immunofluorescence microscopy (Figure 3.4C) and by subcellular fractionation (Figure 3.4D). Importantly, simultaneous abrogation of expression of the core kinase cassette components, MST2 or SAV1 prevented the MARK4-induced cytoplasmic accumulation of YAP/TAZ and restored the expression of YAP/TAZ target, ANKRD1 in MARK4 KO cell lines (Figure 3.5A and B). A similar restoration of ANKRD1 expression by siSAV1 or siMST2 was also observed when MARK4 expression was abrogated using siRNAs (Fig 3.5C).

We next transfected control and MARK4 knockout cells with either wild-type (WT) or kinase-dead (KD) versions of MARK4 and examined YAP/TAZ localization by immunofluorescence microscopy. Reintroduction of wild-type but not kinase-dead, Flag-tagged MARK4 rescued the loss of nuclear YAP/TAZ in MARK4 knockout cells (Figure 3.6A and B). Consistent with this, our TEAD-reporter assay showed that wild-type but not the kinase-dead Flag-MARK4 activates luciferase activity (Figure 3.1C). Collectively these data provide compelling evidence that MARK4 depletion promotes cytoplasmic localization of YAP/TAZ by regulating the Hippo pathway and MARK4 kinase activity is required for Hippo pathway regulation.
Figure 3.4: MARK4 abrogation promotes nuclear to cytoplasmic translocation of YAP/TAZ.

(A) MDA-MB-231 cells stably expressing HA-tagged TAZ(WT) or TAZ(S89A) were transfected with control siRNA or siRNA targeting MARK4, and TAZ localization (green) was determined by immunofluorescence confocal microscopy using anti-HA antibodies. The subcellular localization of TAZ in at least 50 cells for each condition was quantified and is plotted as a percentage of cells displaying the indicated distribution in a representative experiment of two independent experiments. Scale bar, 15 μm. (B) Multiple independent MARK4-knockout (KO) clones were generated in MDA-MB-231 cells using CRISPR/Cas9-mediated gene editing. MARK4 knockout (KO) efficiency and YAP phosphorylation on Ser127 was determined by immunoblotting on regular SDS-PAGE gel using the indicated antibodies (top panels). YAP/TAZ phosphorylation was also assessed by immunoblotting using PhosTag gels (bottom panels). The data are representative of four independent experiments. (C) YAP localization (green) was assessed in control and MARK4-KO cells by immunofluorescence confocal microscopy, and subcellular localization of YAP in at least 30 cells per clone was quantified and plotted as percentage of cells displaying the indicated distribution in a representative of two independent experiments. Scale bar 15 μm. (D) Subcellular localization of YAP/TAZ was evaluated in two control (Cas9) and two MARK4 knockout (KO) clones by immunoblotting after biochemical fractionation, and the cytoplasmic to nuclear YAP/TAZ ratio in the blots was quantified (right panels) for each independent experiment.
Figure 3.5: MARK4 regulation of YAP/TAZ is Hippo pathway dependent.

(A-B) Control (Cas9-Vector) and MARK4 knockout (KO) cells were transfected with control siRNA or siRNAs targeting MST2 or SAV1. (A) YAP localization was determined by immunofluorescence confocal microscopy. The subcellular localization of YAP in at least 50 cells for each condition was quantified and is plotted as a percentage of the mean +/- SD of at least two independent clones (*p = 0.001 as compared to Cas9-Vec siCTL, **p = 0.003 as compared to MARK4-KO siCTL; n.s., not significant, calculated using Student’s two-tailed t-test). Scale bar, 20 μm. (B) The expression of the YAP/TAZ target gene, ANKRD1, and knockdown efficiencies of MST2 and SAV1 in two control and two MARK4-KO clones were assessed by qPCR and are plotted as the mean +/- SEM for two independent experiments each performed with three technical replicates. (C) MDA-MB-231 cells were transfected with control siRNA or siRNAs targeting MARK4, MST2 or SAV1. The expression of ANKRD1 and knockdown efficiencies of MARK4, SAV1 and MST2 were assessed by qPCR and are plotted as the mean +/- SEM of six independent experiments each performed with three technical replicates (**p < 10^{-4}, n.s., not significant, calculated using Student’s two-tailed t-test).
Figure 3.6: Expression of MARK4 rescues nuclear YAP/TAZ localization in MARK4 knockout cells.

The effect of MARK4-KO on YAP localization was rescued by re-introduction of MARK4. (A) Control (Cas9-vector) and MARK4-KO cells were transfected with wild-type or kinase-dead (KD) Flag-MARK4 and YAP/TAZ (green) and MARK4 (red) localization was determined by immunofluorescence confocal microscopy using anti-YAP or anti-Flag antibodies, respectively. Scale bar, 15 μm. (B) The subcellular localization of YAP in at least 30 transfected and adjacent non-transfected cells for each condition was quantitated and plotted as a percentage of the mean +/- SD from three independent experiments (*p = 0.006-0.008, as compared to Cas9-Vec non-transfected, **p = 0.001 as compared to MARK4-KO non-transfected, calculated using Student’s two-tailed t-test).

3.4.4 MARK4 interacts with and phosphorylates MST kinases

To determine the molecular mechanism of MARK4 function in Hippo signalling, we first tested for physical interactions between MARK4 and the Hippo pathway MST core kinase using LUMIER (Barrios-Rodiles et al. 2005). For this, MST variants fused to Firefly luciferase were coexpressed with wild-type (WT) or kinase-dead (KD) Flag-tagged MARK4 and interactions examined by anti-Flag immunoprecipitation followed by measurement of luciferase activity. This analysis revealed that WT and KD MARK4 interacted with both WT and KD versions of MST1 and MST2 (Figure 3.7A). Corroborating the associations detected by LUMIER, an interaction was also observed by immunoprecipitation of Flag-MST2 and immunoblotting for HA-MARK4 (Figure 3.7B), and by immunoprecipitating Flag-MARK4 and immunoblotting for endogenous MST1 (Figure 3.7C). Similarly, an interaction between endogenous MST1 and MARK3 in HEK293T cells was also detected (Figure 3.7D).

Given that MARK4 is a kinase, we speculated that MARK4 might inactivate the Hippo pathway by phosphorylating and thereby inhibiting MST activity. To test whether MARK4 could phosphorylate MST, HA-tagged MARK4 was coexpressed with Flag-MST2 in HEK293T cells, and MST2 mobility on a PhosTag SDS-PAGE gel was examined. Flag-MST2 expressed alone showed multiple bands indicative of the existence of multiple phosphorylation site variants when
Figure 3.7: MARK4 interacts with and phosphorylates MST kinases.

(A) HEK293T cells were transfected with Firefly luciferase tagged wild-type or kinase-dead variants of either MST1 or MST2 and Flag-MARK4. Cell lysates were subject to anti-Flag immunoprecipitation and the presence of MST1/2 was assessed by luciferase assay. Cells transfected with Luciferase tagged MST1/2 alone were used as negative controls and total expression was confirmed by luciferase assay and plotted as the mean +/- SD of triplicate samples from a representative of two independent experiments. (B) Lysates from HEK293T cells transfected with Flag-MST2 and HA-MARK4, were subject to anti-Flag immunoprecipitation and the presence of MARK4 was determined by anti-HA immunoblotting. Equivalent protein expression levels were confirmed (Totals). The data are representative of three independent experiments. (C-D) MARK3 and MARK4 interact with endogenous MST1 and SAV1. (C) HEK293T cells were transfected with either empty vector or Flag-MARK4. Cell lysates were subject to anti-Flag immunoprecipitation and binding to endogenous MST1 and SAV1 was determined by immunoblotting using specific antibodies. Total cell lysates were analyzed by immunoblotting. (D) Cell lysates from HEK293T cells were subjected to anti-IgG or anti-MST1 immunoprecipitation and the presence of endogenous MARK3 and SAV1 was determined by immunoblotting using specific antibodies. Total cell lysates were analyzed by immunoblotting. The data (C-D) are representative of two independent experiments. (E-F) MARK4 phosphorylates MST2. HEK293T cells were co-transfected with wild-type or kinase-dead HA-tagged MARK2, MARK3 (E) or MARK4 (E,F) along with wild-type or kinase-dead Flag-tagged MST2 constructs, as indicated. MST2 phosphorylation was assessed by analyzing mobility shifts by anti-Flag immunoblotting using PhosTag SDS-PAGE gels. Total cell lysates were analyzed by immunoblotting by regular SDS-PAGE. The data (E-F) are representative of three independent experiments.

expressed in mammalian cells (Figure 3.7E). Coexpression of wild-type but not kinase-dead MARK4 caused additional upshifts in MST2 demonstrating that the MARK4 kinase activity was required for the appearance of the additional phosphorylated variants of MST2 (Figure 3.7E). The MARK4-induced upshifts were also observed in the presence of kinase-dead MST2, indicating that MST2 autophosphorylation was not involved. Analysis of two other members of the MARK family showed that similar to MARK4, overexpression of MARK2 and 3 caused the appearance of MST2 bands of slower mobility (Figure 3.7F). Thus, the ability to phosphorylate MST2 is conserved among MARK family members. To further confirm phosphorylation of MST2 by MARK4, we performed an in vitro kinase assay using Flag-MST2 immunoprecipitates and
purified MARK4 protein produced in Sf9 cells. When expressed alone, addition of ATP to wild-type but not kinase-dead MST2 resulted in a prominent upshift on PhosTag gels, indicating robust autophosphorylating activity (Figure 3.8A). Thus, to avoid ambiguities due to MST kinase activity in our analysis of MARK4-mediated phosphorylation, we focused on using the kinase-dead variant of MST2. Addition of purified GST-MARK4 to kinase-dead MST2, in the presence of ATP resulted in the appearance of an upshifted MST2 band, consistent with our results in HEK293T cells (Figure 3.8A). Thus, MARK can directly phosphorylate MST2.

