Understanding the Hippo-LATS pathway in tumorigenesis

By

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ABSTRACT

The Hippo-LATS signaling pathway originally identified in *Drosophila* is conserved in mammalian systems and serves essential roles in mediating size control as well as tumorigenesis. In humans, the core kinase cassette consisting of adaptor proteins WW45 and MOB1, and Ser/Thr kinases MST1/2 and LATS1/2 signal by phosphorylating and inactivating transcriptional co-activators YAP and TAZ, causing cell growth arrest. As the central kinases within the Hippo-LATS pathway, examining the cellular and molecular phenotypes of LATS1 and LATS2 (LATS) will provide insight into the role of this pathway in tumorigenesis.

By simultaneously knocking down both LATS1 and LATS2, genes that were differentially expressed were identified through a whole human genome microarray screen. The multitude of genes identified including *CYR61, MYLK, CDKN1A, SLIT2,* and *TP53INP1* not only provide further evidence for the role of LATS in cell proliferation and apoptosis, but also implicate LATS in novel functions such as cell motility. Loss of LATS1 and/or LATS2 enhances cell migration whereas overexpression of LATS1 dramatically inhibits cell migration in multiple cell lines. The ability of LATS to regulate cell migration occurs through two potential mechanisms. Firstly, LATS functions through its kinase substrates YAP and/or TAZ, or alternatively, LATS1 directly binds actin and inhibits actin polymerization. Thus, through loss of functions studies, we identified a novel role for LATS in regulating cell migration as well as novel mechanisms of LATS function.

As an important signaling molecule within the cell, LATS and the Hippo-LATS pathway are tightly regulated. Using clues from the *Drosophila* pathway, we examined
how the previously uncharacterized gene, hEx, functions within this pathway.

Importantly, this thesis characterizes hEx as a putative tumor suppressor showing that it can inhibit cell proliferation, sensitize cancer cells to Taxol treatment as well as inhibit tumor growth in nude mice. However, unlike *Drosophila* expanded, hEx functions independently of the Hippo-LATS pathway, suggesting that the mammalian signaling pathway is more complicated.

The research findings from this thesis enhance our knowledge of the Hippo-LATS pathway in tumorigenesis by elucidating new functions and mechanisms of LATS functions as well as by exploring how upstream components function in relation to this pathway.
ACKNOWLEDGEMENTS

My years as a graduate student have been both challenging and rewarding. My ability to succeed at this stage in my academic career is a result of the tremendous amount of support I have received from several sources.

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PUBLICATIONS

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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>AGC</td>
<td>Protein kinase A (PKA)/PKG/PKC</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>C-ERMAD</td>
<td>C-terminal FERM/ERM binding domain</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin kinase inhibitor</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>Crbs</td>
<td>Crumbs</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td><em>Drosophila melanogaster</em></td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dIAP</td>
<td><em>Drosophila</em> inhibitor of apoptosis</td>
</tr>
<tr>
<td>Dlg</td>
<td>Discs large</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin/Radixin/Moesin</td>
</tr>
<tr>
<td>Ex</td>
<td><em>Drosophila</em> expanded</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1/ezrin/radixin/moesin</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemaglutinin</td>
</tr>
<tr>
<td>hEx</td>
<td>Human Expanded</td>
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<td>HS</td>
<td>Horse serum</td>
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<tr>
<td>Hpo</td>
<td>Hippo</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LATS</td>
<td>Large tumor suppressor</td>
</tr>
<tr>
<td>Lgl</td>
<td>Lethal giant larvae</td>
</tr>
<tr>
<td>MATS</td>
<td>Mob as tumor suppressor</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Mer</td>
<td>Merlin</td>
</tr>
<tr>
<td>MOB</td>
<td>Mps one binder</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MST</td>
<td>Mammalian sterile-20 like</td>
</tr>
<tr>
<td>NDR</td>
<td>Nuclear Dbf2-related</td>
</tr>
<tr>
<td>NF2</td>
<td>Merlin</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidat P-40</td>
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<td>OA</td>
<td>Okadaic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PPXY</td>
<td>P, Proline; X, any amino acid; Y, tyrosine</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<td><em>S. cerevisiae</em></td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>S. pombe</td>
<td><em>Saccharomyces pombe</em></td>
</tr>
<tr>
<td>Sav</td>
<td>Salvadador</td>
</tr>
<tr>
<td>Scrib</td>
<td>Scribble</td>
</tr>
<tr>
<td>Sd</td>
<td>Scalloped</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labeling by amino acids in cell culture</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interference RNA</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>TAZ</td>
<td>Transcriptional co-activator with PDZ domain</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Trc</td>
<td>Tricornered</td>
</tr>
<tr>
<td>WW45</td>
<td>WW domain protein 45, human Salvador</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-associated protein</td>
</tr>
<tr>
<td>Yki</td>
<td>Yorkie</td>
</tr>
</tbody>
</table>

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**S. pombe**  
*Saccharomyces pombe*

Sav: Salvadador  
Scrib: Scribble  
Sd: Scalloped  
SD: Standard deviation  
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis  
shRNA: Short hairpin RNA  
SILAC: Stable isotope labeling by amino acids in cell culture  
siRNA: Small interference RNA  
SRB: Sulforhodamine B  
TAZ: Transcriptional co-activator with PDZ domain  
TCA: Trichloroacetic acid  
Trc: Tricornered  
WW45: WW domain protein 45, human Salvador  
YAP: Yes-associated protein  
Yki: Yorkie
PART I: GENERAL INTRODUCTION

CHAPTER 1: INTRODUCTION

Cancer is a leading cause of death in both men and women worldwide and can be thought of as a disease of malfunctioning cells (1). With this knowledge, investigating how healthy cells and the proteins within them normally function can enhance our understanding of the process of tumorigenesis and thus our ability to fight this disease.

The rapidly expanding field of cellular signaling programs has shown that the theory from Beadle and Tatum of "one gene, one protein, one function" no longer holds true. The growing number of proteins and the growing number of functions they perform has created a complex network of interplaying molecules. Therefore, the disruption of one gene during tumorigenesis, for example, can affect multiple signaling programs. Understanding how each protein fits within these networks will aid in our understanding of tumorigenesis. In mammalian systems, the emerging family of proteins, LATS (Large Tumor Suppressor), consisting of LATS1 and LATS2 (herein referred to as LATS unless stated otherwise), has increasingly been shown to be important in maintaining cellular homeostasis.

LATS is primarily recognized as a central player in the Hippo-LATS pathway, originally identified in Drosophila with functional homologs present in mammalian systems. The Hippo-LATS pathway consists of a group of conserved tumor suppressors and oncogenes including NF2, hEx, Kibra, MST1/2, LATS1/2, YAP and TAZ (for current review, see Zhao et al.(2)). Research shows that deregulation of most of these proteins can lead to malignant phenotypes (3-12). Therefore, understanding how the
Hippo-LATS pathway, in particular the central player LATS, operates and is regulated is key to understanding tumorigenesis.

Functioning as Ser/Thr kinases, LATS1 and LATS2 possess both broad regulatory functions such as the maintenance of genetic stability in addition to more specific roles in modulation of cell cycle checkpoints and induction of apoptosis. The work of the Yang lab and this thesis expands the number of functions attributed to LATS to include transcriptional regulation and modulation of cytoskeletal dynamics and cell migration. The importance of this work is highlighted by the fact that several groups have also implicated LATS in these new functions, especially in cell migration, since this project began. Furthermore, this thesis describes novel mechanisms of LATS function to include both kinase-independent and Hippo-independent signaling events.

Understanding how the Hippo-LATS pathway is regulated has become a highly investigated area of study. Within the last year alone, several novel negative regulators of the Hippo-LATS pathway have been identified (13-15). Working with clues from the Drosophila system and the proteins known to activate this pathway in that system, the work of this thesis characterizes hEx functions both with respect to its putative tumor suppressor function as well as its relationship to the Hippo-LATS pathway in human systems.

Therefore, the timely work of this thesis both enhances our understanding of the functions of LATS as well as how the Hippo-LATS pathway is regulated. Knowing that this pathway is critical in inhibiting tumorigenesis, this work may aide in the development of novel therapeutics targeting this pathway for cancer treatment.
CHAPTER 2: LITERATURE REVIEW

2.1 Molecular mechanisms of tumorigenesis

Normal cells compose the highly structured tissue system that functions efficiently and effectively for organismal survival. In contrast, tumors arise as a result of the breakdown of this highly ordered system and its normal cellular functions (1). Although inherently complex, simplified tumorigenesis can be viewed as a series of steps beginning with an initial mutating event converting a normal cell into a pre-malignant stage. Subsequent mutations lead to progressively aggressive stages ultimately ending with the metastatic state (16). This multistage progression generally leads to the acquisition of several key hallmarks including replicative immortality, sustained proliferation, escaping cell death, evading tumor suppressors, enhanced angiogenesis, activated invasion and metastasis. More recently, immune avoidance, genomic instability, tumor-promoting inflammation, and aberrant metabolism were also shown to be overarching qualities of tumor cells (17). Typically, these accumulated genetic alterations include inactivation of tumor suppressors combined with activation of oncogenes. Common targets in human cancers include the oncogene RAS or tumor suppressors p53 and pRb, among others (18, 19).

2.1.1 Tumor suppressor phenotypes. Tumor suppressors are classically defined by their loss of function such that loss of the tumor suppressor can lead to the formation of tumors (20). Because aberrant proliferation is observed in all tumors and because the cell cycle (G1, S, G2, M) is the tightly regulated biological process controlling cell proliferation, it is not surprising that most tumor suppressors play a role in regulating the cell cycle. Indeed, the first two tumor suppressors identified, pRb and p53, are intimately
linked to cell cycle progression. Firstly, subsequent characterization of the pRB gene after Knudson’s identification of its link to the development of retinoblastoma (19) showed it to be a critical regulator at the restriction point of the cell cycle. pRb inhibits the activity of the E2F family of transcription factors responsible for the transcription of pro-proliferation genes such as the E-type and A-type cyclins. When cells receive mitogenic signals, pRb is phosphorylated, leading to its release of the E2F transcription factors, upregulation of Cyclin E and Cyclin A and subsequent commitment to cell cycle progression (21).

Secondly, p53, the most widely disrupted gene in human cancers (19), can block cell cycle progression in response to DNA damage. Functioning as a transcription factor, p53 induces or represses the transcription of numerous genes involved in cell cycle progression. One of the most widely recognized upregulated targets is the p21<sup>Cipl</sup> cyclin kinase inhibitor (22). Other genes include 14-3-3σ, reprimo, and GADD45 (23). In this way, p53 is able to arrest cells at several points in the cell cycle including the G2-M transition and the G1-S transition (23).

More recently, tumor suppressors are proving to be influential in mediating a wide variety of cellular functions apart from their roles in regulating cell cycle progression and cellular proliferation. These new functions can include apoptosis and the development of drug resistance as well as cell migration and the development of metastasis. For example, p53 induces apoptosis in response to irreparable DNA damage by increasing the transcription of numerous pro-apoptotic genes (24). In line with this, increased expression of p53 correlated with increased sensitivity to Taxol in a cohort of ovarian cancer patients (25), although conflicting reports have been cited regarding the role p53
plays in drug sensitivity. Despite the complexity of cellular signaling programs, several other tumor suppressors also play a positive role in mediating a drug response to Taxol. For example, the well-known tumor suppressor APC (adenomatous polyposis coli) which protects against the initiation of colorectal tumors mediates sensitivity to Taxol. In addition to the development of mitotic defects upon loss of APC, when APC is knocked down, U20S cells are significantly more resistant to Taxol treatment (26). Similarly, expression of RASSF1A, a tumor suppressor downregulated by hypermethylation in a majority of tumors (27), acts synergistically with microtubule-damaging agents such as Taxol to increase cell death in lung cancer cell lines (28). Like RASSF1A, the well-known tumor suppressor BRCA1 in breast cancer increases sensitivity to Taxol. In this study, inducible expression of BRCA1 in a breast carcinoma cell line increased sensitivity 100-fold, whereas siRNA-mediated knock down lead to Taxol resistance (29). Therefore, tumor suppressors are important regulators of a drug response and their loss can lead to drug resistance.

Metastasis is often the fatal step in tumorigenesis and is responsible for the majority of cancer-related deaths (18). The enhanced ability of tumor cells to adopt migratory characteristics is a key feature of metastasis, and thus cell migration serves as a useful tool in understanding how molecules are dysregulated during metastasis. Importantly, tumor suppressors have been shown to play critical roles in controlling cell migration and thus metastasis. Again, p53 is a prime example of a tumor suppressor that, when deregulated, can drive cell migration and metastasis. Wildtype p53 influences the Rho GTPase family of cytoskeletal proteins essential for cell motility through its transcriptional activity. Consequently, loss of p53 enhances cell migration (30-32).
Similarly, loss of tumor suppressors such as APC (33), NF2 (34, 35), and RASSF1A (36) can enhance migration by interacting with various cytoskeletal proteins.

Therefore, in addition to their canonical function in regulating the cell cycle and cell proliferation, tumor suppressors are an important class of proteins that can affect multiple stages of tumor progression. Understanding their precise signaling networks and their functions will aide in our understanding of the tumorigenic process with the goal of creating improved therapeutics.

2.2 The Hippo-LATS pathway in tumorigenesis

Like other tumor suppressor networks, the Hippo-LATS pathway (Figure 1.1) is also involved in regulating multiple phenotypes. Originally identified in Drosophila and primarily recognized as a regulator of proliferation and apoptosis, more recent evidence has also emerged implicating this pathway in the control of cell polarity and migration. Importantly, the Hippo-LATS pathway is essential in regulating tumorigenesis since many of its components function as either tumor suppressors or oncogenes (reviewed in (12, 37)).