### 3.4.5 MARK4 phosphorylates SAV and this is enhanced by MST2

MST directly binds to and phosphorylates the adaptor protein Salvador (SAV) and this promotes the interaction of MST with the downstream kinase LATS (Pan 2010; Halder and Johnson 2011; Avruch et al. 2012; Meng et al. 2016). Thus, we sought to determine whether MARK4-mediated phosphorylation of MST alters the ability of MST to phosphorylate SAV. When expressed alone, Flag-SAV1 is predominately unphosphorylated and consistent with published results (Wu et al. 2003a; Park and Lee 2011), coexpression of WT but not kinase-dead MST2 induces an upshift of SAV on a PhosTag gel, indicative of SAV phosphorylation (Figure 3.8B). Co-expression of MARK4 did not cause a marked change in MST-induced phosphorylation of SAV suggesting that although MARK4 can phosphorylate MST, this did not alter MST kinase activity towards SAV. Consistent with this, MARK4 phosphorylation of MST2 did not alter intrinsic MST2 kinase activity as assessed in an in vitro MST2 autophosphorylation assay using an activation loop phospho-MST1/2 (T180/183) antibody (Figure 3.8C) or by evaluating the MOB1 phosphorylation using antibodies that recognize the MST-targeted site, Thr35 (Figure 3.8D). However, when wild-type MARK4 was co-expressed with MST2 and SAV in mammalian cells, we noticed the appearance of a new Flag-SAV1 upshifted band in both PhosTag and regular SDS-PAGE gels that was not detected in the presence of kinase-dead MARK4 (Figure 3.8B lanes 5-8). This suggested that MARK4 can phosphorylate SAV.
Figure 3.8: MARK4 does not change MST2 kinase activity.

(A and C) MST2 phosphorylation and kinase activity in the presence of MARK4 was assessed by an in vitro kinase assay using purified GST-MARK4. HEK293T cells were transfected with wild type (WT) and kinase dead (KD) Flag-MST2 and cell lysates were subjected to anti-Flag immunoprecipitation. Immunoprecipitated Flag-MST2 was incubated with GST-MARK4 in the presence or absence of ATP, as indicated. MST2 phosphorylation was analyzed by immunoblotting using PhosTag SDS-PAGE gels (A), and MST2 kinase activity was assessed by immunoblotting for autophosphorylation using phospho-MST1/2 (T180/183) antibody. The data are representative of three independent experiments. (B). (C) The effect of MARK4 expression on MST2 kinase activity was further analyzed by evaluating MOB1 phosphorylation as the MST1/2 direct substrate. HEK293T cells were transfected with Flag-MOB1 and HA-MST2 along with wild type (WT) or kinase dead (KD) versions of HA-MARK4. MOB1 phosphorylation at the MST1/2 phospho-site Thr35, was assessed by immunoblotting using the specific antibody. The data is representative of two independent experiments. (D) Flag-SAV1 was transfected alone or in various combinations with wild-type (WT) or kinase-dead (KD) MST2 in the presence or absence of wild-type (WT) or kinase-dead (KD) HA-MARK4, as indicated. Immunoblotting was used to analyze SAV phosphorylation, as MST substrate by assessing mobility shift on PhosTag gels.

To investigate this further, we co-expressed Flag-SAV1 with HA-MARK4 alone or in the presence or absence of HA-MST2 and assessed SAV upshifts using regular SDS-PAGE gels (Figure 3.9A). Expression of wild-type MARK4 alone resulted in the appearance of a more slowly migrating variant of Flag-SAV1 even in the absence of MST2 (Figure 3.9A, lane 3), while expression of MST2 alone did not alter the migration of SAV on these gels (Figure 3.9A, lane 4). Of note, when MARK4 was co-expressed with either wild-type or kinase-dead MST2, there was an increase in the levels of upshifted Flag-SAV1 (Figure 3.9A, lanes 6 and 8). This suggested that MST2 might increase MARK4-mediated phosphorylation of SAV by enhancing the interaction of SAV with MARK4 and that this effect was independent of MST kinase activity. We confirmed that MARK4 can directly phosphorylate SAV, by performing an in vitro kinase assay using Flag-SAV1 immunoprecipitates and purified GST-MARK4. Addition of MARK4 in the presence of ATP induced SAV phosphorylation as determined by immunoblotting of lysates separated on regular SDS-PAGE gels (Figure 3.9B). Of note, GST-MARK4 showed a slower migration in the presence of ATP, suggesting that MARK4 autophosphorylation also occurs.
Figure 3.9: MARK4 binds to and phosphorylates the adaptor protein SAV.

(A) Analysis of SAV phosphorylation by MST2 in the presence or absence of MARK4. Flag-SAV1 was transfected alone or in various combinations with wild-type (WT) or kinase-dead (KD) MST2 in the presence or absence of wild-type (WT) or kinase-dead (KD) HA-MARK4, as indicated. Immunoblotting was used to analyze SAV phosphorylation by assessing mobility shift on regular SDS-PAGE gels. (B) Analysis of SAV phosphorylation by MARK4 using an in vitro kinase assay. HEK293T cell were transfected with Flag-tagged SAV1 and cell lysates were subject to anti-Flag immunoprecipitation. Immunoprecipitated Flag-SAV1 was incubated with GST-MARK4 in the presence or absence of ATP, as indicated, and SAV1 phosphorylated was analyzed by immunoblotting. (C-E) SAV binds to MARK4 and the presence of MST1/2 kinases enhances SAV interaction with MARK4. (C) HEK293T cell were transfected with Flag-SAV1, HA-MARK4 and HA-MST1 or HA-MST2. Cell lysates were subject to anti-Flag immunoprecipitation and the presence of HA-MARK4 and HA-MST1/2 was determined by anti-HA immunoblotting. Equal protein expression levels were confirmed (Totals). Cell lysates from MDA-MB-468 (D) and HEK293T (E) cells were subject to anti-SAV1 immunoprecipitation and binding to MARK3 and MST1 was determined by immunoblotting using specific antibodies. Equal protein expression levels were confirmed (Totals). The data (A-E) are representative of three independent experiments.

The MARK4-induced upshift of SAV was increased in the presence of MST, thus, we sought to investigate whether the presence of MST enhances binding of MARK4 to SAV. We observed a weak interaction between SAV and MARK4 as determined by Flag-SAV1 immunoprecipitation followed by immunoblotting for HA-MARK4 (Figure 3.9C). Of note, the presence of either MST1 or MST2 enhanced the interaction between SAV and MARK4 (Figure 3.9C). The ability of endogenous SAV1 to interact with both MARK3 and MST1 in MDA-MB-468 and in HEK293T cells was also confirmed (Figure 3.9C and D, and see also Figure 3.7D). Altogether these results show that MARK4 can bind to and phosphorylate SAV and that MST enhances the interaction between MARK4 and SAV.
3.4.6 MARK4 disrupts complex formation between Hippo components MST/SAV and LATS

We speculated that phosphorylation of MST and SAV by MARK4 might inhibit MST and SAV interaction. To test this possibility, we analyzed the interaction between Flag-SAV1 with either transfected or endogenous MSTs in the presence or absence of MARK4. Flag-SAV1 efficiently interacted with both HA-MST2 and with endogenous MST1 and this was not altered in the presence of HA-MARK4 (Figure 3.10A and B). SAV acts as an adaptor that binds to MST and promotes complex formation between MST and LATS (Pan 2010; Halder and Johnson 2011; Avruch et al. 2012; Meng et al. 2016). Thus, we next examined whether MARK4 might attenuate the interaction of the MST/SAV complex with the downstream kinase LATS by immunoprecipitation followed by immunoblotting. Consistent with the proposed function of SAV as an adaptor protein, the interaction between LATS and MST was markedly enhanced in the presence of SAV (Figure 3.10C). Notably, co-expression of wild-type MARK4 but not the kinase-dead version significantly decreased the association of MST with LATS in the presence of SAV (Figure 3.10C). Altogether our data suggest that MARK4 can phosphorylate MST and SAV, and that expression of MARK4 in a kinase-dependent manner, attenuates complex formation between Hippo core components MST/SAV with LATS (Figure 3.10D). We did not detect any changes MST-mediated phosphorylation of endogenous LATS upon loss of MARK4 expression (Figure 3.3D), suggesting that activation of a small pool of LATS is sufficient to induce cytoplasmic YAP/TAZ.
Figure 3.10: MARK4 attenuates formation of a MST/SAV/LATS complex.

(A-B) HEK293T cells were transfected with combinations of Flag-SAV1 and HA-MST2 in the presence or absence of HA-MARK4. Cell lysates were subjected to anti-Flag immunoprecipitation followed by immunoblotting using anti-HA (A) and anti-MST1 (B) antibodies respectively. (C) HEK293T cells were transfected with Flag-LATS1 and HA-tagged MST2, SAV1 and wild-type (WT) or kinase-dead (KD) MARK4 as indicated. Cell lysates were subjected to anti-Flag immunoprecipitation and the interaction of MST with SAV was determined by anti-HA immunoblotting. Equal protein expression levels were confirmed (Totals). The data (A-C) are representative of three independent experiments. (D) Model for MARK4 function in regulating the Hippo pathway. MARK phosphorylates the Hippo core components MST and SAV and disrupts complex formation between MST/SAV and LATS in a kinase dependent manner, which inhibits the Hippo kinase cascade.