2.2.1 Hippo-LATS pathway in Drosophila. The use of genetic studies in Drosophila led to the identification of many of the signaling components within this pathway. The lats gene was first identified in 1995 by Tian Xu at Yale University in his search for tumor suppressor genes using genetic mosaics of Drosophila (D.) melanogaster(38), which identifies mutant tissue due to their dramatic overproliferation phenotype. He showed that dlat is a putative Ser/Thr kinase with homologs in budding yeast Dhf20, and Dhf2. Around the same time, a similar study identified a novel tumor
Figure 1.1: The Hippo-LATS pathway in *Drosophila* and mammals. The evolutionarily conserved Hippo-LATS signaling pathway components in *Drosophila* (A) and (B) mammals. Activation and inhibition are denoted by arrows or blunt-end arrows, respectively. Abbreviations as follows: ADJ, adherens junctions; BLJ, basolateral junctions; Crbs, Crumbs; dJub; Dlg, discs giant large; Ajuba; ex, expanded; hEx, human Expanded; Hpo, Hippo; Lgl, lethal giant larvae; Mats, Mob as tumor suppressor; Mer, merlin; NF2, Merlin; Sav, Salvador; Sd, scalloped; Scrib, Scribble; WW45, human Salvador; Yki, Yorkie.
suppressor called *warts* that, when lost due to mitotic recombination, produced a wart-like phenotype characterized by excessive overproliferation in imaginal disc epithelial cells of *D. melanogaster* (39). As *warts* and *dlats* encode the same gene, for consistency, we will use the *lats* notation.

Within several years, the remaining proteins making up the core cassette were identified, all of them sharing the characteristic mutant overgrowth phenotype. Both the adaptor protein salvador (*sav*) and the Ser/Thr kinase hippo (*hpo*) also inhibit cell cycle exit and induce apoptosis (40-43). The similarity of the overgrowth phenotype between *dlats*, *sav*, and *hpo* suggested that these three genes functioned within the same pathway and may form a complex. Indeed, *sav* binds *dlats* through its WW domains (40) and *hpo* binds and phosphorylates both *sav* and *dlats* (41-43). The final core member identified, *Mob* as tumor suppressor (*Mats*) (44) also directly interacts with *dlats* (45) and *hpo* (46). Therefore, the core signaling cassette in *Drosophila* consists of the adaptor proteins *Mats* and *sav*, both of which can be phosphorylated by the Ser/Thr kinase *hpo*. These phosphorylation events enhance association of *dlats* with *hpo* and subsequent activation of *dlats* and the Hippo-LATS pathway (Figure 1.1A).

The proliferative and apoptotic functions characteristic of this signaling pathway depend on its transcriptional regulation of particular genes such as *cyclin E* and *diap1*. Coupling the transcriptional output with the kinase cassette is Yorkie (*Yki*), a transcriptional regulator which binds to and activates the transcription factor Scalloped (*Sd*). Not only was *Yki* originally identified as a member of this pathway due to its overgrowth phenotype upon overexpression but was also the first kinase substrate of *dlats* to be identified. By phosphorylating *Yki*, *dlats* inhibits the transcriptional activity of *Yki*
(47). Because Yki belongs to a class of transcriptional regulators that cannot directly bind DNA, the identification of Scalloped as a key transcription factor mediating Hippo-LATS signaling provided the final link between this pathway and its cellular effects (48, 49).

In *Drosophila*, external growth signals and regulation of this pathway involves several complexes. Directly upstream lay the FERM domain cytoskeletal crosslinking proteins Merlin (Mer) and Expanded (Ex). These two adaptor proteins function together to enhance dlats and yki phosphorylation. Characteristically, loss of both mer and ex leads to tissue overgrowth and increased expression of Cyclin E (50, 51). More recently, Kibra was added to this complex, binding both mer and ex as well as dlats and hpo/sav (52, 53). In this way, Kibra acts as a scaffold promoting the assembly of mer and ex at the apical membrane and enhancing the activation of hpo and dlats. Although dlats is the central kinase on which many signals converge, recent evidence has also suggested that expanded can interact directly with yki through its PPXY (P, proline; X, any amino acid; Y, tyrosine) motifs, thereby bypassing dlats. This interaction prevents yki from translocating to the nucleus and eliciting its pro-proliferative effects (54).

Linking the Mer-Ex-Kibra complex to external cues are two signaling networks: the fat pathway and the crumbs polarity complex (Fig. 1.1A). The atypical cadherin and tumor suppressor fat was originally identified as a member of the Hippo-LATS pathway due to its shared transcriptional targets and its characteristic tissue overgrowth in genetic mutants (55). Fat activates the Hippo-LATS pathway through ex as well as in parallel to ex. In the former scenario, Fat binds ex, localizing it to the plasma membrane for
activation (56-58). In the latter scenario, Fat inhibits the ability of the unconventional myosin dachs to decrease dlats protein expression (55) (Fig 1.1A).

The second branch linking Mer-Ex-Kibra to external cues is the Crumbs (Crbs) polarity complex. The role of Crbs in regulating the Hippo-LATS pathway is controversial. One study shows that overexpression of Crbs causes mislocalization of ex and subsequent inactivation of the Hippo-LATS pathway leading to tumor growth (59) while another study shows that loss of Crbs leads to the characteristic tissue overgrowth due to its effects on ex (60). Interesting, another study showed how when the Hippo-LATS pathway is inactivated there is an increase in expression of certain polarity proteins including crbs (61), further linking these two signaling networks.

Another polarity complex consisting of Lethal giant larvae (Lgl), discs large (Dlg) and Scribble (Scrib) proteins have also been shown to modulate the Hippo-LATS pathway. Lgl mutant clones exhibit overproliferation similar to Hippo-LATS components (62). Unlike the Crbs complex, Lgl directly acts on the core cassette by affecting localization of Hpo and dRassf.

Other proteins known to regulate the Hippo-LATS pathway in Drosophila include dRassf and djub (Fig. 1.1A). The Ras-associated family member dRassf antagonizes the Hippo-LATS pathway by competing with Sav for Hpo binding, thereby inhibiting the activation of dlats (63). Secondly, the Ajuba protein djub in Drosophila directly interacts with dlats and sav to inhibit phosphorylation of Yki, thereby enhancing cell proliferation and inhibiting apoptosis (64). Therefore, in D. melanogaster, there are multiple modes of regulation within the Hippo-LATS pathway.
The elucidation of the Hippo-LATS pathway in *Drosophila* has provided innumerous clues about the regulation and downstream effectors of the core kinase cassette. Multiple signaling branches converge on this pathway to ultimately control cell proliferation and apoptosis.

### 2.2.2 Hippo-LATS pathway in mammals.

Much of the Hippo-LATS pathway is conserved in mammalian systems. In particular, the core kinase cassette consists of functional homologs signaling in an analogous manner (Fig. 1.1B).

Mammalian homologs for each of the core members include MST1/MST2 (Hpo), WW45 (Sav), LATS1/LATS2 (dlats), and MOB (Mats). Importantly, the overgrowth phenotype in Hpo, Mats, and dlats mutant flies can be rescued by expression of their mammalian counterparts: MST2, LATS1, and MOB1A, respectively (42, 44, 65). In addition, these proteins also function in an analogous manner in mammalian systems. Association between MST1/2 and WW45 is essential for MST1/2 phosphorylation and activation of LATS1/2 (66-68). Of the 7 human MOB proteins, only MOB1A and MOB1B are preferentially phosphorylated by MST1/2 (69) and can interact with and enhance activation of LATS1 and LATS2 (70). Since membrane-bound MOB1 has been shown to activate LATS1 (71), most likely LATS1 is recruited to the membrane by MOB1 to enhance its interaction and activation by MST1/2.

As in *Drosophila*, LATS signals through transcriptional co-activators in mammalian systems. YAP is the closest homolog to *Drosophila* Yki and its expression rescues the mutant phenotype in flies (47). By binding to YAP WW domains, both LATS1 (72) and LATS2 (73) can phosphorylate multiple Ser residues on YAP, based on the LATS consensus phosphorylation sequence HXRXXS/T (H, histidine; X, any amino
acid; R, arginine; S, serine; T, threonine) (72). Similarly, the YAP paralog TAZ, which shares 50 percent identity with YAP, is also phosphorylated by LATS (74). Of the 5 phosphorylation sites in YAP and the four sites in TAZ, one conserved phosphorylation site is essential for LATS function: S127 in YAP (72, 73), and S89 (74) in TAZ. Phosphorylation by LATS on these sites enhances association of YAP or TAZ with the cytoplasmic anchor 14-3-3, thereby inhibiting YAP/TAZ nuclear activity. In addition to inhibiting their translocation to the nucleus, LATS also stimulates their degradation. Recent evidence shows that LATS phosphorylation recruits the CK1ε kinase, leading to further phosphorylation within a phosphodegron and recruitment of the SCFβ-TRCP E3 ubiquitin ligase, followed by YAP (75) and TAZ (76) ubiquitylation and subsequent degradation. Thus, the signaling output of the Hippo-LATS pathway is tightly regulated by LATS.

Once in the nucleus, YAP and TAZ activate the *Drosophila* Sd homologs, the TEAD/TEF family of transcription factors, of which there are 4 mammalian homologs, TEAD1-4 (Fig 1.1B). Both YAP and TAZ activate members of the TEAD family (77-79). For example, YAP induces expression of *CTGF* and *ITBG2* through TEAD1 as part of its growth conducive function (78). In addition, TAZ induces *CYR61* and *CTGF* expression through TEAD4 to maintain a drug resistant phenotype in TAZ-overexpressing MCF10A cells (80). Interestingly, the key genes mediating the effects in *Drosophila*, cyclin E and *diap1*, have not been identified as transcriptional targets in mammalian systems, suggesting some divergence between *Drosophila* and mammalian systems.
Unlike the *Drosophila* system, the key factors regulating the activity of the Hippo-LATS pathway and linking it with external growth cues is not well understood. For example, although human homologs exist for ex (hEx/FRMD6), fat (FAT4), Lgl (Lgl1-2), and Crbs (Crumbs1-3), their relationship to the Hippo-LATS signaling pathway has not been studied.

On the other hand, the homologs RASSF1A (dRassf), Ajuba/LIMD1/WTIP (djub), Merlin (mer), and KIBRA (Kibra) can regulate the Hippo-LATS pathway. The closest dRassf homolog, RASSF1A, functioning as a tumor suppressor in human cancer, complexes with MST2, WW45 and LATS1 and activates MST2 and LATS1 (81). The activation of the Hippo-LATS pathway due to RASSF1A is in contrast to the inhibitory effect of dRassf. However, another more distant dRassf homolog, RASSF6, also binds MST2 in a way that inhibits MST2 and LATS1 activity (82). Thus the regulation of the Hippo-LATS pathway in mammalian systems is complicated by the presence of multiple homologs for each *Drosophila* gene.

The Ajuba family members, Ajuba, LIMD1, and WTIP have all been shown to act like their *Drosophila* counterparts by binding LATS1 and LATS2 and inhibiting YAP S127 phosphorylation (64). Alternatively, expression of Merlin enhances phosphorylation of MST2 and LATS2 (83) and human KIBRA can bind and enhance NF2-mediated phosphorylation of both LATS1 and LATS2, thereby enhancing phosphorylation of YAP (52, 84). Human Merlin also directly binds YAP and inhibits its translocation and nuclear activity (85). Although these conserved components have been shown to regulate the Hippo-LATS pathway, the precise molecular mechanisms mediating these events are not known.
Other proteins, such as CD44 (86), Itch(14), NPHP4 (13), and ASPP1 (15) have very recently been shown to act as negative regulators of the Hippo-LATS pathway. The conservation of these new members of the Hippo-LATS pathway with Drosophila systems is not known but points to increased complexity of signaling patterns in mammalian systems.

2.2.3 The Hippo-LATS pathway in human cancer. The significance of this signaling pathway is solidified by its essential role in human tumorigenesis. Many of the core signaling components function as either tumor suppressors or oncogenes.

Preliminary evidence suggests that MOB1A/1B, MST1/2, and WW45 are tumor suppressors. Low mRNA MOB1A expression has been found in colorectal and lung cancer samples (87, 88) and a mutation has been found in a human melanoma cell line (44). Similarly, mutations in WW45 have been reported in colon and renal cancer cell lines (40) and protein expression is downregulated post-transcriptionally in kidney cancer cell lines (89). In addition, mice with liver-specific conditional WW45 knockout have increased number of liver tumor foci and significantly enlarged tumors (10, 90). Both MST1 and MST2 have also been implicated in liver tumorigenesis in mice. Although loss of only one protein has no effect on liver tumorigenesis, loss of both MST1 and MST2 leads to the development of hepatocellular carcinoma (10, 90-92). More importantly, expression of either MST1 or MST2 are downregulated in human colorectal cancer or soft tissue sarcomas, respectively (93, 94). The loss of MST1/2 or WW45 and the development of liver tumors is associated with increased activation of YAP (10, 90), highlighting the importance of the Hippo-LATS pathway in cancer progression. The above evidence suggests that these core Hippo-LATS pathway components may function
as tumor suppressors in human cancer, but more genetic and clinical analysis is needed to verify their role in human tumorigenesis.