3.4.7 MARK4 depletion attenuates proliferation and migration in breast cancer cells

Numerous studies have established the importance of Hippo signalling in tumorigenesis and have shown that increased activity of YAP/TAZ is associated with enhanced cell proliferation and migration (Pan 2010; Halder and Johnson 2011; Harvey et al. 2013; Yu et al. 2015). MDA-MB-231 cells are aggressive triple-negative breast cancer cells, which have undergone epithelial to mesenchymal transition and have bypassed cell-cell contact inhibition. The Hippo pathway is dampened in these cells and YAP/TAZ are predominantly nuclear, contributing to their tumorigenic properties (Cordenonsi et al. 2011; Heidary Arash et al. 2014). Our data shows that MARK4 expression strongly activates YAP/TAZ and that abrogation of MARK4 expression leads to loss of nuclear localization and transcriptional activity of YAP/TAZ. Therefore, we sought to determine if loss of MARK4 would alter tumorigenic properties of MDA-MB-231 cells. Thus, we first evaluated the role of MARK4 in the growth of MDA-MB-231 cells by abrogating expression of MARK4 with siRNAs. We observed that loss of MARK4 expression resulted in a marked decrease in cell proliferation as measured by SRB assay (Figure 3.11A and B). This reduction was comparable to the decrease observed upon loss of TAZ (Figure 3.11A and B) or YAP (Kim et al. 2011; Heidary Arash et al. 2014).
A. Relative Cell Growth

B. Relative Expression of MARK4 and TAZ

C. Unoccupied Area (%)

D. Unoccupied Area (%)

E. Relative Expression of MARK4, TAZ, and YAP

F. Relative Cell Growth

G. Unoccupied Area (%)

H. Relative Cell Growth

I. Migration

Images show experimental results with various treatments and time points.
**Figure 3.11:** Abrogation of MARK4 expression attenuates cell growth and cell migration in breast cancer cells.

MDA-MB-231 cell growth was assessed by SRB assay and cell migration was determined using the wound healing scratch assay by live-cell phase-contrast microscopy. (A-E) MDA-MB-231 cells were transfected with control siRNA or siRNAs targeting MARK4, TAZ or YAP. (A) Cell growth was assessed and plotted as the mean +/- SEM of three independent experiments each performed with six replicates. (B) Knockdown efficiency was determined by qPCR and is plotted as the mean +/- SD of three independent experiments each performed with three technical replicates. (C-E) Cell migration was monitored and the cell free area was quantified for a representative experiment (C) or for the average of three independent experiments (D) with four independent fields per condition and is plotted as the mean +/- SD or the mean +/- SEM, respectively. In D, p-values (***) for siCtl compared to siMARK, siTaz and siYAP were 0.0004, 0.0001 and 0.0003, respectively. (E) Knockdown efficiency was determined by qPCR and plotted as the mean +/- SD of three independent experiments each performed with three technical replicates. (F-G) Loss of MST2 or SAV1 rescues the effect of MARK4 knockdown on cell growth and migration. (F) MDA-MB-231 cells were transfected with control siRNA or siRNA targeting MARK4, MST2 or both and cell growth was assessed and plotted as the mean +/- SEM, of three independent experiments each performed with six replicates, (*p=0.05 and **p=0.009). (G) Cell migration was assessed and the cell-free area was quantitated as the mean +/- SEM of three independent experiments, in four independent fields per condition (*p=0.02, **p=0.002, ***p<10^-4) (H) Cell growth in two control (Vec) and two MARK4 KO cell clones was assessed and plotted as the mean +/-SEM of three independent experiments, each performed with six replicates (**p=0.002). (I) Cell migration in control (Vec) and MARK4 KO cells was assessed and the cell-free area was quantitated as the mean +/- SEM of three independent experiments in four independent fields per condition (**p=0.009). Representative images are shown (right). Scale bar 126 μm. All p-values were calculated using Student’s two-tailed t-test.

Next, we examined the effect of loss of MARK4 expression on MDA-MB-231 cell migration using the wound healing scratch assay. MARK4 depletion resulted in a marked decrease in cell migration similar to TAZ knockdown, though less dramatic than that observed upon loss of YAP (Figure 3.11C-E). This is consistent with a recent study showing that MARK4 downregulation by miR-515-5p in breast and lung cancer cells reduces cell migration and metastasis (Pardo et al. 2016). To demonstrate that the MARK4 effects on cell proliferation and migration are through the Hippo pathway, we sought to rescue the effects by blocking the expression of MST and SAV.
Importantly, concomitant abrogation of SAV1 or MST2 expression overcame the inhibitory effects of siMARK4 on cell growth and migration indicating that MARK4 functions through SAV1/MST2 (Figure 3.11F and G). We also examined MARK4 knockout cells generated by CRISPR and similar to siRNA-mediated knockdown, MARK4 knockout cells showed a decrease in cell growth and migration (Figure 3.11H and I). Thus loss of MARK4 in MDA-MB-231 breast cancer cells decreases YAP/TAZ activity and attenuates cell growth and migration. Taken together our findings show that MARK4 disrupts complex formation between Hippo core components and thereby functions as a negative regulator of Hippo signalling to promote cell migration and proliferation of breast cancer cells.
3.5 Discussion

In this study, we performed a high throughput TEAD-luciferase reporter screen to uncover genes that modulate YAP/TAZ activity upon overexpression and identified multiple members of MAP/microtubule regulating kinase (MARK) family as top hits. We demonstrate that expression of MARK family members leads to robust activation of the TEAD reporter, an indicator of YAP/TAZ transcriptional activity and that depletion of MARK4 expression in breast cancer cells results in loss of YAP/TAZ nuclear localization and a marked decrease in target gene expression. Mechanistically, we show that MARK4 binds to and phosphorylates the core components of the Hippo pathway, MST and SAV and that MARK4, in a kinase dependent manner, inhibits the assembly of core the Hippo kinase cassette by disrupting the interaction of MST and SAV with LATS.

The mammalian MARK family is comprised of four kinases, MARK1-4 (also referred to Par-1c, Par-1b, Par-1a and Par-1d, respectively) which are orthologs of the Drosophila Par-1 protein. Thus, MARK/Par-1 kinases are evolutionarily conserved proteins with diverse roles in embryonic development, asymmetric cell division, and cell polarity regulation (Tassan and Goff 2004; Hurov and Piwnica-Worms 2007; Naz et al. 2013; Dumont et al. 2015). MARKs belong to the larger AMPK family, which includes multiple Ser/Thr kinases with diverse cellular and physiological functions including the prototypic AMP-activated protein kinase (AMPK) as well as Salt-inducible kinases (SIKs) (Tassan and Goff 2004; Hurov and Piwnica-Worms 2007). AMPK is a master regulator of energy balance in the cell and responds to nutrient availability and intracellular energy levels to regulate metabolic pathways (Shackelford and Shaw 2009; Mihaylova and Shaw 2011). Recent studies have shown that several members of the AMPK family have divergent roles in regulating Hippo signalling. For example, activation of AMPK in response to glucose deprivation increases LATS1/2 kinase activity to restrict YAP/TAZ function (DeRan et al. 2014; Mo et al. 2015; Wang et al. 2015). In contrast, a study in Drosophila has shown that SIKs inhibit the Hippo kinase cascade to activate Yorki (Yki, the YAP/TAZ ortholog) and promote tissue growth by phosphorylating SAV (Wehr et al. 2013). Although the SIK phosphorylation site is not conserved in mammals, overexpression of human SIK2 in HEK293T cells activated the TEAD-reporter, suggesting that SIK may have retained an inhibitory function on the Hippo pathway in mammals (Wehr et al. 2013). Together with our findings showing that MARKs inhibit the Hippo pathway, these studies highlight the role of AMPK family members as differential but important upstream
regulators of the Hippo signalling pathway. Understanding how and whether MARKs and AMPK family members link to other Hippo regulatory pathways such as mechanotransduction would be a pertinent avenue for future studies.

Our work has shown that abrogation of MARK4 expression either transiently using siRNAs or genomically, using CRISPR/Cas9 promotes the cytoplasmic localization of YAP/TAZ and inhibits target gene expression. While we did not detect any changes MST-mediated phosphorylation of endogenous LATS upon loss of MARK4 expression (Figure 3.3D), it may be that activation of a small pool of LATS that is not efficiently detected by the antibodies is sufficient to induce cytoplasmic YAP/TAZ. In Drosophila, and consistent with our findings, loss of Par-1 (the MARK ortholog) also leads to activation of the Hippo pathway and results in a marked decrease in eye and wing imaginal disc size that is dependent on Yorki (Huang et al. 2013). In mice, shRNA-mediated loss of two other MARK family members, Mark2 and Mark3 [Par-1a/Par-1b], also promotes cytoplasmic localization of Yap in mouse trophoblast cells during early embryonic development (Hirate et al. 2013). While we focused on MARK4 due to a prominent effect in MDA-MB-231 cells, overexpression of MARK2, 3 and 4 activates the TEAD-luciferase reporter (Figure 3.1D) and siRNA-mediated depletion of MARK3 [PAR-1a] in MDA-MB-468 cells also promotes YAP/TAZ phosphorylation and decreases expression of target genes (Figure 3.2C and 3.3E). Thus, multiple MARK family members have a similar ability to positively regulate YAP/TAZ activity. A recent study showed that LKB1-deficient tumors display high levels of nuclear YAP and that LKB1 promotes Hippo pathway activity by regulating the localization of the basolateral protein, Scribble (Mohseni et al. 2014). In contrast to findings that MARKs enhance YAP/TAZ activity (herein and (Hirate et al. 2013; Huang et al. 2013)), in this study it was proposed that MARKs act downstream of LKB to inhibit YAP/TAZ. The differing roles of MARKs in regulating Hippo signalling in distinct contexts will require further investigation.

MARKs are Ser/Thr kinases and our analysis of the rescue of the MARK4 knockout phenotype clearly demonstrates that MARK4 kinase activity is required to regulate YAP/TAZ localization. Concordantly, our investigation of whether MARK4 regulates YAP/TAZ by phosphorylating any of the Hippo core components revealed that it binds to and phosphorylates both MST and SAV. Moreover, we demonstrated that MARK4, in a kinase-dependent manner, inhibits the assembly of core Hippo kinase cassette by disrupting the interaction of MST and SAV with LATS (Figure 3.10C). Epistasis analysis of Par-1 in Drosophila placed Par-1 upstream of the Hippo (Hpo) core
kinase while molecular analysis showed that Par-1 could phosphorylate and decrease Hpo kinase activity (Huang et al. 2013). Although we observe an increase in MST upshift in PhosTag gel upon MARK4 expression, indicative of phosphorylation, we did not observe a change in MST kinase activity towards its substrate SAV. However, we also observed MARK-dependent phosphorylation of SAV and a decrease in complex formation between the Hippo core components MST and SAV with LATS. Of note, analysis of Drosophila Salt-inducible kinases (SIKs) describe a similar finding, namely that SIKs phosphorylate Sav and that this phosphorylation disrupts the interaction between Hpo and Sav with Wrts, the orthologs of mammalian MST, SAV and LATS, respectively (Wehr et al. 2013). Thus, the mechanism proposed for SIKs is consistent with that we observed for MARK4 (Figure 3.10D). Although MARKs and SIKs are distinct kinases, they are both AMPK family members, and the similarity of mechanisms in flies and mammalian cells suggests that modulation of complex formation between Hippo pathway core components is an evolutionarily conserved mechanism for regulating Hippo signalling. Altogether these data suggest that although there are similarities between Drosophila and mammalian Par-1 in regulating Hippo signalling, there appears to be a divergence in the mechanism of action.

Numerous studies have firmly established the role of YAP/TAZ during tumorigenesis. YAP/TAZ activate a growth promoting gene expression program and increased activity of YAP/TAZ has been associated with cell proliferation, migration, resistance to apoptosis, transformation of normal epithelial cells, increased cancer stem cell content and higher resistance to chemotherapeutic agents (Pan 2010; Harvey et al. 2013; Yu et al. 2015). MDA-MB-231 cells are aggressive triple-negative breast cancer cells with an inactive Hippo pathway. These cells show predominantly nuclear YAP/TAZ, which contributes to their tumorigenic characteristics, thus providing a suitable cell line model to study the biological impact of YAP/TAZ modulators (Cordenonsi et al. 2011; Kim et al. 2011). In agreement with a role for MARK4 in promoting YAP/TAZ dependent transcription, we demonstrate that MARK4 loss of function either transiently by siRNA-mediated knockdown or permanently by CRISPR/Cas9-mediated knockout, attenuates proliferation and migration in MDA-MB-231 breast cancer cells. Given that depletion of MARK3, in a different breast cancer line, similarly induced cytoplasmic localization of YAP/TAZ, our findings suggest that targeting MARK activity might provide therapeutic benefit in breast cancer by restricting YAP/TAZ activity. In future work, it would be interesting to investigate whether MARKs also regulate YAP/TAZ in other types of cancers.
Chapter 4
Investigating the role of GIT1 and DLG5 in Hippo pathway regulation

The data related to GIT1 presented in Figures 4.1 to 4.4 are not published. The data related to DLG5 presented in Figure 4.5 has been published in:


I performed all the experiments presented in this chapter.
4 Investigating the role of GIT1 and DLG5 in Hippo pathway regulation

4.1 Abstract

βPIX and MARK kinases have been identified as positive and negative regulators of the Hippo kinase cascade. In many contexts βPIX acts as part of a multiprotein complex to organize downstream signalling events such as focal adhesion dynamics and cell migration. GIT1 is the constitutive binding partner for βPIX that is involved in most processes regulated by βPIX. DLG5 on the other hand has been identified through affinity purification-mass spectrometry to bind to Hippo pathway core components MST-SAV and MARK kinases. Thus to determine whether GIT1 and DLG5 are also involved in regulating the Hippo pathway, I examined subcellular localization and transcriptional activity of YAP/TAZ upon overexpression or abrogation of GIT1 and DLG5. Interestingly, increased expression of GIT1 represses transcriptional activity and promotes cytoplasmic localization of YAP/TAZ, whereas GIT1 depletion results in higher expression of YAP/TAZ target genes. Conversely, DLG5 abrogation leads to increased phosphorylation, loss of nuclear localization and decreased expression of YAP/TAZ targets. These findings suggest that similar to their binding partners, GIT1 and DLG5 might act as positive and negative regulators of the Hippo pathway respectively.
4.2 Introduction

The Hippo signalling pathway is composed of a core Ser/Thr kinase cascade that regulates the co-transcription factors YAP/TAZ in response to a variety of upstream stimuli such as cell-cell contact and junctional complexes, actin cytoskeleton dynamics, G-protein coupled receptors, energy status of the cell and AMPK signalling (Hansen et al. 2015). Despite growing knowledge about the upstream regulators of the Hippo signalling, the mechanistic details and the molecular mediators that connect these signals to the core of the pathway are not fully understood.

We have previously identified βPIX and microtubule-affinity regulated kinases (MARKs) as positive and negative regulators of the Hippo kinase cascade respectively. Both βPIX and MARK can associate with several partners and often exert their biological functions as part of a multiprotein complex. Given the importance of protein-protein interactions in Hippo pathway regulation, we sought to determine whether binding partners of βPIX and MARKs might also be involved in Hippo signalling.

βPIX, through interactions with various proteins such as PAK, Rac1, Cdc42, Scribble and GIT1, is involved in the regulation of a variety of cellular processes including focal adhesion dynamics, cytoskeleton rearrangement and cell migration (Frank and Hansen 2008). Although many of these interactions are dynamic and transient, βPIX shows a strong binding to GIT1 and in many contexts PIX-GIT function as a complex (Rosenberger and Kutsche 2006; Frank and Hansen 2008).

G-protein coupled receptor kinase-interactor 1 (GIT1) is a versatile protein with a complex domain structure that can bind multiple and functionally diverse proteins. As the name implies, GIT1 was first identified as a protein that binds GPCR kinases (GRKs), a family of Ser/Thr kinases responsible for terminating GPCR signalling by phosphorylating active GPCRs that in turn triggers arrestin binding and receptor internalization (Premont et al. 1998; Moore et al. 2007). At the same time GIT1 was shown to be localized in focal adhesions by binding to paxillin (Turner et al. 1999). In response to Rac1/Cdc42 activation, the paxillin-binding domain of GIT1 becomes exposed and this leads to recruitment of the PIX-GIT complex to focal adhesions (Brown et al. 2002; Manabe et al. 2002). GIT1 is also a GTPase activating protein (GAP) for ARF GTPases, which are involved in vesicular trafficking and endosomal recycling. It has been reported that GIT1 can travel between different cellular compartments and regulate endosomal recycling, however the exact role and mechanisms of action in this context are not clear (Di Cesare et al. 2000; Matafora et al. 2001).
MARKs also demonstrate extensive protein interactions that are important for biological functions. Affinity purification followed by mass spectrometry has recently identified disc large homology (DLG5) as MARK and MST1/2 binding partner (Kwan et al. 2016). DLG5 encodes a large coiled-coiled MAGUK (membrane associated guanylate kinase) protein that contains CARD, Duff, four PDZs, SH3 and GUK domains (Nechiporuk et al. 2007). The domain structure of DLG5 suggests that it might have a scaffolding function in signalling pathways. It has been shown that DLG5 is required for the maintenance of apico-basal polarity and DLG5 knockout results in loss of polarity in neuronal progenitors and epithelial cells lining the kidney collecting ducts (Nechiporuk et al. 2007; Nechiporuk et al. 2013). Furthermore, DLG5 is required for proliferation and maintenance of neuronal progenitors, however the molecular mechanisms through which proliferation and progenitor cell homeostasis are regulated by DLG5 are not clearly understood.

Here, I demonstrate that GIT1 and DLG5 negatively and positively regulate the transcriptional activity of YAP/TAZ, respectively. I show that GIT1 depletion induces the expression of YAP/TAZ target genes and promotes cell growth in mammary epithelial cells. Furthermore, I demonstrate that GIT1 interacts with Hippo core kinase LATS, and overexpression of GIT1 co-localizes with LATS and YAP in vesicular structures. Loss of DLG5 on the other hand, inhibits YAP/TAZ activity by promoting phosphorylation, degradation and cytoplasmic localization.
4.3 Materials and Methods

4.3.1 Cell Culture, Plasmids and Transfection

For cell culturing, MCF10a cells were grown in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone and 100 ng/ml cholera toxin, NMuMG in DMEM with 10% FBS and 10 μg/ml insulin, HEK293T cells in DMEM with 10% FBS, and MDA-MB-231 cells in RPMI with 5% FBS. The Git1 construct was generated by PCR using mouse Git1 (NM_001004144.1) as template and tagged with Flag or HA in Gateway destination vectors. Flag- or HA-tagged constructs for βPix, LATS1, TAZ and YAP in pCMV5 were previously described in Chapter 2. Cells were transfected with Dharmacon siGENOME pools of four individual siRNAs (Thermo Scientific) using Lipofectamine RNAiMAX (Life Technologies) or with cDNAs, using Lipofectamine 3000 (Life Technologies) according to the manufacturer’s instructions.

4.3.2 Immunoblotting, Immunoprecipitation and antibodies

Cells were lysed in lysis buffer [50 mM tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X100, 1 mM DTT containing phosphatase and protease inhibitors]. Lysates were separated on SDS-PAGE gels and immunoblotting was performed using standard protocols as described in Chapters 2 and 3. Phos-Tag gels, using reagents purchased from Waco Chemicals, were prepared according to manufacturer’s instructions. For immunoprecipitations, cell lysates were subject to anti-Flag immunoprecipitation and proteins collected using protein G-Sepharose prior to analyses by immunoblotting. The antibodies used were: YAP (Cell Signaling #4912); TAZ (Cell Signaling #2149); LATS1 (C66B5; Cell Signaling #3477); MST1 (Cell Signaling #3682); DLG5 (Nechiporuk et al., 2013); rat anti-HA (Roche #1867423) and anti-Flag M2 (Sigma-Aldrich #F1804).