Preliminary evidence also suggests that FAT4 functions as a tumor suppressor since it is downregulated in breast cancer (95). Of all the other upstream regulatory components of the Hippo-LATS pathway, only RASSF1A and NF2 have been shown to function as tumor suppressors in mammalian systems. RASSF1A is frequently hypermethylated and subsequently downregulated in the majority of human tumors including lung, breast, colorectal, hepatocellular, and leukemia, to name a few (27). The NF2 gene was initially discovered as the gene responsible for the development of the Neurofibromatosis type 2 cancer syndrome where patients develop various tumors of the nervous system. Since then, loss of NF2 function has been associated with the development of thyroid, hepatocellular, and colorectal carcinomas as well as melanoma (96). Like MST1/2 and WW45, inactivation of Merlin enhances YAP expression and activity in certain tumor cell lines (97).

The two downstream targets of LATS, YAP and TAZ, primarily function as oncogenes. Although widespread analysis of TAZ in human cancer has not been done yet, TAZ has been shown to be upregulated in 21 percent of breast cancers (98), and increasing TAZ protein levels correlate with increasing invasiveness in breast cancer cell lines (80). Recently, TAZ expression was also shown to be upregulated in human lung cancer cell lines (99). YAP is a more widely recognized oncogene and its expression is associated with cancers of the ovary, esophagus, colon, pancreas, liver, lung, as well as pancreatic, glioblastoma, oral squamous-cell carcinoma (100-106).
As the central signaling kinases within the Hippo-LATS pathway, LATS1 and LATS2 play important roles in tumorigenesis and much evidence exists for the tumor suppressor function. Mouse studies provided the first evidence that the LATS proteins function as tumor suppressors. Although loss of Lats2 is embryonic lethal (107, 108), mice which lack Lats1 develop ovarian or soft tissue sarcomas both spontaneously or in response to carcinogenic treatment (109).

Since then, an explosion of studies explicitly addressed the role of LATS in human tumors. LATS1 and/or LATS2 have been implicated in soft tissue sarcomas, leukemia, astrocytoma, and in cancers of the breast, prostate, lung, liver, and esophagus (3-11). Decreased expression of LATS2 is seen in prostate cancer tissues (8) and levels of phosphorylated LATS1/LATS2 are reduced in tumor susceptible mst1/2 liver-specific knockout mice (10). Classically, tumor suppressor loss of function occurs through mutations. However, mutations in LATS genes are rare. Single mutations have only been found in LATS2 in esophageal (11), lung cancer (9) or mesothelioma (110), and the rates of these mutations are small. Only 1/60 esophageal tumors had a LATS2 mutation, and 9/50 non-small cell lung carcinomas (NSCLC) had a mutation in the kinase domain. In mesothelioma, 10/45 malignant tumors carried inactivating deletions or mutations. However, despite limited mutations in LATS2 in lung cancer, expression of LATS2 is frequently downregulated, which implies alternate mechanisms of gene silencing.

Of the soft tissue sarcomas examined, myxoid liposarcoma, leiomyosarcomas, and malignant fibrous histiocytoma had downregulated LATS1 gene expression (3). Specific examination of the alterations in these tumors showed that the majority of these tumors had hypermethylated CpG islands in the promoter region. Indeed,
hypermethylation appears to be a major mechanism of LATS1 and LATS2 downregulation. Both LATS1 and LATS2 are hypermethylated in astrocytoma (6) as well as acute lymphoblastic leukemia (4) and breast cancer (111). Furthermore, in the cases of leukemia and breast cancer, the expression levels of LATS1 or LATS2 exhibited prognostic and/or predictive factor capabilities. Firstly, in acute lymphoblastic leukemia, it was shown that expression of LATS2 was the most important prognostic factor in predicting both disease-free survival and overall survival when surveying potential prognostic factors (4). In addition, loss of LATS2 leads to decreased sensitivity to two commonly used chemotherapeutic drugs, doxorubicin and etoposide, in leukemic cell lines (112), suggesting that expression of LATS2 might also serve as a valuable predictive factor.

In breast cancer, both LATS1 and LATS2 are downregulated by promoter hypermethylation and this decreased expression is associated with aggressive breast tumors characterized by large tumor size, high lymph node metastasis, and estrogen and progesterone receptors negative status. However, in this study, only LATS1 expression correlated with disease outcome and could serve as a prognostic factor (111). A following study showed that LATS1, along with 4 other genes, were underexpressed in node-positive breast tumors and together could serve as prognostic factors for breast cancer (5). LATS can also provide details about how a patient is likely to respond to a given treatment. In one study, patients with low LATS2 expression were significantly more likely to respond positively to etoposide plus cyclophosphamide (EC) treatment, whereas low levels of LATS1 were also associated, but not significantly, with better response rates to EC treatment. Importantly, neither LATS1 nor LATS2 levels showed
any significant association with response rates to docetaxel (7), suggesting that levels of LATS expression, particularly LATS2, can be used to determine treatment options for breast cancer patients.

The above evidence demonstrates the integral role of the Hippo-LATS pathway in controlling tumorigenesis. Critical to our understanding of the role of this pathway in tumor suppression, however, is our understanding of the mechanisms of LATS-mediated functions.

2.3 LATS: Large Tumor Suppressor

As a central player in the Hippo-LATS signaling pathway, elucidating the molecular functions and regulation of LATS1 and LATS2 (LATS) is essential to understanding both their role within the Hippo-LATS pathway as well as their interaction with other signaling networks. Although LATS1 and LATS2 may possess separate functions as suggested by their distinct phenotypes in knockout mice, the high degree of homology between LATS1 and LATS2 suggest that they also possess similar functions.

Mammalian homologs to dlat were identified independently by two groups. Firstly, by screening a cDNA library using a dlat probe under low-stringency hybridization conditions, Xu's group from Yale University also isolated mouse (Lats1) and human (LATS1) genes (65). To prove LATS1 is conserved and functionally homologous to dlat, they expressed a LATS1 transgene in dlat mutant flies, showing that expression of LATS1 was sufficient to rescue tumor growth in these mutant flies (65). Another group in Japan performed sequence database searching using a BLAST algorithm to identify the human homolog to dlat and mapped LATS1 to chromosome 6q24-25.1 (113). Similarly, human LATS2 was independently identified in two
laboratories. The first group used a cDNA library extraction method from mouse testis, identifying first mouse Lats2, and then subsequently isolating human LATS2 cDNA. LATS2 maps to the 13q11-q12 chromosomal region, a region shown to exhibit loss of heterozygosity in primary cancers of breast, liver, and lung (114), suggesting that, like LATS1, LATS2 may be a tumor suppressor gene. The second group, through screening human myeloid cell lines by degenerate PCR to identify novel protein kinases expressed in human hematopoietic progenitor cells, identified a kinase that was phosphorylated during mitosis, calling it kpm (kinase phosphorylated during mitosis) (115). kpm and LATS2 are identical genes, so again, for consistency, we will use the LATS2 notation throughout this thesis.

Since its discovery in Drosophila, LATS has been shown to be an evolutionarily conserved gene with homologs in yeast (dbf2, cbk1, orb6, Sid2p), C. elegans (cLats), mice (Lats1, Lats2), and humans (Ndr1, Ndr2, LATS1, LATS2), to name a few (65, 114, 115) (Fig. 1.2). Their encoded proteins belong to the AGC family of Ser/Thr protein kinases based on sequence alignments of their catalytic kinase domains (116). Although their C-terminal kinase domains are well-conserved and harbor considerable sequence similarity, the N-terminal regions of these proteins varies significantly and can account for functional differences. Specifically, the human Ndr proteins which possess truncated N-termini in comparison to LATS1 and LATS2 have only been implicated in centrosomal duplication, neurite outgrowth, and fear conditioning (117, 118) in contrast to the broader functional capacity of LATS proteins.
**Figure 1.2: Evolutionarily conserved homologs of LATS.** Several LATS homologs are shown along with their percent identity and similarity of the kinase domain compared to the original *Drosophila lats* gene.
2.3.1 Structure, expression, and subcellular localization. Compared to other members of the LATS kinase subfamily of AGC kinases, human LATS1 and LATS2 are most homologous to each other. In addition to the conserved kinase domain, both the amino- and carboxy-termini share regions of similarity (Fig. 1.3). Within the kinase domain, both LATS1 and LATS2, as well as their subfamily of AGC kinases, possess a unique insertion of 30-60 amino acids between subdomains VII and VIII, which likely plays an autoinhibitory function (116). Other conserved features include a protein binding domain (PBD) that lies just upstream of the catalytic domain which binds several proteins including LIMK1 (119), MOB1 (120), and Zyxin (121), as well as the two LATS conserved domains, LCD1 and LCD2. Although the functional significance of LCD1 and LCD2 are not fully understood, one study showed that deletions of either of these regions in LATS2 abolished its tumor suppressor activity on NIH3T3/v-ras cells, suggesting that these domains are essential for LATS2 tumor suppressor function (122). In addition LATS1 and LATS2 both possess PPXY motifs that bind WW domain proteins such as YAP and TAZ (72-74) and an ubiquitin associated domain (UBA) on their N-terminal regions.

LATS1 and LATS2 also possess unique features not present in the other homolog. For example, LATS1 possesses a P-stretch, a region rich in proline residues (113) which could be applied for protein-protein interaction. In contrast, LATS2 possesses seven repeats of alternating proline-alanine resides (PAPA repeat), which may be involved in distinct protein-protein interactions (114). These seemingly small differences in domain structure could account for potential differences in protein function.
Figure 1.3: Structure of LATS1 and LATS2. LATS1 and LATS2 share several conserved domains including the C-terminal Ser/Thr kinase domain (LATS1: aa708-1130; LATS2: aa670-1108), a Protein Binding Domain (PBD) (LATS1: aa656-758; LATS2: aa618-720), two LATS Conserved Domains (LCD1 and LCD2) (LATS1: aa13-167, aa458-523; LATS2: aa1-160, aa403-463, respectively), and an ubiquitin binding domain (UBA) (LATS1: aa101-138; LATS2: aa99-133). In addition, both LATS1 and LATS2 have at least one PPxY motif (LATS1: Y376, Y559; LATS2: Y518). As distinct proteins, LATS1 possesses a P-stretch (aa236-266), whereas LATS2 possesses a PAPA repeat (aa467-480).
In terms of tissue expression, both LATS1 and LATS2 are ubiquitously expressed (65, 113-115). However, levels of expression differ slightly: LATS1 expression is highest in the adult ovary (65), whereas LATS2 appears to have highest expression in heart and muscle tissues (114, 115). Furthermore, LATS1 and LATS2 have different expression patterns during development. Prominent expression of LATS1 was found in tissues of ectodermal origin such as the neural tube and neuroepithelium, whereas highest LATS2 expression was found in tissues of mesodermal origin including the cardiac outflow tract and heart (107). Furthermore, some evidence exists for multiple splice variants of both LATS1 and LATS2. A smaller gene transcript exists in the retina for LATS1 (123) and in testis for LATS2 (114, 115). However, neither of these smaller transcripts has been shown to exhibit any specific functions, so the significance of these smaller transcripts is unclear. From this analysis, it is clear that LATS1 and LATS2 exhibit distinct expression patterns and these differing levels of expression in both developing and adult tissues could suggest that LATS1 and LATS2 may possess distinct roles.

Although some studies have shown nuclear localization of LATS2 (114, 122), most studies clearly demonstrate centrosomal localization of both LATS1 and LATS2 during interphase (107, 113, 121, 124-128) and association with the mitotic apparatus in mitotic cells (113, 119, 121, 122, 124-127). However, Yang et al. show diffuse cytoplasmic staining of LATS1 when cells were fixed without detergent pre-extraction (119), suggesting that the use of detergents may wash out most cytoplasmic proteins, leaving behind centrosomal proteins. Several other laboratories confirmed that both LATS1 (125) and LATS2 (127, 128) primarily localize within the cytoplasm. As
discussed, regulation of LATS localization is a potential mechanism for regulating LATS activity; therefore, LATS may translocate to different subcellular locations in response to different stimuli.

**2.3.2 Biochemical Function as Ser/Thr Kinase.** The first studies to identify *dlats* in *Drosophila* showed that this gene possesses a conserved Ser/Thr kinase domain (38, 39), and early studies used autophosphorylation of LATS to indicate kinase activity (68, 115). Although several studies implicated the kinase function of LATS as essential for LATS-mediated cellular effects (122, 125, 129, 130), the specific substrates for LATS1 and LATS2 remain elusive. Only recently, due to homologous interactions in *Drosophila* lats, have two substrates been identified in mammalian systems. In parallel studies, Zhao et al. and Hao et al. identified the transcriptional co-activator YAP (Yes-associated protein) as a phosphorylation target of LATS1 and LATS2. In mapping the phosphorylation sites within YAP, the consensus phosphorylation sequence for LATS1 and LATS2 was determined to be HX(R/H/K)XX(S/T) (H, histidine; R, arginine; K, lysine; S, serine; T, threonine; X, any amino acid) (72, 73). A homolog of YAP in mammals, TAZ (Transcriptional Co-activator with PDZ domain), was later identified as a substrate of LATS2, in which phosphorylation by LATS2 inhibited TAZ function (74).

YAP and TAZ only mediate some of the effects of LATS1 or LATS2, suggesting that LATS must possess multiple substrates. Although the consensus motif is similar to other AGC kinases (72), the LATS family is the only family to require a histidine at the –5 position, which implies specificity in LATS phosphorylation targets. The recent elucidation of the consensus phosphorylation sequence of LATS is the essential stepping stone to the identification of numerous potential substrates. Importantly, several new
kinase substrates for LATS1 or LATS2 have been identified, including the transcriptional repressor FOXL2 (131), the pRb/DREAM associated kinase DYRK1A (132), 14-3-3γ, and the p53-binding partner ASPP1 (133). The identification of these new substrates has provided new mechanisms through which LATS1 and/or LATS2 may regulate transcription, senescence, and apoptosis. The identification of additional kinase substrates will not only aid in understanding the specific mechanisms mediating LATS functions, but they may also point to novel functions.