4.3.3 Immunofluorescence Microscopy

Cell were plated in 4-well Lab-Tek chambers (#154526) and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were permeabilized with 0.5% Triton-X100 in PBS for 10 min at room temperature. Samples were washed three times with 0.01% PBS-Tween, and then blocked in 2% BSA-PBS for 30 min before treating samples with primary
antibody overnight at 4°C. The primary antibodies used in this chapter and the concentrations are:
mouse anti-YAP 1:300; Santa Cruz sc-101199; rabbit anti-Flag 1:500, Sigma F7425; or rat anti-
HA 1:500, Roche #1867423  Samples were then washed three times with 0.01% PBS-Tween  and
were incubated with the secondary antibodies, goat anti-rabbit Alexa-fluor 488 (Life Technologies
#A11305, 1:1000 in 2% BSA-PBS); goat-anti-mouse Alexa Fluor 546 (Life Technologies
#A11029, 1:1000 in 2% BSA-PBS) or goat anti-rat Alexa-Fluor 488 (Life Technologies #A11006,
1:1000) for 1-2 h at room temperature. Slides were washed three times with 0.01%PBS-Tween
and once with PBS and mounted with ProLong Gold Antifade Reagent (Life Technologies
#P36035). Cell nuclei were visualized by DAPI staining. Images were captured using a spinning
disk confocal scanner (CSU10, Yokogawa) on Leica DMI6000B microscope and Volocity
software was used for image acquisition and processing

4.3.4 Quantitative Real-Time PCR

Total RNA was purified using PureLink RNA Mini Kit (Life Technologies. cDNA was
synthesized using 1 µg of purified RNA using Oligo-dT primers and M-MLV Reverse
Transcriptase (Invitrogen #28025-013). Real-Time PCR was performed using the SYBR Green
master mix (Applied Biosystems) on the ABI Prism 7900 HT system (Applied Biosystems).
Relative gene expression was quantified by ΔΔCt method and normalized to Gapdh or HPRT. The
sequence of the primers used for qPCR in this study are listed below:

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-Gapdh</td>
<td>ACATCAAGAAGGTGGTGAAGCAGG</td>
<td>ACGAATTGGCTACAGCAACAGGG</td>
</tr>
<tr>
<td>m-Ctgf</td>
<td>GGGCCTCTTCTGCGATTTC</td>
<td>ATCCAGGCAAGTGCATTGGTA</td>
</tr>
<tr>
<td>m-Ankrd1</td>
<td>TGCGATGAGTATAACCGGACG</td>
<td>GTGGATTCAAGCATATCTCGGAA</td>
</tr>
<tr>
<td>m-Cyr61</td>
<td>CGAGGTGGAGTTGACGAGAAACCA</td>
<td>CTTTGAGCACTGGGACCATGAAGT</td>
</tr>
<tr>
<td>m-Git1</td>
<td>CCTGGGACGACACATCTCC</td>
<td>GATGGGGTGGACTTTATCTTGG</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Sequence</td>
<td>Reverse Sequence</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>h-ANKRD1</td>
<td>AGTAGAGGAACCTGCTCCTGG</td>
<td>TGGGCTAGAGTGTCTTCAGAT</td>
</tr>
<tr>
<td>h-CTGF</td>
<td>AGGAGTGCGCTGTGGGACGA</td>
<td>CCAGGCGAGTTGGCTCTCAATC</td>
</tr>
<tr>
<td>h-CYR61</td>
<td>CGAGGTGGAGTTGACGAGA</td>
<td>CTTTGAGCAGTGGGACCATGA</td>
</tr>
<tr>
<td>h-YAP</td>
<td>TGCGTACARCGTTACCA</td>
<td>GGTGCCACTGTGAAGGA</td>
</tr>
<tr>
<td>h-TAZ</td>
<td>GTATCCCAGCCCAAATTCGATG</td>
<td>CAGCGCATTGGGCACTACATCAG</td>
</tr>
<tr>
<td>h-GIT1</td>
<td>GAGTCACCGCAACGACCTC</td>
<td>CAGCCCCATACTACAAGCA</td>
</tr>
<tr>
<td>h-HPRT</td>
<td>ATGGACAGGACTGACTCTTTC</td>
<td>TTGAGCACACAGAGGCTACAATG</td>
</tr>
</tbody>
</table>
4.4 Results

4.4.1 GIT1 regulates YAP/TAZ transcriptional activity

Given that βPIX and GIT1 display strong physical interactions and functional cooperation in various contexts, I asked whether GIT1 might also be involved in regulating the Hippo pathway. For this, I initially performed a TEAD-reporter luciferase assay to evaluate the effect of GIT1 expression on YAP/TAZ transcriptional activity. Similar to βPIX and LATS2, overexpression of GIT1 led to a marked decrease in TEAD-reporter activity in NMuMG mammary epithelial cells and HeLa cells, suggesting that GIT1 restricts YAP/TAZ function (Figure 4.1A).

To further investigate the role of GIT1 in YAP/TAZ regulation, I evaluated the effect of GIT1 depletion on expression of YAP/TAZ target genes by real-time PCR. siRNA-mediated abrogation of GIT1 in NMuMG mammary epithelial cells resulted in a marked increase in the expression level of well-known YAP/TAZ target genes CTGF, ANKRD1 and CYR61 (Figure 4.1B). To determine whether GIT1 can also regulate YAP/TAZ in other cell types, I examined the effect of GIT1 abrogation on the expression of target genes in C3H10T fibroblastic mesenchymal cell, HCT116 colorectal cancer cells and MCF10a mammary epithelial (Figure 4.2). Loss of GIT1 in these cells led to a marked increase in the expression of YAP/TAZ target genes, suggesting that GIT1 role in regulating YAP/TAZ is not restricted to one cell or tissue type.

4.4.2 GIT1 regulates cell proliferation in mammary epithelial cells

YAP/TAZ are co-transcription factors that induce a growth promoting gene expression program, and increased activity of YAP/TAZ promotes cell survival, proliferation, migration and ultimately tumorigenesis (Hansen et al. 2015). Given the effect of GIT1 knockdown in promoting YAP/TAZ transcriptional activity, I speculated that GIT1 depletion might result in increased cell proliferation. To test this, GIT1 was abrogated in MCF10a cells using siRNA and cell proliferation was monitored by counting cell numbers. I also depleted YAP and TAZ and compared their effect on cell proliferation with that of GIT1 knockdown. Concordantly, loss of GIT1 promoted cell proliferation whereas YAP depletion attenuated proliferation and TAZ knockdown had a modest effect (Figure 4.3A).
Figure 4.1: Git1 regulates YAP/TAZ transcriptional activity.

(A) NMuMG or HeLa cells were transfected with a TEAD-luciferase reporter and plasmids encoding Git1, LATS2 and βPIX or pCMV5 as control. YAP/TAZ transcriptional activity was measured by luciferase assay and data are shown as the mean ± standard deviation of triplicate samples. (B) NMuMG cells were transfected with control siRNA or a pool of siRNAs targeting Git1. The mRNA levels of Yap/Taz target genes, Ankrd1, Ctgf and Cyr61 were measured by qPCR and Git1 knockdown efficiency was confirmed. (C) Deconvolution of Git1 siRNAs. NMuMG cells were transfected with single oligonucleotides comprising the siRNA pool. Git1 knockdown efficiency and Ctgf relative expression was determined by Real-time qPCR.
Figure 4.2: Loss of GIT1 induces YAP/TAZ activity in multiple cell types.

C3H10T (A), HCT116 (B) and MCF10a (C) cells were transfected with siControl or siGIT1 and the relative expression of YAP/TAZ target genes, ANKRD1 and CTGF were measured by Real-time PCR.
Figure 4.3: GIT1 interacts with the Hippo core kinase and regulates cell growth.

(A) MCF10a mammary epithelial cells were transfected with control siRNA or siRNA targeting GIT1 and cell proliferation was monitored by counting the cells during a 96 hour time period. (B) HEK293T cells were transfected with HA-tagged GIT1 and Flag-tagged YAP, TAZ or LATS1. Cell lysates were subjected to anti-Flag immunoprecipitation and the presence of GIT1 was evaluated by anti-HA immunoblotting. Equal protein expression was determined (Totals).

4.4.3 GIT1 interacts with LATS1 and co-localizes with Hippo components on vesicular structures

The Hippo kinase cascade is the major determinant of YAP/TAZ subcellular localization and transcriptional activity. However, it has been reported that YAP/TAZ can also be regulated in Hippo-independent manners (Hansen et al. 2015). For instance, AMPK inhibits YAP activity by directly phosphorylating YAP at Ser94 and disrupting YAP-TEAD interactions (Mo et al. 2015). Furthermore changes in actin cytoskeleton dynamics have been suggested to directly modulate YAP/TAZ independent of the Hippo kinase cascade (Dupont et al. 2011). It was shown in Chapter 2 of this thesis that the GIT1-intercating partner, βPIX, forms a complex with both LATS kinase and YAP/TAZ to promote YAP/TAZ phosphorylation in response to various upstream cues. To gain more insight into the possible mechanisms of GIT1 function, I examined the physical interactions between GIT1 and Hippo core components LATS, YAP and TAZ.

For this, Flag-tagged YAP, TAZ and LATS1 were co-expressed with HA-GIT1 and the interactions were examined by anti-Flag immunoprecipitation followed by anti-HA immunoblotting (Figure 4.3B). This analysis showed that GIT1 interacts with LATS1 kinase but not YAP and TAZ, suggesting a possible LATS-mediated mechanism for YAP/TAZ regulation. While suggestive, this data does not prove that GIT1 regulates the Hippo kinase cascade and further analysis such as evaluation of YAP and LATS phosphorylation are required.

To further evaluate the relationship between GIT1 and the Hippo core components, I asked whether GIT1 expression regulates the subcellular localization of YAP and/or LATS. Transient expression of Flag-Git1 promoted a clear loss of nuclear YAP, which is consistent with the TEAD reporter and knockdown experiments (Figure 4.1).
Notably transfected Flag-Git1 showed a prominent punctate localization pattern on what appear to be vesicle-like structures with a striking co-localization of YAP on these structures (Figure 4.4A). Moreover, I also observed a similar co-localization of overexpressed Flag-LATS1 with HA-Git1 on vesicle-like structures (Figure 4.4B). These observations are noteworthy in light of recent findings in *Drosophila*, where protein interaction mapping identified multiple members of the endosomal pathway as interacting partner of the Hippo core components (Kwon et al. 2013). In addition, overexpression of an arrestin family related protein *leash*, was shown to promote the localization of the YAP ortholog, Yki to vesicular structures (Kwon et al. 2013). In this study, the vesicles were identified as lysosomes and early endosomes. Elucidating the identity of these vesicles in mammalian cells and determining their role in Hippo pathway regulation warrants further studies.
**Figure 4.4:** GIT1 overexpression promotes cytoplasmic YAP and co-localizes with YAP and LATS on vesicular structures.

(A) NMuMG cells were transfected with Flag-GIT1 and the subcellular localization of transfected GIT1 and endogenous Yap were analyzed by confocal immunofluorescence microscopy using anti-Flag and anti-Yap antibodies, respectively. (B) NMuMG cells were co-transfected with HA-tagged GIT1 and Flag-tagged LATS1, and the subcellular localization of transfected proteins was determined by confocal immunofluorescence microscopy using anti-HA and anti-Flag antibodies, respectively.

### 4.4.4 DLG5 regulates YAP phosphorylation and subcellular localization

DLG5 was identified as a binding partner of MST1/2 and MARK3 by Dr. Emili’s laboratory through affinity purification followed by mass spectrometry (AP/MS) (Kwan et al. 2016). Investigation of Hippo pathway activity in Dlg5-/− knockout mice revealed a prominent activation of the Hippo pathway in neuronal progenitor cells in Dlg5-/− compared to wild-type mice (Kwan et al. 2016). Through biochemical analysis, Kwan and colleagues demonstrated that DLG5 act as a scaffolding protein that brings MARK kinases to the Hippo core components MST1/2, thereby facilitating the inhibitory phosphorylation of MST1/2 by MARKs.

To determine whether the role of DLG5 in regulating Hippo pathway also extends to human cancer cells, I analyzed the effect of DLG5 abrogation in breast cancer MDA-MB-231 cells and liver cancer HepG2 cells. Disruption of DLG5 expression resulted in a decrease in YAP/TAZ protein levels in both MDA-MB-231 and HepG2 cells (Figure 4.5A), however YAP phosphorylation was only increased in MDA-MB-231 cells as assessed by immunoblotting on PhosTag gels (Figure 4.5A). In HepG2 cells YAP was highly phosphorylated even in cells transfected with siControl, suggesting that Hippo pathway is more active in these cells compared to MDA-MB-231 cells, hence DLG5 disruption wouldn’t further increase YAP phosphorylation. MST and LATS levels also showed a modest decrease upon DLG5 knockdown which may be due to the feedback mechanisms that are involved in the Hippo pathway homeostasis (Chen et al. 2015; Moroishi et al. 2015). In addition, DLG5 knockdown promoted cytoplasmic localization of YAP in MDA-MB-231 cells as analyzed by immunofluorescence microscopy (Figure 4.5B).
Figure 4.5: Loss of DLG5 activates the Hippo pathway.

(A-C) MDA-MB-231 and HepG2 cells were transfected with control siRNA or siRNA targeting DLG5. (A) DLG5 regulates YAP subcellular localization. YAP localization in MDA-MB-231 cells was evaluated by confocal immunofluorescence microscopy using anti-YAP antibodies. (B) Cell lysates separated on normal or PhosTag SDS-PAGE gels, were analyzed by immunoblotting using the indicated antibodies. Total levels of YAP, TAZ, MST1, LATS1, DLG5 and GAPDH as the loading control were determined as indicated. YAP phosphorylation was assessed by mobility shift on PhosTag gel. (C) Loss of DLG5 inhibits YAP/TAZ transcriptional activity. The relative expression of YAP/TAZ and YAP/TAZ target genes, ANKRD1, CTGF and CYR61 was determined by Real time-qPCR. DLG5 knockdown efficiency was also confirmed.

Changes in subcellular localization and phosphorylation of YAP was corroborated by a decrease in transcriptional activity. DLG5 abrogation led to a marked decrease in the expression of YAP/TAZ target genes, ANKRD1, CTGF and CYR61 in both MDA-MB-231 and HepG2 cells as assessed by real-time PCR (Figure 4.5C).

Altogether these findings show that DLG5 functions as a negative regulator of the Hippo pathway and loss of DLG5 results in increased phosphorylation, cytoplasmic retention and subsequent degradation of YAP/TAZ.
4.5 Discussion

We have previously shown that βPIX and MARKs regulate YAP/TAZ activity in response to multiple upstream cues. In this study we examined the role of GIT1 and DLG5, the binding partners of βPIX and MARKs, respectively, in Hippo signalling and we demonstrate that similar to their binding partners, GIT1 and DLG5 function as negative and positive regulators of YAP/TAZ, respectively. We show that loss of GIT1 induces the expression of YAP/TAZ target genes in multiple cell types. Moreover, GIT1 depletion in mammary epithelial cells results in an increase in cell proliferation, consistent with the known growth promoting effects of higher YAP/TAZ activity. Conversely, abrogation of DLG5 leads to a marked decrease in the expression of YAP/TAZ target genes concomitant with a decrease in total protein levels, higher YAP phosphorylation and cytoplasmic localization of YAP/TAZ, indicative of Hippo pathway activation.

The Hippo signalling pathway senses and integrates multiple microenvironmental cues such as cell density, physical properties of extracellular matrix, GPCRs and energy status of the cell. Although the characterized upstream regulators of the Hippo pathway have been studied separately, these processes are highly interconnected. This suggests the existence of molecular mediators that might receive and integrate multiple upstream signals to regulate Hippo kinase cascade. GIT1 and DLG5 in association with βPIX and MARKs are involved in regulating various cellular processes.

GIT1 is a versatile protein that has been reported to interact with GPCR kinases (GRKs) with possible roles in feedback regulation of G-protein coupled receptor signalling. It is also localized at the focal adhesions, connecting stimuli from extracellular matrix to actin cytoskeleton dynamics. G-protein coupled receptors are known to regulate Hippo pathway and it has been suggested that GPCRs modulate LATS1/2 kinases through actin cytoskeleton remodeling, although the mechanistic details are not clear. Intriguingly we find that GIT1 interacts with the Hippo core kinase LATS, suggesting that GIT1 might be able to integrate signals from GPCR signalling and actin remodeling to the Hippo kinase cascade. Whether GIT1 actually plays a role in these contexts require further investigation. Moreover, we observed that GIT1 shows a distinct vesicular-like localization pattern when overexpressed in mammary epithelial cells. GIT1 is a GTPase-activating protein (GAP) for Arf GTPases that are involved in vesicular trafficking and endosomal recycling.
pathway, however the role of GIT1 in this context is not clear. Interestingly, a recent study with the aim of characterizing the Hippo pathway interactome in *Drosophila* uncovered a strong association between endocytosis and vesicular trafficking complexes, including multiple Arf and Rab GTPases, with Hippo core components. Further characterization of interactors identified a member of the arrestin family, *leash* as an inhibitor of Yki. When overexpressed, *leash* was shown to localize and recruit Yki to vesicular structures that were identified as lysosomes and early endosomes using specific markers. Notably, Git has a conserved role in regulating the Hippo pathway in *Drosophila*. It has been shown that Git, in complex with Pix, acts as a scaffold for the Hippo core kinase Hpo and promotes the kinase activity (Dent et al. 2015). However, the contexts and upstream stimuli that regulate Git in the fly were not investigated in this study. Whether Git is involved in regulating the endosomal pathway in *Drosophila* has not been documented, and the exact role of the endosomal pathway in Hippo pathway in *Drosophila* and mammals awaits further investigations. Nonetheless the resemblance between YAP and Yki localization in vesicles upon GIT1 expression and the connection between GIT1 and Arf GTPases makes GIT1 an interesting candidate to study in this context.