2.3.3 Regulation of LATS Activity. Regulation of this kinase activity is vital to maintaining homeostasis as inappropriate activation or inhibition can often lead to tumor development. There are several established mechanisms of regulation which can be applied to the regulation of LATS: regulation of expression both at the transcription and protein levels, as well as post-translation modifications such as phosphorylation, control of localization, and conformational regulation. Firstly, as part of the AGC family of kinases, full activation of LATS1 and LATS2 involves phosphorylation of two conserved regulatory motifs: the activation segment within the kinase domain and the hydrophobic motif that lies just outside the catalytic unit on the C-terminus (116). The residues responsible in LATS1 are S909 and T1079; in LATS2, S871 and T1041 represent the activation loop and hydrophobic motif residues, respectively.

Several studies have begun to elucidate a signaling cascade, known as the Hippo-LATS pathway, leading to LATS phosphorylation and activation in mammalian systems. The protein complex consisting of the scaffolding proteins WW45 and MOB1A/1B, the Ras-effector protein RASSF1A, and the Ser/Thr kinase MST1/2 interact with LATS1 and LATS2, leading to phosphorylation by MST1/2 and subsequent activation of LATS (68,
MST2 and its close homolog MST1, but not a more distant homolog MST4, can phosphorylate both LATS1 and LATS2. Of the two conserved phosphorylation sites within LATS1, T1079 is specifically phosphorylated by MST2 and S909 is the site of autophosphorylation. Importantly, mutation of either of these residues renders LATS1 inactive (68). Through extensive biochemical work, a signaling cascade dictates that activation of RASSF1A by stimulation of the Fas death receptor (134) or by DNA damage caused by ionizing radiation (135) dissociates the inhibitory Raf1-MST2 complex. The release of MST2 allows it to bind and activate LATS1. Both WW45 and MOB1A/1B most likely aid in stabilizing this complex to enhance MST2 and LATS1 activations (71, 81). Although these studies primarily focused on activation of LATS1, because MST2 was shown to also phosphorylate LATS2, a similar mechanism most likely occurs. In Drosophila, membrane associated proteins, Merlin and Expanded, and recently a new addition to the pathway, Kibra, have also been shown to activate this pathway (50, 53, 136). The conservation of this upstream signaling complex is not yet clear in mammalian systems. However, recent studies have shown that human Merlin can increase LATS2 phosphorylation (83) and this is enhanced by co-expression of Kibra (52, 84). Kibra is a WW domain protein which can bind the PPXY motifs of LATS (84). Other kinases, such as Cdc2/Cyclin B, Aurora-A, and Chk1 can phosphorylate LATS1 or LATS2, however, it is not clear if these phosphorylations specifically enhance LATS kinase activity (113, 124, 126, 137). Therefore, the formation of the Hippo-LATS complex appears to be a major mechanism of LATS activation.
When discussing the negative regulation of LATS kinase activity, anything that negatively regulates MST2, for example, has potential to indirectly affect LATS as well. Interestingly, one such negative regulator is RASSF6, a family member to the LATS activator RASSF1A (82). Like RASSF1A, RASSF6 binds MST2, but instead of activating it, RASSF6 inhibits MST2 activity and subsequent phosphorylation and activation of LATS kinase. More importantly, however is the identification of proteins that directly inhibit LATS kinase activity. Only within the last year have such negative regulators been identified. For example, our lab identified the E3 ubiquitin ligase ITCH as a direct negative regulator of the Hippo-LATS pathway by increasing LATS1 degradation (14). Additionally, NHPH4, a cilia-associated protein, and the p53-associated protein ASPP1 have been found to bind LATS1 and/or LATS2, thereby competing with YAP/TAZ and decreasing their LATS-mediated phosphorylation, effectively inhibiting the Hippo-LATS pathway (13,15).

In addition to differential phosphorylation as a mechanism controlling proper activation, precise subcellular localization allows for both specific activation and direct targeting of proximal proteins. One study has shown that LATS1 activity can be controlled by directing LATS1 to distinct regions within the cell. Co-expression of MOB1 with LATS1 enriches LATS1 in the membrane fraction, and membrane targeted MOB1 significantly enhances LATS1 phosphorylation at both its activation segment and hydrophobic motif. This suggests that MOB1 targets LATS1 to the membrane where it can be activated by MST1/2. Conversely mutant LATS1 unable to bind MOB1 cannot translocate to the membrane and cannot be phosphorylated (71). As further evidence, in a recent paper, Chow et al. confirmed that membrane-targeted activation of LATS1 by
MOB1 significantly increases LATS1 kinase activity toward its substrate YAP (70). Although from these studies it is clear that LATS1 kinase activity can be enhanced by membrane-targeting, it is not yet known if this is the only mechanism of LATS1 activation or if the same holds true for LATS2. In addition, since these studies only use ectopic expression of targeted proteins, it would be important to establish the dynamics of endogenous LATS in response to MOB1 expression.

Once activated, LATS can travel to other parts of the cell to elicit its effects. Specific proteins or stimuli, therefore, might recruit LATS1 to specific subcellular regions for particular functions. Aurora-A is responsible for phosphorylating LATS2 on Ser83 during mitosis and this phosphorylation is responsible for localizing LATS2 to the centrosome (126). As Aurora-A was not able to phosphorylate kinase-dead LATS2, this phosphorylation is likely a secondary affect that only occurs after activation of LATS2. In addition, treatment of cells with nocodazole (128), which disrupts the centrosome and leads to mitotic dysfunction, or expression of constitutively active H-Ras (138) causes nuclear localization of LATS2 and activation of p53-dependent events.

Although both mitotic stress and onogenic activation lead to nuclear accumulation of LATS, the precise mechanism mediating this translocation is not fully understood. Other cell cycle regulators such as the cyclin kinase inhibitor p27^Kip1 (139) and tumor suppressor p53 (140) can translocate between the cytoplasm and nucleus to elicit distinct effects. In their cases, the control of localization involves the presence of nuclear localization signals (NLS) or nuclear exclusion signals (NES) and specific protein-protein interactions and/or post-translation phosphorylation events. Similar mechanisms of
nuclear/cytoplasmic translocation could exist with LATS, however, no nuclear localization or exclusion signals have yet been identified within either LATS1 or LATS2.

Protein expression can be regulated through various mechanisms, including epigenetic mechanisms, transcriptional regulation, and post-transcriptional mechanisms such as RNA degradation by miRNA and/or protein degradation through the lysosomal or proteosomal pathways. Although LATS1 and LATS2 are ubiquitously expressed in most tissues and cell lines examined, their protein levels can be modulated at several levels in response to different stimuli.

Firstly, at the transcription level, p53 has been shown to induce LATS2 mRNA levels in response to nocodazole. And p53 is specifically recruited to a p53-response element within the promoter region of LATS2 (128). In addition to nocodazole treatment, constitutive expression of oncogenic H-Ras also leads to a p53-dependent increase in LATS2 transcription (138). Interestingly, the promoter region of LATS1 also harbors p53-response elements, but whether p53 is able to induce LATS1 transcription has not been studied. While, p53 is the only transcription factor identified to regulate LATS2, the CUX1 transcription factor has been shown to upregulate LATS1 expression (141), although the context suggests a contradictory, pro-tumorigenic function for LATS1 and remains to be validated by additional studies. In addition, loss of the FOXP3 transcription factor in prostate cancer is associated with reduced LATS2 gene expression, providing another possible mechanism of LATS2 downregulation in cancer (142).

Identifying additional transcriptional regulators that modulate LATS expression will help in understanding the role of LATS in tumorigenesis.
Post-transcriptionally, miRNAs have increasingly become recognized as one of the most abundant classes of regulatory genes in humans (143). miRNA complexes can bind mRNA based on sequence complementarities, causing inhibition of protein translation and/or degradation of mRNA. Importantly, recent studies have identified several miRNAs which regulate LATS2 expression. miRNA-372 and miRNA-373 have been shown to inhibit LATS2 expression with a corresponding reduction in mRNA and protein levels in testicular germ cell tumors (144), gastric cell lines (145) and esophageal cancer cell lines (146). In addition miRNA-31 has been shown to function as an oncogene by inhibiting the expression of several tumor suppressors including LATS2 (147). In addition, PiwiL2 and its associated Piwi-interacting RNAs (piRNAs), a regulatory protein which interacts with miRNA, has been shown to modulate LATS2 expression as a mechanism in regulating the cell cycle of mesenchymal stem cells (148). Finally, LATS2 mRNA stability is also regulated by tristetraprolin (TTP), which binds AU-rich elements within the 3'UTR to destabilize LATS2 mRNA and leads to subsequent downregulation of protein expression (149), a possible mechanism for regulating LATS2 effects on cell proliferation. Therefore, the expression of LATS2 appears to be tightly regulated by several different miRNAs and mRNA binding proteins. So far no studies have identified any miRNAs responsible for regulating LATS1 expression, although it is likely that they exist as they do for LATS2.

At the protein level, several different genes regulate LATS1 or LATS2 levels. First of all, in senescent cells, sustained activation of reactive oxygen species (ROS)-PKCδ signaling leads to decreased levels of LATS1 protein (150). In addition, the kinase NUAK1, which belongs to the AMPK family, can phosphorylate LATS1 on S464, which
ultimately decreases LATS1 protein stability (151). Mutated LATS1, S464A, which cannot be phosphorylated by NUAK1, had unaffected protein levels, suggesting that this phosphorylation is essential for targeting LATS1 for degradation. Conversely, overexpression of oncogenic H-Ras increases LATS2 protein expression apart from its role in upregulating LATS2 transcription. H-Ras is able to increase LATS2 protein stability through a signaling cascade involving the ATM-related kinase, ATR, and its target Chk1 (138). Therefore, signaling from PKCδ, NUAK1, and H-Ras can all modulate LATS protein stability. However, none of these studies examined the proteins directly involved in increasing or decreasing LATS1 or LATS2 stability. The heat-shock proteins act as chaperones, stabilizing protein expression. One such protein, HSP90, directly targets LATS1 and LATS2, stabilizing their protein expression (152). In addition, ubiquitin or ubiquitin-like moieties generally target proteins for direct degradation through the proteosomal or lysosomal pathways (153). Many E3 ubiquitin ligases such as the Nedd4-like family of E3 ubiquitin ligases including Nedd4, Itch/AIP1, WWP1, WWP2, Smurf1, Smurf2, among others, contain WW domains (154) which may bind the PPxY motifs of LATS, thereby targeting them for degradation. As discussed, work in our lab has shown that the E3 ubiquitin ligase Itch can bind, ubiquitinate and degrade LATS1 protein (14).

2.3.4 Functions of LATS. LATS1 and LATS2 possess an ever expanding and primarily overlapping collection of functions. Some of these functions are expressed through specific phenotypes, such as cell proliferation, apoptosis, and cell migration. Other functions imply a broader, more governing role of LATS. These functions include
its ability to regulate genetic stability, transcription, and protein stability, which in themselves can lead to a myriad of phenotypes.

2.3.4.1 Cell Proliferation. Since the identification of LATS, its role in cell proliferation has been at the forefront of research studies. We and others have shown that overexpression of LATS1 or LATS2 dramatically inhibits both cell proliferation (122, 125, 129, 130, 155) and anchorage-independent growth (72, 73, 138) in various cell lines. Conversely, downregulation of LATS1 enhances cell proliferation in MCF10A immortalized mammary epithelial cells (156) and loss of LATS2 leads to contact inhibition of growth in Lats2−/− mouse embryonic fibroblasts (MEFs) (107). As expected, any changes in proliferation are correlated with changes in the cell cycle. Both mitogenic and anti-mitogenic signals are relayed to the cell cycle machinery, including the cyclin dependent kinases, their associated cyclins, and the cyclin kinase inhibitors (CKIs), through multiple signaling proteins. For a cell to maintain homeostasis, these positive and negative regulators must be tightly controlled, and any disruption of this machinery can lead to abnormal cell growth. Importantly, both LATS1 and LATS2 have been shown to regulate various aspects of the cell cycle machinery.

We were the first to show that LATS1 inhibits cell proliferation in several cell lines by blocking the G2/M transition (155). We demonstrated that increasing LATS1 leads to an increase in the percentage of cells in G2/M and subsequent decrease in S phase. As the G2/M transition is primarily controlled by Cdc2/Cyclin A/B, we showed that overexpression of LATS1 inhibits the kinase activity of this complex. Consistently, other studies also showed that LATS1 binds Cdc2 and suggested that this prevents Cyclin A or B binding and inhibits its activity (65). Furthermore, in MCF7 breast carcinoma
cells, overexpression of LATS1 leads to downregulation of Cyclin A and Cyclin B protein levels and subsequent inhibition of Cdc2/Cyclin A/B activity, with no effect on the levels of other cell cycle regulators Cyclin E, Cdc2, CDK2, p27^{Kip1}, and p21^{Cip1} (129). Therefore, either by inhibiting Cyclin A and/or Cyclin B expression or by inhibiting their binding to Cdc2, LATS1 inhibits the kinase activity of Cdc2, thereby arresting the cells at G2/M, and inhibiting their proliferation. Importantly, the effect of LATS1 on cell proliferation and subsequent regulation of the G2/M transition is dependent on its kinase activity (129).