In contrast to GIT1, abrogation of DLG5 leads to a marked decrease in the expression of YAP/TAZ target genes concomitant with loss of nuclear YAP/TAZ, increased YAP phosphorylation and a decrease in total protein levels. Interestingly, affinity purification/mass spectrometry identified DLG5 as the binding partner of MARKs and the Hippo core kinases MST1/2 (Kwong et al). Further biochemical analysis demonstrated that DLG5 acts as a scaffold protein, and recruits MARKs to inhibit MST kinase activity. Of note, co-expression of DLG5 and MST, promotes the inhibitory phosphorylation of MST detected on PhosTag gels, reminiscent of the pattern observed upon MARK expression. Indeed, a DLG5 construct lacking a MARK binding domain is unable to induce MST phosphorylation, suggesting that the phosphorylation is mediated by MARKs.
Chapter 5
General Discussions and Future Directions
5 General Discussions and Future Directions

5.1 Summary

The Hippo signalling pathway has emerged as a critical regulator of growth control and organ size during the last decade. Since the initial characterization of this evolutionarily conserved pathway, efforts have been made to better understand i) the biological implication of the Hippo pathway and defects associated with aberrant regulation and ii) to uncover the upstream regulators. This thesis was aimed at identifying novel upstream regulators of the Hippo signalling pathway and to further characterize the mechanisms of action. In Chapter 2, I identified ARHGEF7 also known as βPIX, as a positive regulator of the Hippo pathway that inhibits the YAP/TAZ transcriptional activity in response to cell-cell contact and actin cytoskeleton remodelling by scaffolding the interaction between LAST and YAP/TAZ. In Chapter 3, microtubule affinity regulated kinases (MARKs) were identified as activators of TEAD-reporter luciferase activity in a high-throughput screening. I demonstrated that MARKs inhibit the Hippo kinase cascade and enhance YAP/TAZ transcriptional activity by disrupting complex formation between the Hippo core components, MST, SAV and LATS, hence promoting the tumorigenic properties of breast cancer cells. In Chapter 4, I examined the potential role of the known binding partners of βPIX and MARKs, namely GIT1 and DLG5 in the Hippo signalling. I demonstrated that GIT1 knockdown promotes YAP/TAZ transcriptional activity and cell proliferation in mammary epithelial cells, whereas DLG5 depletion results in loss of nuclear YAP/TAZ and repression of transcriptional activity.
5.2 βPIX regulates the Hippo pathway in response to multiple cues

5.2.1 βPIX interacts with the Hippo core components to restrict YAP/TAZ activity

In Chapter 2, I identified ARHGEF7, also known as βPIX, as novel upstream regulator of the Hippo pathway that positively regulates the Hippo core kinase, LATS to restrict YAP/TAZ activity. In a high throughput protein-protein interaction screen conducted in the laboratory, βPIX showed strong interactions with the Hippo core components LATS1/2 and YAP, which were further confirmed by immunoprecipitation followed by immunoblotting. Through loss of function experiments, I showed that βPIX depletion results in higher YAP/TAZ transcriptional activity and promotes nuclear accumulation of YAP/TAZ in response to high cell density and loss of actin stress fibers. Further, I demonstrated that loss of βPIX attenuates YAP phosphorylation in both contexts, suggesting a role for βPIX in regulating the Hippo kinase cascade. Indeed, LATS1/2 are required downstream of βPIX to promote cytoplasmic YAP localization upon overexpression. Mechanistically, I demonstrated that βPIX functions as a scaffolding protein to enhance the interaction between LATS and YAP/TAZ, thereby promoting LATS-mediated phosphorylation and cytoplasmic accumulation of YAP/TAZ. Using a number of mutant constructs for interaction mapping, I showed that the KER domain, a region enriched in charged amino acids at the C-terminus of βPIX, mediates LATS-YAP binding. This region is also required for interaction with GIT1, suggesting that βPIX-GIT1 interaction might be important in regulating the Hippo pathway. Interestingly, a study by Dent and colleagues demonstrated that Pix has an evolutionarily conserved role in Hippo signalling in Drosophila (Dent et al. 2015). This study revealed that Pix in complex with Git, functions as a scaffold to activate Hpo kinase in a GEF-activity independent manner. Concordantly, I showed that the βPIX mutant constructs defective in GEF activity, are capable of binding and inhibiting YAP/TAZ, suggesting that GEF activity is dispensable in regulating the Hippo pathway. Although the point of regulation in the Hippo kinase cascade has diverged from Hpo, the MST ortholog, to LATS in mammals but scaffolding function of βPIX has been conserved.
5.2.2 The connection between βPIX and focal adhesions in regulating the Hippo pathway

βPIX regulates the Hippo-YAP signalling downstream of multiple cues such as cell-cell contact and actin cytoskeleton reorganization, however the mechanisms that engage βPIX to regulate the Hippo pathway are not known. βPIX has a well-established role in focal adhesion dynamics and actin cytoskeleton remodeling downstream of cell-matrix interactions (Kim et al. 2001; Rosenberger and Kutsche 2006; Frank and Hansen 2008). Interestingly, major components of the focal adhesions such as FAK, SRC and integrin-linked kinase (ILK) have recently been shown to play critical roles in regulating YAP and TAZ through various mechanisms (Serrano et al. 2013; Kim and Gumbiner 2015; Taniguchi et al. 2015; Li et al. 2016). As an important component of the focal adhesion, βPIX in complex with GIT1 is recruited to focal contacts upon cell-matrix engagement to organize downstream signalling events and to regulate focal adhesion turnover, actin cytoskeleton remodeling and cell migration (Rosenberger and Kutsche 2006; Frank and Hansen 2008). In a study to characterize the protein composition and dynamics of focal contacts, Kuo and colleagues revealed an antagonistic relationship between βPIX and actin stress fibers during focal adhesion maturation (Kuo et al. 2011). Accordingly, nascent focal contacts showed an enrichment for βPIX and its binding partners, whereas mature focal adhesions were depleted of βPIX and rather associated with actin stress fiber components such as RhoA, ROCK and contractile Myosin II (Kuo et al. 2011).

Interestingly, a recent study reveals that the exclusivity between βPIX and components of mature focal adhesions, plays a role in regulating the Hippo-YAP pathway in response to matrix stiffness and mechanotransduction (Chakraborty et al. 2017). Accordingly, culturing cells on stiff matrix promotes focal adhesion formation by activating Focal Adhesion Kinase (FAK), and at the same time βPIX is excluded from focal adhesions and cortical regions of the cells. In contrast βPIX displays a cortical localization when cells are cultured on soft matrix with concomitant recruitment of Hippo core components, which is consistent with the scaffolding function of βPIX proposed in Chapter 2 of this thesis (Chakraborty et al. 2017). In spite of these findings, the mechanisms that regulate subcellular localization of βPIX and engagement with the Hippo core components in response to either mechanotransduction or cell-density sensing are not known and warrant further investigations.
One plausible mechanism is posttranslational modifications in response to mechanical cues and focal adhesion dynamics. It is known that βPIX can be phosphorylated by SRC tyrosine kinase and PKA (Feng et al. 2006; Chahdi and Sorokin 2008b), both of which have been implicated in regulating the Hippo-YAP pathway, although with opposing effects (Kim et al. 2013; Yu et al. 2013; Kim and Gumbiner 2015; Taniguchi et al. 2015; Li et al. 2016). Thus, it would be worthwhile to determine whether these phosphorylation events might affect βPIX subcellular localization or interaction with components of the Hippo pathway.

Investigating the protein interaction dynamics of βPIX in response to cell-cell contacts or mechanical cues might also provide insight into the biochemical events that engage βPIX with the Hippo pathway core components in response to these upstream signals. For instance, βPIX and GIT1 form a multiprotein complex that is dynamically regulated in response to cell-ECM contacts and focal adhesion formation and subsequently organize downstream signalling events. As I demonstrated in Chapter 4, GIT1 also interacts with the Hippo core kinase LATS1 and loss of GIT activates YAP/TAZ transcriptional activity. Affinity purification followed by mass spectrometry can be utilized to characterize the dynamics of βPIX binding partners and potentially identify novel interactor that are important in regulating βPIX in the context of Hippo signalling.

5.2.3 Investigating the role of βPIX in tumorigenesis

As it was shown in Chapter 2, βPIX overexpression in MDA-MB-231 cells which are aggressive breast cancer cells attenuates tumorigenic features such as cell proliferation and migration. However, these assessments were done by in vitro assays over a short period of time. It would be worthwhile to evaluate the effect of βPIX expression on tumorigenesis using in vivo assays such as xenograft transplantation or matrigel colony formation assays. It needs to be noted that βPIX is also a GEF for Rac and Cdc42, both of which have well-known tumorigenic functions (Sahai and Marshall 2002). Higher activity of Rac1/Cdc42 promotes cell motility and invasiveness of cancer cells. Moreover, they have been implicated in cell cycle progression downstream of growth factors (Sahai and Marshall 2002). Thus, long term expression of βPIX might contribute to tumor progression by activating Rac1/Cdc42 GTPases, confounding the inhibitory effects on YAP/TAZ. Therefore, it is essential to distinguish between the GEF activity and YAP/TAZ inhibitory functions, when evaluating the role of βPIX in tumorigenesis in vivo. This can be achieved using
different mutant constructs of βPIX. As I demonstrated in Chapter 2, the GEF activity of βPIX is dispensable for inhibiting YAP/TAZ activity, while deletion of KER domain at the C-terminal region of βPIX induces cytoplasmic YAP/TAZ without disrupting the GEF activity. These constructs can be stably expressed in cancer cells to investigate their effects on tumorigenesis compared with the wild-type βPIX construct.

5.3 MARK4 as a negative regulator of the Hippo kinase cascade

5.3.1 MARK4 promotes YAP/TAZ activity

In an attempt to identify positive regulators of YAP/TAZ transcriptional activity we undertook a functional screening using TEAD-luciferase reporter. Multiple members of the microtubule affinity regulated kinases (MARKs) strongly activated the TEAD reporter in this screen, suggesting that MARKs act as negative regulators of the Hippo kinase cascade to promote YAP/TAZ transcriptional activity. Using loss of function experiments, I demonstrated that MARK4 knockdown in MDA-MB-231 breast cancer cells results in a marked decrease in the expression of YAP/TAZ target genes, concomitant with loss of nuclear localization as assessed by immunofluorescence microscopy. This was paralleled by LATS-mediated phosphorylation of YAP, as determined by immunoblotting, providing evidence that MARK4 regulates YAP/TAZ activity through the Hippo kinase cascade. The importance of LATS-mediated phosphorylation downstream of MARK4 knockdown was further corroborated using MDA-MB-231 cells, stably expressing a mutant version of TAZ(S89A), which is refractory to LATS-mediated phosphorylation. TAZ(S89A) mutant shows a constitutive nuclear localization in MDA-MB-231 cells, and loss of MARK4 does not promote cytoplasmic localization.