Like LATS1, LATS2 also regulates the G2/M transition. Using an inducible system for overexpression of LATS2, it was shown that LATS2 arrests HeLa cells at G2/M with a decrease in the cell population at G1. Overexpression of LATS2 did not affect the expression of cell cycle regulators, but did inhibit the phosphorylation and kinase activity of the G2/M regulator, Cdc2/Cyclin B. Cdc25C, which is inactivated by phosphorylation, is the main phosphatase responsible for dephosphorylating and activating Cdc2. Expression of LATS2 increased the phosphorylated inactive form of Cdc25C, which is unable to activate Cdc2/Cyclin B (130). Interestingly, using a different cell system, LATS2 also affects the other major cell cycle transition, G1/S. Cell cycle analysis of tumorigenic NIH3T3-v-ras cells overexpressing LATS2 showed that these cells enter the S-phase of DNA replication much later than control cells with no change in their rate of DNA synthesis, suggesting that LATS2 blocks the G1/S transition. Mechanistically, overexpression of LATS2 inhibits the kinase activity of the major regulator of the G1/S transition, the Cyclin E/CDK2 complex, but has no effect on expression levels of these proteins or other cell cycle regulators Cyclin A, p21^{Cip1},
p27Kip1, or p57Kip2 (122). As with LATS1, the effects of LATS2 on both G1/S and G2/M require its kinase activity, as kinase-dead mutants were unable to affect cell proliferation or cell cycle changes (122, 130).

By inhibiting the kinase activity of two essential cyclin kinase complexes, LATS1 and LATS2 arrest cells in either G1/S or G2/M and inhibit cell proliferation. Although mechanisms have been proposed, little is actually understood about how LATS and its kinase activity directly affect the cell cycle machinery. Despite the fact that LATS can negatively regulate the cell cycle regulators Cdc25C, Cdc2/Cyclin A/B, or Cdk2/Cyclin E, these proteins cannot actually be phosphorylated by LATS (Yang, unpublished). Since YAP and TAZ are the only substrates of LATS identified so far, indirectly, LATS could induce cell cycle arrest through YAP and/or TAZ. However, although YAP (156) and TAZ (74) have been shown to affect LATS-mediated modulation of colony formation or proliferation, respectively, there is no evidence that YAP or TAZ directly regulate the cell cycle machinery, including Cdc2 or Cdk2. Therefore, the identification of cell cycle proteins that are directly regulated by LATS is essential to understanding LATS-mediated cell cycle arrest.

2.3.4.2 Apoptosis and Senescence. As a mechanism against cancer progression, tumor suppressors induce apoptosis, or programmed cell death, in response to DNA damage, serum deprivation, oxidative stress, or oncogene activation. As an alternative, tumor suppressors may trigger senescence to prevent the promotion of tumor cells (157, 158). Growing evidence points to the critical role of LATS in mediating these responses.

Some of the earliest studies with LATS showed that overexpression of either LATS1 or LATS2 could induce apoptosis (129, 130, 155), although mechanistically
LATS1 and LATS2 differ. Whereas LATS1 upregulates pro-apoptotic proteins p53 and Bax (155, 159), LATS2 downregulates anti-apoptotic proteins Bcl-X\textsubscript{L} and Bcl-2 (159). In terms of the two classical pathways of apoptosis (the intrinsic or extrinsic pathways), these early studies suggest that LATS functions within the intrinsic pathway. Any cellular stress caused by irreparable DNA damage, UV irradiation, chemotherapeutics, oncogenic activation, or growth factor withdrawal activates this pathway through modulation of the Bcl-2 family of proteins (157), including activation of Bax by LATS1 or downregulation of Bcl-2 or Bcl-X\textsubscript{L} by LATS2. Indeed, LATS2 has been shown to promote apoptosis in response to mitotic stress (128), oncogene activation (138) and UV damage (137). In the latter situations, oncogenic H-Ras-V12 expression activates the DNA-damage pathway, including ATR, which phosphorylates Chk1, ultimately leading to an increase in LATS2 expression and p53-dependent transcription of pro-apoptotic proteins (138). Chk1 can also phosphorylate LATS2 leading to phosphorylation of and translocation of 14-3-3\gamma by LATS2 in response to UV damage (137). Another potential mechanism of LATS2-mediated cell death in response to oncogenic activation is LATS2-mediated phosphorylation of the ASPP1 protein, which directs ASPP1 to the nucleus. LATS2 and ASPP1 function together to direct p53 to pro-apoptotic promoters, such as the BAX promoter (133). In the intrinsic pathway, activation of the caspase cascade begins with caspase 9, and ectopic expression of LATS2 increases processing and activation of this specific caspase (159). In addition, through a domain specific association, LATS1 enhances the activation of the serine protease Omi/HtrA2 which, upon release from the mitochondria, activates apoptosis through both activation of the caspase cascade as well as through its serine protease activity in a caspase-independent manner.
mechanism (160). Importantly, for both the regulation of Bcl-2 proteins and the activation of Omi/HtrA2, the kinase activity of LATS is essential, although the specific substrates mediating LATS-mediated apoptosis are not clear.

In addition to the intrinsic pathway of apoptosis, LATS can also induce apoptosis through the extrinsic, or death receptor pathway. This pathway is triggered from outside the cell through the activation of specific receptors such as Fas, TNF, or TRAIL receptors. Ultimately these receptors activate the caspase cascade, beginning with caspase 8 (157). In this context, LATS activity is enhanced by stimulation of the Fas death receptor through RASSF1A and MST2 (134). This suggests that the Hippo-LATS pathway may be important for the induction of apoptosis through the extrinsic pathway of apoptosis, whereas additional LATS signaling pathways are central to the intrinsic pathway of cell death.

Many antitumor therapies utilize the inherent cell death pathways to exert their effects. Consequently, aberrant alterations within these pathways can often lead to drug resistance (161). Since LATS is integral to induction of apoptosis, preliminary studies have shown that loss of LATS may result in drug-resistance. For example, loss of LATS2 was associated with resistance to two chemotherapeutic drugs doxorubicin or etoposide in leukemogenic cells (112). This study alludes to the potential predictive value of LATS in determining treatment outcomes.

As an alternative to apoptosis, tumor suppressors such as p53 can trigger senescence in response to oncogene or DNA damage induced stress (158). For example, oncogenic H-Ras-V12 induced senescence is mitigated by the downregulation of p53, thereby ultimately leading to transformation. Similarly, in a recent study, oncogenic H-
Ras-V12-induced senescence was completely abrogated by knockdown of LATS2 (138). In this study, H-Ras-V12 overexpression increased LATS2 protein stability, suggesting that oncogenic stress activates LATS2 in an attempt to protect against transformation. In another study searching for miRNAs that would cooperate with H-Ras-V12 to induce transformation, miR372 and miR373, were identified and shown to modulate LATS2 levels (144). Importantly, expression of miR372 and miR373 lead to reduced LATS2 expression and inhibited oncogene-induced senescence. In a recent study, LATS2 was also shown to participate in pRB-induced senescence by phosphorylating DYRK1A, a kinase involved in the DREAM E2F repressor complex (132). Paradoxically, LATS1 seems to have the opposite effect on senescence, potentially through its regulation of cytokinesis. Activation of the p16-Rb pathways leads to elevated levels of reactive oxygen species and activation of PKCδ in senescent cells. This positive feedback loop reduces LATS1 levels, thereby inhibiting cytokinesis and maintaining senescence (150). Similarly, loss of LATS1 induces senescence in response to ectopic expression of NUAK1 (151). The discrepancy between LATS1 and LATS2 function in the context of senescence is the first real evidence that LATS1 and LATS2 may possess distinct functions.

2.3.4.3 **Cytoskeletal dynamics.** Driven by the dynamic changes of the actin and microtubule cytoskeleton, cell migration is a vital process involved in many cellular responses such as embryonic development, immune response, and wound healing (162). When deregulated, cytoskeletal dynamics and cell migration can often direct tumor metastasis (163).
Since its discovery, several lines of evidence have suggested that LATS modulates cytoskeletal dynamics. First of all, multiple LATS homologs such as \textit{S. cervisiae} Cbk1, Dbf2, Dbf20, and \textit{S. pombe} Orb6 and Sid2 play pivotal roles in orchestrating various aspects of cell morphogenesis including the organization of the actin and tubulin cytoskeletons (164). In addition, the \textit{Drosophila} Ndr kinase, Trc, is essential for the development of epidermal hairs, sensory bristles, and dendrite arborization, all processes that require the activation of both actin and tubulin cytoskeletons (165, 166). Importantly, human LATS1 and LATS2 can interact with various cytoskeletal proteins. LATS1 interacts with Zyxin, a component of the focal adhesion complex important for actin polymerization (121). Moreover, LATS1 colocalizes with F-actin at the contractile ring along with the cytoskeletal protein LIMK1 (119). By binding LIMK1, we showed that LATS1 is able to inhibit the kinase activity of LIMK1 and its effects on actin dynamics. LATS2, on the other hand, associates with the microtubule-associated Ajuba, recruiting \(\gamma\)-tubulin to the centrosome (127). Therefore, LATS is implicated in regulating both actin and microtubule cytoskeletal components necessary for efficient cell migration.

\textbf{2.3.4.4 Protein Stability.} In addition to transcriptional regulation of gene expression, recent evidence has suggested that LATS may also directly regulate protein stability by targeting certain proteins to the ubiquitin-proteasome pathway. In particular, phosphorylation of YAP on Ser381 by LATS1 primes YAP for subsequent phosphorylation by CK1 in a phosphodegron, which then recruits the E3 ligase SCF-\(\beta\)TRCP to ubiquitinate and target YAP for degradation (75). A similar mechanism occurs for LATS1-mediated degradation of TAZ (76). In addition, LATS has been shown to
affect the stability of other proteins, particularly p53, as well as the mitotic regulators Aurora-B and PLK1 (108) and apoptotic proteins Bcl-2, Bcl-XL, and Bax (159). The precise mechanisms mediating LATS-regulated protein stability for these genes remain unanswered. This new function adds another layer to the complexity of LATS signaling and how its broad functions can dramatically affect cellular homeostasis.

### 2.3.4.5 Maintenance of Genetic Stability.

In addition to responding to mitogenic or anti-mitogenic cues, the cell cycle is also a system of checkpoints. If these checkpoints malfunction, genetic instability, characterized by DNA mutations and/or chromosomal abnormalities, including aneuploidy, results (18, 167). Significantly, LATS1 and LATS2 not only respond to mitogenic cues, but are also active participants in multiple cell cycle checkpoints to ensure genetic stability.

The significance of LATS in maintaining genetic stability is evidenced by the gross abnormalities observed in cells with decreased LATS1 or LATS2 expression. Loss of LATS2 results in loss of diploidy, enhanced number of structural chromosomal rearrangements, and defects in chromatin condensation (107). Likewise, loss of LATS2, both in knockout mice as well as through siRNA-mediated knockdown, leads to chromosomal misalignment, enlargement of nuclei, and the presence of micronuclei (108). Alternatively, overexpression of kinase-dead LATS1 acting in a dominant-negative fashion results in lagging chromosomes, as well as enlarged or multiple nuclei, and production of tetraploid cells (125).

Aneuploidy is often associated with centrosomal defects, which include centrosome amplification characterized by centrosome overduplication, supernumerary centrioles, or centrosome fragmentation. Importantly, as discussed previously, both
LATS1 and LATS2 localize to the centrosome (107, 113, 121, 124-128), and as such can influence centrosome function. In two separate studies, both LATS2−/− embryos and MEFs were shown to possess multiple copies of centrosomes (107, 108). Furthermore, overexpression of LATS2-wildtype, but not LATS2-kinase-inactive, rescues the centrosomal defect in LATS2−/− MEFs (107). Significantly, centrosomal amplification was attributed to increased centrosomal fragmentation and/or defects in cytokinesis (108). Since the centrosome is responsible for forming the spindle poles during mitosis and the spindle poles are responsible for the equal distribution of chromosomes, the spindle assembly checkpoint is vital for the maintenance of chromosomal stability. Here, too, LATS is involved. Loss of LATS1 or expression of dominant-negative, kinase-inactive LATS1 leads to prolonged activation of and a defective response by the spindle assembly checkpoint (125).

Not only can centrosomal and spindle dysfunction lead to chromosomal abnormalities and genetic instability, but aberrant mitotic progression can also result in the missegregation of chromosomal content. If cells exit mitosis too fast, if mitosis is delayed and cytokinesis is missed, improper segregation of cellular components and chromosomes can occur. The overexpression of a truncated N-terminal LATS1 fragment, presumably functioning in a dominant-negative fashion, prolongs mitosis (121). This effect can be attributed to its interaction with the LIM protein Zyxin, which is targeted to the mitotic apparatus in response to LATS1 binding. Similarly, loss of LATS1 or its binding partner, MOB1A, prolongs telophase or exit from mitosis, whereas moderate overexpression of LATS1 facilitates mitotic exit, an effect dependent on expression of MOB1A (120). LATS2 also promotes mitotic progression in a similar manner through
its interaction with and activation by MOB1 (108). In addition, LATS2⁻/⁻ MEFs have decreased expression of several mitotic regulators such as Aurora-B, and PLK1 (108).

LATS1 and LATS2 regulate cytokinesis to ensure the complete and equal separation of two daughter cells. We have previously shown that LATS1 colocalizes at the contractile ring with LIMK1 and F-actin (119). This interaction between LATS1 and LIMK1 inhibits the kinase activity of LIMK1 and subsequently reduces the production of LIMK1-induced polynucleate cells. Microinjection of LATS1 or LIMK1 reverts this LIMK1-induced cytokinesis failure (119), implicating LATS1 in the regulation of cytokinesis. In addition, LATS2⁻/⁻ MEFs fail to undergo cytokinesis (108), suggesting that LATS2 is also essential for ensuring complete cell division.

When cells progress uninhibited through the spindle assembly checkpoint and mitosis, they acquire an increased likelihood for developing aneuploidy. At this point, the G1 tetraploidy checkpoint serves as a final opportunity for correction. Here, damaged cells will either undergo repair or be targeted for apoptosis, and importantly, LATS regulates this response. Cells overexpressing kinase-inactive LATS1 which have prolonged activation of the spindle assembly checkpoint and develop tetraploid cells also fail to undergo G1 arrest, whereas wildtype cells undergo apoptosis (125). Significantly, in these cells, p53 is not induced, suggesting that LATS1 activation of the G1 checkpoint occurs through p53. LATS2 also appears to activate the G1 tetraploidy checkpoint through p53. For example, in response to nocodazole-induced microtubule damage in which cells exit mitosis without completing cytokinesis, LATS2 was shown stabilize p53 by binding and inhibiting Mdm2 (128). Normal cells treated with nocodazole bypass the G1 checkpoint as these cells have 4N content and even 8N content. However, expression
of wild-type LATS2, but not kinase-dead LATS2, equips these cells with proper checkpoint function and thereby produces diploid cells. Furthermore, using siRNA-mediated knockdown of LATS2 and/or p53, it was established that LATS2 is necessary for p53 activation to prevent polyploidization (128). Therefore, both LATS1 and LATS2 function through p53 to ensure proper function of the G1 tetraploidy checkpoint.

From the above discussion, it is clear that LATS1 and LATS2 function in similar fashion to act as master regulators of numerous checkpoints within the cell cycle with the ultimate aim of maintaining genetic stability. As with all the other functions of LATS, the kinase activity appears to be essential for regulation of genetic stability, but the precise substrates mediating this effect remain undefined.

2.3.4.6 Other. LATS may possess other functions in addition to its roles in proliferation, apoptosis, migration, transcriptional regulation, and cell cycle checkpoints. Briefly, the Hippo-LATS pathway has been implicated in organ size control (168) and LATS2 has been directly implicated in the control of cardiac myocytes by reducing their size through moderating the levels of AKT and 70S6K phosphorylations, two proteins that act as positive regulators of hypertrophy (169). It is understood that organ size control is essential to tissue homeostasis, and as with any other essential process, deregulation often leads to cancer.

2.3.5 LATS Conclusion. The above discussion has shed light on the magnitude of LATS1 and LATS2 signaling within the cell, suggesting that LATS is a novel governor of cellular homeostasis. Although LATS1 and LATS2 possess minor differences in expression patterns and mechanisms of action, most studies only investigate one or the other protein with respect to specific activities. More evident is
that both LATS1 and LATS2 share many functions and thus comprise a new family of tumor suppressors within the cell. Not only is LATS tightly regulated through numerous mechanisms including control of expression both at the transcription and protein level, as well as post-translational phosphorylation events, and control of localization, but it possesses broad regulatory functions such as transcriptional regulation and the monitoring of genetic stability in addition to its specific effects on cell proliferation, apoptosis, and migration. The deregulation of LATS and the development of tumorigenesis in a multitude of tissues is further evidence of the importance of this tumor suppressor. All of this suggests that LATS plays an essential role in the cell and understanding the specific mechanisms of LATS function is imperative to understanding cellular signaling in relation to disease.

2.4 FERM domain proteins

Two proteins within the Hippo-LATS signaling pathway contain the FERM domain: Merlin and Expanded. The approximately 300 amino acid FERM domain is named after the first four proteins that contain this cytoskeleton-crosslinking domain: 4.1, ezrin, radixin, moesin. Now, the FERM domain, located mainly at the N-terminus, is associated with a large number of proteins including kinases, phosphatases, actin-binding proteins, as well as other less characterized proteins (170). The FERM domain is primarily responsible for linking its proteins to the membrane and as such, can stabilize membrane-cytoskeletal structures as well as coordinate signaling events (171).

2.4.1 ERM proteins: Ezrin and Merlin. The ERM subfamily of the FERM domain superfamily in mammals consists of the closely related Ezrin, Radixin, and
Moesin as well as the less related, but still homologous Merlin. Merlin and the ERM
possess the conserved FERM domain in their N-terminus, followed by a coiled-coiled
domain and less conserved C-terminal which includes a FERM binding region (C-
ERMAD) (Fig. 1.4).

The NF2 gene, encoding the protein Merlin, was initially identified as the gene
mutated in the germ line of patients with the neurofibromatosis type II cancer syndrome
(172). As for other FERM domain proteins, Merlin is conformationally regulated
through intramolecular interactions between its FERM domains and their C-terminal
regions. The closed form of Merlin is responsible for its growth-suppressive functions.
Specifically, Merlin is phosphorylated on S518 by the p21-activated kinase, PAK1,
among other Ser/Thr kinases, in response to signals from the Rho GTPases Rac and
Cdc42(173, 174). This phosphorylation releases intramolecular associations within
Merlin and renders Merlin inactive. Conversely, activation of Merlin in response to
growth arrest signals involves dephosphorylation of S518, which can be mediated by the
myosin phosphatase MYPT1-PP1δ (175, 176).

Merlin functions as a tumor suppressor, mediating contact-inhibition of growth
(174, 177) and inhibiting metastasis (178, 179) although the precise mechanisms are
unclear. Of the potential tumor suppressor mechanisms, Merlin can inhibit Ras and Rac
mediated tumor growth (180), inhibit membrane receptors such as EGFR by inducing
their internalization (181) or by directly binding and inhibiting membrane receptors such
as Erb2 (182) or transmembrane proteins such as CD44 (177).

One branch of the tumor suppressive function of Merlin involves the Hippo-
LATS pathway. Like Merlin (183), the Hippo-LATS pathway is activated through cell-
Figure 1.4: FERM domain proteins. The conserved FERM domain is present in many different proteins. Ezrin, Radixin, and Moesin make up the ERM subfamily of the FERM domain superfamily. Merlin is highly related to the ERM subfamily. *Drosophila* expanded also contains the FERM domain but has a divergent C-terminus. Abbreviations: A/FBD, ERM, actin, FERM-binding domain; CC, coiled-coiled region; FBD, FERM binding domain; P, proline-rich region. (Adapted from (171)).
cell contact (73, 77, 184). Several recent studies show that Merlin can activate this pathway. First of all, in glioma cells, Merlin expression increased phosphorylation of MST1/2 as well as phosphorylation of LATS2 and YAP (83). Similar results were seen in HEK293 cells upon ectopic expression of Merlin and further enhanced with expression of its binding partner KIBRA (52). A more direct effect of Merlin on YAP has been proposed. In this way, Merlin binds YAP, inhibiting its nuclear translocation (185) and preventing its nuclear transcriptional co-activator activity (85). Importantly, loss of YAP in NF2−/− livers greatly suppressed their overgrowth phenotypes (186), which provides evidence that Merlin functions through YAP in the maintenance of liver homeostasis. A conflicting report showed that liver overgrowth observed in the NF2−/− livers was not due to Hippo-LATS signaling, but instead was a result of increased EGFR signaling (187). These reports show that the mechanism of Merlin tumor suppression is complex and most likely involves the integration of multiple signaling networks.

2.4.2 Expanded. Like Merlin, Expanded also belongs to the FERM domain superfamily (Fig. 1.4) and has been shown to function upstream of the Hippo-LATS pathway in Drosophila systems. A mutated expanded (ex) gene was originally identified in 1926 and shown to enhance the growth of imaginal discs in Drosophila, suggesting that ex functions as a tumor suppressor (188). Since then, ex has been shown to restrict growth, differentiation, and enhance apoptosis in the wings and eyes of flies (189-191). Due to the similar overgrowth phenotypes in the imaginal discs of flies exhibiting mutations in the core Hippo-LATS pathway components, ex was added as a key tumor suppressor in this pathway (50).
As FERM domain proteins, ex and mer colocalize at adherens junctions in the apical membrane and directly interact with each other (51, 53). In this way, mer and ex function together to elicit their tumor suppressor effects. Although loss of either mer or ex leads to proliferative and apoptotic defects, loss of both genes leads to dramatic overgrowth phenotypes reminiscent of Hippo-LATS mutants (50, 51). Together, mer and ex act synergistically to increase dIats and yki phosphorylation (50). In addition to the Hippo-LATS pathway, mer and ex also negatively regulate expression of cell surface receptors such as EGFR as part of their tumor suppressor function (192).

Intriguingly, mer and ex also possess independent functions in Drosophila. For example, ex affects Wingless protein levels, which is not shared by mer (193), and ex has been shown to directly bind and inhibit Yki in Drosophila cells (54). Finally, upstream components of the Hippo-LATS pathway, Fat and Crbs affect ex localization (57, 59, 60), but have not been shown to affect mer. These studies point to both the synergistic as well as differential signaling mechanisms of mer and ex.

Little is known about the mammalian homolog of ex. Only recently, a human homolog was identified. This homolog (hEx/FRMD6/Willin) was shown to localize at the plasma membrane and co-localize with actin(194). Although tissue expression analysis of hEx, along with related Ezrin and Moesin in head and neck squamous carcinoma has been performed, the results for hEx expression are inconclusive (195). Therefore, much needs to be learned about hEx in terms of its cellular and molecular functions. The ability of hEx to function as a tumor suppressor in human systems and whether hEx functions analogously with its Drosophila counterpart as activator of the Hippo-LATS pathway are areas of ongoing research.
CHAPTER 3: HYPOTHESIS AND OBJECTIVES

LATS is the central kinase within the Hippo-LATS signaling pathway. Because LATS and the Hippo-LATS pathway play a vital role in maintaining cellular homeostasis, understanding the molecular mechanisms of its functions and regulation is imperative to our understanding of human disease, specifically tumorigenesis.

By knocking down endogenous protein levels, this project aims to elucidate new functions and potential mechanisms of LATS activity. Furthermore, taking clues from the Drosophila Hippo-LATS pathway, we hope to elucidate potential mechanisms through which LATS is regulated, thereby linking this important tumor suppressor to external growth cues.

3.1 Hypothesis

As an important signaling component within the cell, LATS must possess a broad range of cellular functions including an ability to mediate the transcription of a variety of genes, an ability to modulate a drug response, as well as an ability to regulate cytoskeletal dynamics and cell migration. Furthermore, as part of the Hippo-LATS signaling pathway, the regulation of LATS1 and LATS2 could be controlled by upstream signaling components, namely through human Expanded.

3.2 Objectives

3.2.1 Elucidating the cellular functions of LATS

Previous studies on LATS1 and LATS2 have used overexpression as a means to study their independent cellular functions. We will use RNAi to downregulate LATS1,
LATS2, or both LATS1 and LATS2 to examine their roles in regulating proliferation and drug resistance. In addition, using our RNAi systems, we will examine how LATS modulates transcription, cytoskeletal dynamics and cell migration. To elucidate differentially expressed genes regulated by LATS, we will employ a whole human genome microarray screen. The identity of novel genes regulated by LATS will also point to novel mechanisms mediating LATS functions.

3.2.2 Characterizing the functions of hEx and its effects on the Hippo-LATS signaling pathway.

The upstream components regulating the Hippo-LATS pathway in Drosophila have been well-characterized. However, how the Hippo-LATS pathway in mammalian systems is regulated is currently unknown. In Drosophila, expanded functions as a tumor suppressor and as a key molecule processing external signals from distinct membrane associated proteins to activate the Hippo-LATS pathway. However, functions of the human homolog of Drosophila expanded, hEx, are uncharacterized. Using a combination of overexpression and RNAi experiments, we will elucidate the putative tumor suppressor function of hEx and assess its ability to function through the Hippo-LATS pathway.

In summary, through the identification of novel functions, novel mechanisms of LATS functions, and characterization of upstream signaling components, this project hopes to more fully understand the role the Hippo-LATS pathway plays in tumorigenesis.
PART II: IDENTIFICATION OF NOVEL FUNCTIONS OF THE HIPPO-LATS PATHWAY

CHAPTER 4: IDENTIFICATION OF NOVEL GENES MEDIATING LATS PHENOTYPES

4.1 Contributions

The following people have contributed to the work in this chapter: Paul Savage, an undergraduate student under my supervision, performed the western blots showing activation of the Hippo-LATS pathway in response to Taxol (Fig. 4.4B). The microarray was performed by Hong Guo in Dr. Feilotter’s lab and analysis of the results was performed by myself using a program designed by Babak Rashidi, a former summer student in the laboratory. Parts of this chapter have been published in Gene. (Visser et al. Gene, 2010, 449:22-29.)

4.2 Introduction

Although tumor cells generally possess several well-known hallmarks of cancer (17), the inherent genetic instability of tumor cells ensures that each cell has the ability to mutate on its own, leading to the heterogeneity observed both within a tumor mass as well as within the population. Arguably, the fundamental trait of all tumor cells is their capacity for uncontrolled cell proliferation. Cell proliferation is tightly controlled through mitogenic and anti-mitogenic signals that ultimately converge on the cell cycle
and its regulatory machinery including the cyclins, cyclin-dependent kinases (CDKs), and the cyclin kinase inhibitors (CKIs). The proper expression, localization, and activity of these regulatory proteins are essential for maintaining homeostasis.

In addition to evading cell cycle regulation, tumor cells must also acquire the ability to evade the inherent cell death pathways both during the initiation of tumorigenesis as well as during chemotherapy treatments. Although multiple classes of drugs exist, one commonly used chemotherapeutic is Taxol (paclitaxel). Unfortunately, its use in clinical settings is hindered by the development of resistance. This microtubule inhibitor functions by stabilizing microtubules during mitosis which prevents the formation of normal mitotic spindles, leading to cell cycle arrest and subsequent cell death (196). However, the mechanisms mediating cell death in response to Taxol are still an area of intense research. Understanding the molecular mechanisms underlying the cells’ response to Taxol will aide clinicians in determining which patients will favourably respond.