5.3.2 Determining phosphorylation sites on MST and SAV

In order to determine the mechanisms by which MARK4 regulates Hippo kinase cascade, I demonstrated that MARK4 binds to the Hippo core components, MST and SAV. Furthermore, I showed that MARK4 is capable of phosphorylating MST and SAV, as assessed by upshift on
PhosTag gels and by an *in vitro* kinase assay. However, to definitively prove that MARK4 phosphorylates MST and SAV, it is important to identify the phosphorylation sites and to further investigate the role of these phosphorylation events in the Hippo signalling by site-directed mutagenesis. In an attempt to do this, I investigated the potential phosphorylation sites on MST2 by mutating Serine residues to Alanine and examining the effect of MARK4 expression on MST2 phosphorylation using PhosTag gels. Interestingly, mutating Ser444 dramatically diminished but did not completely abolish the MARK4 induced upshift on the PhosTag gel (unpublished data). This observation suggests that MARK4 phosphorylates MST2 at other residues that have yet to be determined. Of note, it has been shown that Par-1 in *Drosophila* can also inhibit the Hippo pathway by phosphorylating the MST ortholog, Hpo which results in reduced Hpo kinase activity and diminished Hpo-Sav binding (Huang et al. 2013). However, MARK4 has no effect on MST kinase activity as evaluated by MST autophosphorylation and phosphorylation of MOB1 as the MST substrate. Furthermore, there is no evidence that Par-1 can phosphorylate SAV in *Drosophila*. Thus, the MARK/Par-1 mechanism of action in the Hippo pathway in *Drosophila* and mammals has diverged.

5.3.3 MARK4 disruption of complex formation between Hippo components parallels observations with other AMPKs

I demonstrated that MARK4 inhibits the Hippo signalling pathway by disrupting complex formation between the Hippo core components, MST-SAV-LATS in a kinase dependent manner. Notably, Salt-inducible kinases (SIKs), which belong to the AMPK related family, have been shown to phosphorylate the adaptor protein Salvador in *Drosophila*, and disrupt complex formation between Sav, Hpo and Wrts, the orthologs of SAV1, MST1/2 and LATS1/2 respectively (Wehr et al. 2013). Although the identified SIK phosphorylation sites on Salvador are not conserved in mammals, loss of interaction between the Hippo core components resembles the mechanism of action of MARK4. Altogether, findings presented in Chapter 3 of this thesis and the aforementioned studies demonstrate that MARK/Par-1 proteins and other AMPK family members have evolutionarily conserved roles in regulating the Hippo pathway, the mechanisms of action have diverged throughout evolution.
5.3.4 Potential mechanisms of MARK regulation

As discussed in Chapter 1, MARK along with 13 other closely related kinases that belong to the family of AMPK related kinases can be phosphorylated by LKB1, leading to the activation of kinase activity (Lizcano et al. 2004). However, LKB1 also phosphorylates and activates AMPK which has an opposing effect on the Hippo pathway compared to the MARKs (Mo et al. 2015; Wang et al. 2015). Therefore, it is not clear whether activation by LKB1 plays a role in regulating MARK kinase activity in the context of the Hippo kinase cascade. Given the well-known tumor suppressive function of LKB1, it does not seem very plausible that MARKs become activated by LKB1 to promote the activity of oncogenic YAP/TAZ. Indeed, recent studies have shown that LKB1 activity leads to Hippo pathway activation in mammals and in Drosophila, repressing YAP/TAZ or Yki activity, respectively (Mohseni et al. 2014; Gailite et al. 2015).

On the other hand, aPKC as part of the PAR complex is capable of phosphorylating and inhibiting MARK/Par-1 kinase activity (Chen et al. 2006; Hurov and Piwnica-Worms 2007). The PAR complex as an essential regulator of the apical polarity establishment in epithelial cells by cooperating with tight junction components such as the Crumb complex which has been implicated in Hippo pathway regulation (Hurov et al. 2004; Suzuki et al. 2004; Varelas et al. 2010b). Thus, it is plausible that the PAR complex, by antagonizing MARK/Par-1 activity promotes Hippo pathway activation upon tight junction formation. Moreover, the identification of DLG5 polarity protein, as a novel binding partner of MARK and a negative regulator of the Hippo kinase cascade provides further evidence for a potential role of cell polarity in regulating MARK kinases (Kwan et al. 2016).

5.4 GIT1 and DLG5 in regulating YAP/TAZ

5.4.1 GIT1 inhibits YAP/TAZ transcriptional activity

Given the strong association between βPIX and GIT1 and functional similarities in various contexts, I sought to determine whether GIT1 also has a role in regulating the Hippo-YAP signalling in mammalian cells. Through loss-of-function experiments using siRNA mediated knockdown, I showed that GIT1 depletion leads to a marked increase in the expression of
YAP/TAZ target genes in mammary epithelial cells. This suggests that GIT1 also acts as a positive regulator of the Hippo pathway to restrict YAP/TAZ activity. Interestingly, it has been shown that Git, in association with Pix, regulates the Hippo pathway in *Drosophila* (Dent et al. 2015).

To gain insight into the mechanism of mammalian GIT1 function, I investigated the physical interactions between GIT1 and the Hippo pathway core components, LATS1 and YAP. Notably, GIT1 showed a specific binding to LATS1 but not YAP, suggesting that GIT1 might regulate the LATS kinase to inhibit YAP/TAZ transcriptional activity. To further investigate the role of LATS in mediating GIT1 function, it will be necessary to evaluate YAP phosphorylation at Ser127 or Ser397, which are LATS phosphorylation sites. Moreover, it would be pertinent to examine the effect of GIT1 knockdown on LATS kinase activity by analyzing phosphorylation at Ser909 which is the autophosphorylation site.

5.4.2 GIT1 subcellular localization and the possible role for endosomal pathway in the Hippo signalling

To investigate the role of GIT1 expression on YA/TAZ localization, I overexpressed GIT1 in mammary epithelial cells and analyzed GIT1 and YAP localization using confocal immunofluorescence microscopy. Notably, GIT1 showed a distinct vesicular localization in transfected cells and promoted cytoplasmic YAP. Interestingly the cytoplasmic YAP shows a partial co-localization with GIT1 at vesicular structures. Similarly, co-expression of LATS1 resulted in co-localization with GIT1 at vesicular structures. It would be worthwhile to determine the nature of these vesicles using specific endosomal or lysosomal markers and to further investigate the role of endosomal-lysosomal pathway in the Hippo-YAP signalling. Of note it has been shown that Yki, the YAP ortholog in *Drosophila* can be recruited to, and degraded by the lysosomal vesicles (Kwon et al. 2013). Interestingly, a recent study has identified YAP as an inhibitor of innate antiviral immunity that can be degraded by the lysosomal-endosomal pathway in response to viral infections (Wang et al. 2017). Whether this pathway is a common mechanism for regulating YAP abundance and activity is not known and would be worth investigating.
5.4.3 Investigating the role of GIT1 in cell proliferation and migration

Given the inhibitory role of GIT1 on YAP/TAZ activity, I hypothesized that GIT1 depletion would promote cellular processes associated with higher YAP/TAZ activity such as proliferation or migration. Indeed, GIT1 abrogation led to a noticeable increase in the proliferation of mammary epithelial cells while YAP knockdown markedly attenuated cell number. To confirm that the increase in cell proliferation upon GIT1 knockdown is due to higher YAP activity and not due to other pathways, it will be important to determine whether concomitant disruption of YAP expression would rescue the observed effect. Furthermore, the effect of GIT1 depletion on cell migration should be evaluated using in vitro and in vivo assays and further rescued by concomitant YAP or TAZ deletion.

5.4.4 Investigating the connection between DLG5-MARK and the Hippo core components

In an affinity purification followed by mass spectrometry (AP/MS) screen conducted by Dr. Emili’s laboratory, DLG5 was identified to bind to MST1/2 and MARK kinases, suggesting a role for DLG5 in the Hippo pathway through the MARK kinases. Functionally, DLG5 has a similar role as MARKs in restricting the Hippo kinase cascade and promoting YAP/TAZ transcriptional activity (Kwan et al. 2016). I demonstrated that DLG5 depletion results in loss of nuclear YAP/TAZ localization and a marked decrease in target gene expression. Mechanistically, DLG5 has been shown to induce MST phosphorylation in a MARK dependent manner, suggesting DLG5 acts as a scaffold to promote MST-MARK interaction and facilitate inhibitory phosphorylation of MST (Kwan et al. 2016). Interestingly, DLG5 showed interaction with SAV in the original AP/MS screen, however the relationship between DLG5 and SAV was not further pursued. It would be worthwhile to determine whether this interaction results in MARK mediated phosphorylation of SAV and to further investigate the importance of SAV-DLG5 binding in the Hippo pathway. The connection between DLG5 and MARKs is interesting given the role of DLG5 in apico-basal polarity and MARK function in microtubule dynamics. It is not known how the DLG5-MARK axis is being regulated and it would be important to determine whether changes in microtubule dynamics or cell polarity complexes are involved in modulating DLG5-MARK complex formation and thereby inhibition of the Hippo kinase cascade.
5.5 Conclusion and significance

The Hippo signalling pathway is at the core of organ size regulation from *Drosophila* to mammals. This evolutionarily conserved pathway is composed of Ser/Thr kinases that ultimately regulate the co-transcription factors YAP/TAZ in response to a variety of intrinsic and extrinsic factors. An outstanding question in the field of Hippo signalling is to understand how various upstream stimuli regulate the Hippo kinase cascade. In this thesis, I identified multiple novel upstream regulators of the Hippo signalling pathway that can activate or inhibit YAP/TAZ transcriptional activity, and further provided insight into the mechanisms by which they regulate the Hippo kinase cascade. Many unanswered questions remain to be addressed, especially to better understand the biological significance of the identified Hippo pathway components and the mechanisms through which they are regulated.
References


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