Importantly, some of the earliest studies on LATS1 or LATS2 showed that overexpression of either LATS1 or LATS2 can inhibit cell proliferation or induce apoptosis in various cell lines (122, 129, 130, 155, 159). Because most of the studies on the roles of LATS1 and LATS2 in cell proliferation and apoptosis were shown by overexpressing either LATS1 or LATS2, we wanted to confirm the essential roles of these proteins in these processes by downregulating endogenous LATS1 or simultaneously downregulating both LATS1 and LATS2 in different cell lines and examining the cellular effects on cell proliferation or response to Taxol treatment.
In addition, despite all the research regarding LATS functions, the molecular mechanisms and the proteins mediating its functions remain largely unknown. Several lines of evidence suggest that LATS may regulate gene transcription. Firstly, LATS2 was shown to bind to the androgen receptor transcription factor and thus modulate expression of its target genes (8). Secondly, using the fly eye as a tool to study how cells make cell-fate decisions, it was shown that, in *Drosophila*, *dlats* and PH-domain gene *melted* antagonistically modulate each other’s transcription (197). Finally, as a central player of the Hippo-LATS signaling pathway, LATS is able to modulate two transcriptional activators, YAP, an oncogene that contributes to malignant transformation (72, 73, 104), and TAZ, recently described to stimulate cell proliferation, resistance to Taxol, cell migration and invasion, and reduce contact inhibition of growth (74, 80, 98), thereby suggesting that LATS is responsible for differential gene expression. Indeed, downstream components of the Hippo-LATS pathway have independently been shown to modulate gene expression (73, 77, 78, 156, 168). Therefore, the identification of novel genes differentially regulated by LATS will provide novel insights into the molecular mechanisms mediating LATS functions.

This chapter explores in more detail how loss of either LATS1 or both LATS1 and LATS2 can lead to the acquisition of the two major cancer phenotypes: enhanced proliferation and resistance to cell death. In addition, we identify novel genes regulated by LATS through a whole human genome microarray screen.
4.3 Material and Methods

4.3.1 Cell culture and antibodies. Cells were maintained in DMEM (HeLa, MCF7 and BT20) or 5A McCoy media (SKBR3, HCT116) supplemented with 10% FBS (Sigma) and 1% Penicillin/Streptomycin (Invitrogen). MCF10A cells were maintained in DMEM-F12 supplemented with 5% horse serum (Sigma), 10 µg/ml insulin (Sigma), 20 ng/ml EGF (Sigma), 100 ng/ml choleratoxin (Sigma), 0.5 µg/ml hydrocortisone (Sigma) and antibiotics (Table 4.1). Antibodies used were as follows: anti-LATS1 rabbit polyclonal (Y03), anti-LATS2 rabbit polyclonal (Bethyl laboratories), anti-β-actin mouse monoclonal (Sigma), anti-myc (9E10) mouse monoclonal, or anti-p21Cip1 rabbit polyclonal (BD Pharminogen).

4.3.2 Establishment of LAT51-expressing cell lines and siRNA and shRNA mediated knockdown of LAT51 and/or LAT52. To establish LAT51-overexpressing cells, HeLa cells were infected with lentivirus expressing vector alone (control) or myc-tagged LAT51 (LAT51-myc). To transiently knockdown LAT51 and LAT52, siRNA duplexes targeting human LAT51 or LAT52 were purchased from Integrated DNA Technologies (Coralville, IA, USA). Sense strand sequences are listed in Table 4.2. HeLa or MCF10A cells were transfected with a combination of 10 nM LAT51 siRNA and 10 nM LAT52 siRNA (siRNA-LAT5) or negative control (siRNA-Control) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. For best results, three days post-transfection, protein was extracted and knockdown of LAT5 was confirmed by western blot using anti-LAT51, anti-LAT52, and anti-β-actin antibodies. For stable knockdown of LAT51 or LAT52, lentivirus produced using plasmid constructs
<table>
<thead>
<tr>
<th>Cell Line</th>
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<tr>
<td>HCT116</td>
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</tr>
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containing shRNA against LATS1 and LATS2 or negative control eGFP purchased from Open Biosystems was infected into cells as previously described (72) and selected with 1 µg/mL puromycin. Efficient knockdown was confirmed by western blot as above.

4.3.3 RNA isolation and microarrays. Total RNA was extracted using TRIzol (Invitrogen) according to standard protocol and stored at -80°C until use. For microarray analysis, RNA was extracted from siRNA-LATS-A and siRNA-Control cells and further purified with RNeasy Mini Kit (Qiagen, Valencia, CA). Whole Human Genome Oligo (60-mer) array gene expression analysis was performed with siRNA-LATS-A and siRNA-Control HeLa cells a total of four times. Arrays were as follows: Array 1: siRNA-Control-Cy3-CTP, siRNA-LATS-A-1-Cy5-CTP; Array 2: siRNA-Control-Cy3-CTP, siRNA-LATS-A-2-Cy5-CTP (biological replicate); Array 3: siRNA-Control-Cy5-CTP, siRNA-LATS-A-1-Cy3-CTP (technological replicate for array 1); Array 4: siRNA-Control-Cy5-CTP, siRNA-LATS-A-2-Cy3-CTP (technological replicate for array 2). For each sample, 200ng total RNA was reverse transcribed, linear amplified, and labeled with either Cy3 or Cy5 using Agilent's Low RNA Input Linear Amplification Kit PLUS, according to manufacturer's instructions. After labeling, samples were measured on a Nanodrop microarray module for labeling efficiency and quantification. Samples were then hybridized on Agilent 4 x 44K whole human genome GE arrays (Agilent Design # 014850) at 65°C for 17hrs. After washing in GE washing buffer, the slide was scanned with Agilent Microarray Scanner G2565BA. Feature extraction software (Version 9.5.3.1) was used to convert the image into gene expression data. Data were normalized by the linear lowess method. Genes that were 1.5-fold differentially expressed on 3 of 4 arrays were scored as significant. Furthermore, only genes with a p-value ≤ 0.05 based
on a student's t-test were selected. Mean fold change is mean of 4 arrays. Molecular functions of genes were classified according to Gene Ontology. Microarray expression data is deposited in the Gene Expression Omnibus database (GEO, National Center for Biotechnology Information) under accession number GSE18138.

4.3.4 Quantitative real-time PCR (qRT-PCR). SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) was used to quantify the level of selected differentially expressed genes. In brief, triplicate of 0.2 µg of total RNA extracted were mixed with SuperScript III RT/Platinum, 2x SYBR Green reaction mix and gene-specific forward and reverse primers. qRT-PCR was run using an ABI PRISM 7700 Sequence Detection System with 18S rRNA used as an internal control. The mRNA level of each gene was calculated using the following formula: \( 2^{-\Delta C_t^{\text{Sample}} - \Delta C_t^{\text{Control}}} \), where \( \Delta C_t = \) average cellular gene change Ct normalized to rRNA. The mean and S.D. were calculated from Ct values of triplicate qRT-PCRs for each RNA sample.

4.3.5 Cell proliferation assays. HeLa or MCF10A cells were transfected with siRNA as described above. HeLa cells were plated at 2 x 10^4 cells/well and MCF10A cells were plated at 3 x 10^4 cells/well of a 24-well plate 1 day post-transfection and allowed to proliferate. Cells were counted on days 2, 4, and 6 after plating. Data is mean ± SD of 3 wells. The experiments were repeated at least 3 times.

4.3.6 Cell viability assays. HeLa cells transfected with siRNA as described above were plated in triplicate at 2 x 10^4 cells/well in a 24-well plate 2 days post-transfection. The next day cells were treated with 0, 25, or 100 nM Taxol. 48hrs after treatment, cell viability was quantified using the Trypan blue (Sigma) exclusion method by counting white and blue cells. Percent cell death was calculated as follows: (number of blue
cells/number of blue cells + number of white cells) x 100. MCF7 cells infected with lentivirus containing control shRNA for eGFP (shRNA-Control) or LATS1 shRNA targeting sequences (shRNA-LATS1-A, shRNA-LATS1-B) were plated in triplicate at 2 x 10^4 cells/well of a 24-well plate. The next day, cells were treated with 0, 10, or 40 nM Taxol and 48hrs later, cell viability was counted as for the HeLa cells. These experiments were repeated at least 3 times. The SRB assay (Sulforhodamine B colorimetric assay) was used to measure cell viability of SKBR3 and BT20 cells treated with 0, 0.1, 0.5, 1.0, 2.5, 5, 10, 25, and 50 nM Taxol. Triplicate of 5 x 10^3 SKBR3 or BT20 cells were plated in 96-well plates. The next day cells were treated with Taxol for 72 hrs. To measure cell viability, cells were fixed with 10% TCA for 1 hr at 4 °C and stained with 0.4 % SRB dye for 30 min at RT. Excess dye was removed by repeatedly washing in 1 % acetic acid. The protein-bound dye was dissolved in 10 mM Tris base for OD reading at 510 nM. Data is represented as percent viability compared to 0 nM control.

4.3.7 Analysis of protein phosphorylation after Taxol treatment. In order to determine if LATS1 is phosphorylated in response to Taxol, SKBR3 or BT20 cells were treated with 25 or 100 nM Taxol for 3 days. Untreated cells were lysed for protein extraction at 50 percent confluency using RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% Deoxycholate, 0.1% SDS, 1% NP40, 10 mM EDTA, pH7.4, proteinase inhibitor). To collect total cells, the floating (dead) cells are collected and kept on ice while the remaining attached (alive) cells are trypsinized. The combined trypsinized and floating cells were centrifuged and the cell pellet was lysed in RIPA lysis buffer. Alternatively, floating and attached cells were collected separately. Protein samples were run on 8 or
10 % SDS-PAGE gels and western blots were performed using anti-LATS1 (Y03), anti-pLATS1-T1079, anti-pYAP-S127 (Cell Signaling), anti-YAP (Santa Cruz), and anti-β-actin (Sigma) antibodies

4.3.8 Dual luciferase assay. Triplicates of 1 x 10^5 HeLa, HCT116-WT, or HCT116-p53KO cells were plated in a 24-well plate. HeLa cells were transfected with 0.02 µg p21-luc together with 0.02 µg MST2 and increasing concentrations of LATS1 or LATS2 (0.05 to 0.18 µg) using Lipofectamine 2000 (Invitrogen). HCT116-WT and HCT116-p53KO cells were transfected with 0.02 µg p21-luc together with 0.23 µg LATS1 or LATS2 using Lipofectamine 2000. As an internal transfection control, 5 ng of Renilla luciferase vector (pRL-TK) was also co-transfected in each sample. Luciferase activity was measured 1 day after transfection, using the Dual Luciferase Reporter Assay System (Promega) and the Turner Biosystems 20/20 Luminometer (Turner Biosystems). Briefly, cells were harvested in 250 µl 1x Passive Lysis buffer by incubating for 15 minutes at RT. 20 µl of cell lysate was mixed with 100 µl Luciferase assay reagent, firefly luciferase activity was measured on the luminometer, followed by the addition of 100 µl Stop&Glo reagent and measurement of Renilla luciferase activity. Data is mean ± SD of ratio firefly:renilla.

4.4 Results

4.4.1 LATS is an essential regulator of cell proliferation

To efficiently knockdown both LATS1 and LATS2 (LATS), we utilized RNA-interference technology. As shown in Figure 4.1A, siRNA-mediated knockdown of
LATS1 and LATS2 (siRNA-LATS-A, siRNA-LATS-B) in the human cervical carcinoma HeLa cell line led to a dramatic knockdown of both proteins compared to control cells (siRNA-Control). To eliminate off-target effects, two different siRNA sets targeting both LATS1 and LATS2 were used with consistent downregulation of both LATS1 and LATS2 shown in both. Significantly, downregulation of LATS in HeLa cells increases cell proliferation (Fig. 4.1B) as quantified by counting the cell number over a period of 6 days. In both cell lines with reduced LATS expression, the number of cells on day 6 was 1.6-fold higher than control cells. Importantly, this effect is not cell type specific, since similar downregulation of both LATS1 and LATS2 in the immortalized breast epithelial MCF10A cells (Fig. 4.1C) also enhances cell proliferation (Fig. 4.1D). In this cell line, the downregulation of LATS in the siRNA-LATS-A cell line led to a 1.7-fold increase in total cell number after 6 days and a 1.4-fold increase in the siRNA-LATS-B cell number. Together, these results show that LATS plays a critical role in regulating cell proliferation.

4.4.2 LATS regulates drug response

In addition to proliferation defects, tumor cells also exhibit defects in apoptosis. One of the ways this is manifested in tumor environments is the development of drug resistance. We can functionally show that LATS mediates a drug response, as loss of both LATS1 and LATS2 in HeLa cells leads to resistance to the commonly used chemotherapeutic Taxol (Fig. 4.2A). This effect is significant at both 25 nM and 100 nM with a 2.0 ± 0.3-fold and 2.1 ± 0.95-fold reduction in cell death at 25 nM, and a 2.2 ± 0.19-fold and 1.6 ± 0.9-fold reduction at 100 nM for siRNA-LATS-A and siRNA-LATS-
Figure 4.1: Loss of LATS enhances cell proliferation. (A) Western blot confirming siRNA-mediated knockdown of LATS1 and LATS2 in HeLa cells compared to siRNA control. β-actin is used as an internal loading control. (B) Knockdown of LATS increases cell proliferation in HeLa cells. One day post siRNA transfection, cells were seeded in 12-well plates and total cell numbers were counted on days 2, 4, and 6 post-seeding. Data is mean ± SD. (C) Western blot confirming siRNA-mediated knockdown of LATS1 and LATS2 in MCF10A cells. β-actin is used as an internal loading control. (D) Knockdown of LATS increases cell proliferation in MCF10A cells. Cell proliferation assay was performed as for HeLa cells.
B, respectively. And again, as with proliferation, the ability of LATS to modulate a cell’s response to drug treatment is consistent across cell lines since loss of LATS1 in MCF7 cells also leads to Taxol resistance (Fig. 4.2B,C). In this cell line LATS1 was stably downregulated using lentivirus containing one of two different shRNA sequences targeting LATS1 (Table 4.2) and compared to the control which contained an shRNA sequence targeting the non-mammalian gene eGFP. Compared to the control treated with 10 nM Taxol (22.46 ± 1.84 percent cell death), both shRNA-LATS1-A and shRNA-LATS1-B had significantly less cell death (16.04 ± 2.59 and 14.64 ± 3.29 percent cell death, respectively). Even at a higher drug concentration of 40 nM, both cell lines with reduced LATS1 expression had less cell death (23.39 ± 2.49 and 19.40 ± 3.56 percent cell death, respectively) compared to 26.28 ± 2.24 percent cell death in the control cell line. Therefore, expression of LATS1 and LATS2 is essential for drug-induced apoptosis, and loss of these genes can lead to drug resistance.

To further understand the mechanism by which LATS, particularly LATS1, mediates cell death in response to Taxol treatment, we analyzed the phosphorylation status of LATS1. Because Taxol is a widely used drug in the treatment of breast cancer (198), we analyzed how LATS1 is phosphorylated in two non-invasive breast cancer cells lines: SKBR3 and BT20. Both SKBR3 and BT20 cells were treated with 25 and 100 nM Taxol for 72 hrs before protein extraction. Since the Hippo-LATS pathway can be activated upon cell-cell contact (184), untreated control cells were collected at 50 percent confluency. In the treated samples, both the floating (dead) and attached (alive) cells were collected before protein extraction in RIPA lysis buffer. Intriguingly, upon Taxol treatment, there is a significant band shift in LATS1, indicative of phosphorylation.
Figure 4.2: Loss of LATS leads to Taxol resistance. (A) siRNA-mediated knockdown of LATS1 and LATS2 in HeLa cells decreases cell death after Taxol treatment. 2 days post-transfection, cells were plated in triplicate in 24-well plates. The next day, cells were treated with 0, 25, or 100 nM Taxol. 48hrs after treatment, cell viability was measured using the Trypan blue exclusion method. Data is mean ± SD of triplicate wells. (B) Stable downregulation of LATS1 alone using shRNA-containing lentivirus. MCF7 cells were infected with control lentivirus or virus containing shRNA sequences targeting LATS1. Western blot confirms knockdown of LATS1. β-actin is used as an internal loading control. (C) Knockdown of LATS in MCF7 cells renders cells resistant to Taxol-induced cell death. Cells were plated in triplicate in 24 well plates and the next day treated with 0, 10, or 40 nM Taxol. 48hrs after treatment, cells were collected and cell viability was quantified as before. Data is mean ± SD. * indicates p-value ≤ 0.05 using Student’s t-test.
However, this phosphorylation is specific to SKBR3 since no band shift was observed in the BT20 cells (Fig. 4.3A). Comparing cell viability after Taxol treatment in SKBR3 and BT20 cells using the SRB assay, it is clear that SKBR3 cells are significantly more sensitive to Taxol treatment (Fig. 4.3B). Whereas there is approximately 50 percent cell viability at 50 nM of Taxol in BT20 cells, less than 10 percent of SKBR3 cells survive this same concentration of Taxol. Combined with the specific phosphorylation observed in SKBR3 cells, these results demonstrate that LATS1 phosphorylation correlates with sensitivity to Taxol treatment.

Phosphorylation of LATS1 is essential for activation of LATS1. To date, only MST1/2 have been shown to activate the kinase activity of LATS1 through phosphorylation on T1079 on LATS1 (68). However, as shown in Figure 4.4A, although there is a band shift observed at all concentrations of Taxol, there is no signal using a specific anti-pLATS1-T1079 antibody after Taxol treatment. As a control, phosphorylation of T1079 could be detected in cells treated with okadaic acid (OA), which has been previously shown to enhance phosphorylation and activation of LATS1 (68, 108). Furthermore, no significant phosphorylation of YAP S127, the kinase substrate of LATS1, was observed. However, total YAP levels shows a dramatic band shift, similar to LATS1, upon addition of Taxol. This band shift is most prominent in the floating Taxol-sensitive population but is not evident in the attached Taxol-insensitive cell population (Fig. 4.4B), proving that specific phosphorylation events occur in response to Taxol treatment. Therefore, LATS1 and YAP are both phosphorylated on residues distinct from the canonical Hippo-LATS phosphorylation sites.
Figure 4.3: LATS1 phosphorylation correlates with Taxol sensitivity. (A) LATS1 is specifically phosphorylated in SKBR3 cells. Both SKBR3 and BT20 breast cancer cells were treated with 0, 25, or 100 nM Taxol for 48hrs. Both floating (dead) and attached (alive) cells were collected, run on 8% SDS-PAGE, and blotted for LATS1. β-actin is used as an internal loading control. Molecular weight markers are shown on the right. (B) SKBR3 cells are more sensitive to Taxol compared to BT20 cells. The SRB assay (Sulforhodamine B colorimetric assay) was used to measure cell viability of SKBR3 and BT20 cells treated with 0, 0.1, 0.5, 1.0, 2.5, 5, 10, 25, or 50 nM Taxol. Triplicate of 5 x 10^3 SKBR3 or BT20 cells were plated in 96-well plates. The next day cells were treated with Taxol for 72 hrs. To measure cell viability, cells were fixed with 10% TCA for 1 hr at 4 °C and stained with 0.4 % SRB dye for 30 min at RT. Excess dye was removed by repeatedly washing in 1 % acetic acid. The protein-bound dye was dissolved in 10 mM Tris base for OD reading at 510 nM. Data is represented as percent viability compared to 0 nM control.
Figure 4.4: Activation of LATS1 is independent of the Hippo pathway. (A) LATS1-T1079 is not phosphorylated by Taxol. SKBR3 cells were treated with 0, 10, 25, 50, 100, or 200 nM Taxol. Both floating and attached cells were collected, run on 8% SDS-PAGE, and analyzed by western blot using anti-LATS1, anti-LATS1-T1079, and anti-β-actin as internal control. As a control for LATS1-T1079 phosphorylation, HeLa cells were treated with okadaic acid (OA). Molecular weight markers are shown on the right. (B) YAP is phosphorylated on a site distinct from S127. SKBR3 cells were treated with 100 nM Taxol and floating, attached, or both floating and attached (Total) were collected. Extracted protein was run on 10% SDS-PAGE and analyzed by western blot using anti-LATS1, anti-YAP, anti-YAP-S127, or anti-β-actin as an internal control. Molecular weight markers are shown on the right.
4.4.3 Identification of novel genes differentially regulated by LATS1/2

To identify genes modulated by LATS, including those genes responsible for the proliferation and cell death phenotypes observed in HeLa cells with downregulated LATS expression, the expression profile of siRNA-LATS-treated HeLa cells was investigated using whole Human Genome Oligo (60mer) Arrays (Agilent). Total RNA was extracted from two biological replicates in which both LATS1 and LATS2 were knocked down in independent experiments. A total of 4 arrays were used: two biological replicates and two technological replicates. A minimum of 1.5-fold change in gene expression between siRNA-Control and siRNA-LATS cells on 3 of 4 arrays was scored as significant. In addition, genes were excluded if the average of 4 arrays did not meet both the 1.5-fold change minimum and a p-value cutoff of ≤ 0.05. According to these stringent criteria, a total of 218 genes were downregulated and 219 genes upregulated in siRNA-LATS-treated HeLa cells. Significantly, knockdown of LATS modulated the expression of numerous genes involved in a variety of different cellular processes including adhesion, communication, proliferation, cell cycle, cell death, motility, differentiation, cytoskeletal dynamics, metabolism, and immune response (Fig. 4.5A,B). Of particular interest are those genes involved in tumorigenic processes such as cell proliferation (e.g. CDKN1A, NOV, SPRY4, MYEOV), cell death (e.g. BIRC4BP, CASP9, TP3INP1), cell adhesion or motility (e.g. CYR61, FAT4, SLIT2), as well as cell communication (e.g. ARHGAP9, BDKRB1, IL1B, VAV1) (Table 4.3).

Among the LATS target genes identified by microarray analysis, 13 known genes were selected for validation by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). These genes included 4 upregulated genes (ARHGDIB, VAV1,
Figure 4.5: LATS differentially regulates numerous genes involved in different biological processes. Genes identified in 44K human genome microarray as being differentially expressed by LATS were functionally grouped according to Gene Ontology. (A) Upregulated genes (B) Downregulated genes.
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and 9 downregulated genes (*BIRC4BP, CD82, CDKN1A, FAT4, SLIT2, SPRED2, SPRY2, SPRY4, TP53INP1*). Remarkably, qRT-PCR showed significant correlation with microarray analysis (Fig. 4.6) with a majority of genes being differentially expressed in both microarray and qRT-PCR. Specifically, *CYR61* and *WISP2* were upregulated 5.7–10.0 and 5.8–9.2-fold, respectively in the two siRNA-LATS HeLa cell lines. Downregulated genes include *BIRC4BP, CDKN1A, FAT4, SLIT2, SPRED1, SPRY2, SPRY4, and TP53INP1* whose expressions were decreased 46.2–60.2, 8.5–8.0, 3.3–4.6, 2.6–2.7, 3.5–3.0, 2.6–2.7, and a dramatic 154–206 or 23.2–25.4-fold downregulation, respectively in the two siRNA-LATS treated cell lines compared to siRNA-Control treated cells. Therefore, LATS is responsible for regulating the expression of a large number of genes involved in multiple aspects of tumor progression, including cell proliferation, cell death, cell adhesion, and cell motility, among other processes.

### 4.4.4 LATS1 induces p21<sup>Cip1</sup> expression

As a cell cycle regulator and modulator of apoptosis, p21<sup>Cip1</sup> may mediate some of the proliferative and apoptotic effects of LATS (199). Notably, p21<sup>Cip1</sup> (*CDKN1A*) was identified as a differentially expressed gene in siRNA-LATS-treated HeLa cells. In these cells with downregulated LATS expression, p21<sup>Cip1</sup> mRNA expression was examined by qRT-PCR and a 4.79±3.85-fold decrease observed (Fig. 4.6). To further confirm that p21<sup>Cip1</sup> expression is regulated by LATS, we analyzed p21<sup>Cip1</sup> mRNA and protein levels in LATS1-overexpressing HeLa cells. As expected, p21<sup>Cip1</sup> mRNA expression increased
Figure 4.6: Validation of differentially expressed genes by qRT-PCR. SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) was used according to manufacturer’s instructions. qRT-PCR was run using ABI PRISM 7700 Sequence Detection System. 18S rRNA was used an internal control. Relative mRNA expression was calculated according to the $2^{-\Delta\Delta T}$ method. Data is mean of 3 samples ± SD.
Figure 4.7: LATS induces p21\textsuperscript{Cip1} expression. HeLa cells were infected with lentivirus containing empty vector (control) or vector containing LATS1-myc (LATS1-myc). (A) RNA was extracted with TRIzol reagent and qRT-PCR was performed and analyzed as before. (B) Western blot assessing overexpression of LATS1 with anti-myc 9E10 antibody and p21\textsuperscript{Cip1} expression with anti-p21 antibody.
5.67±1.36-fold (Fig 4.7A), which correlated with an increase in p21\textsuperscript{Cip1} protein level as observed by western blot (Fig. 4.7B).

Although there are multiple mechanisms by which p21\textsuperscript{Cip1} mRNA and protein can be regulated, we show that both LATS1 and LATS2 can induce activation of the p21\textsuperscript{Cip1} promoter in a dose-dependent manner when co-transfected with MST2 using the dual luciferase promoter reporter system (Fig. 4.8A,B). At the maximum concentration of LATS1 or LATS2 plasmid there is a 1.7- and 1.9-fold increase in p21\textsuperscript{Cip1} promoter activity, respectively. This suggests that LATS regulates the transcription of p21\textsuperscript{Cip1}. However, although p21\textsuperscript{Cip1} is a primary transcriptional target of the transcription factor and tumor suppressor p53 (22), in our system, activation of p21\textsuperscript{Cip1} promoter activity by LATS is independent of p53. In both wildtype HCT116 cells with functional p53 and in p53-knockout HCT116 cells, we show that the ability of LATS1 or LATS2 to activate the p21\textsuperscript{Cip1} promoter is unchanged (Fig. 4.9). In wildtype HCT116 cells, LATS1 and LATS2 can activate the p21\textsuperscript{Cip1} promoter 1.58 ± 0.12 and 1.44 ± 0.1 fold, respectively, and in p53-knockout HCT116 cells, p21\textsuperscript{Cip1} promoter activity remains at 1.65 ± 0.21 and 1.39 ±0.03 fold respectively for LATS1 and LATS2. Therefore, this study shows that LATS can regulate p21\textsuperscript{Cip1} transcription and expression independent of p53.

4.5 Discussion

This current study provides convincing evidence for the role of LATS in regulating both cell proliferation and Taxol sensitivity in multiple cell lines. In addition, it provides novel insights into the potential mechanisms mediating these responses.
Figure 4.8: Activation of p21$^{\text{Cip1}}$ promoter by LATS1 and LATS2. HeLa cells were transfected with luciferase reporter plasmid expressing p21$^{\text{Cip1}}$ promoters (p21$^{\text{Cip1-luc}}$) together with vector or MST2 and LATS1 (A) or LATS2 (B). Luciferase assays were carried out using a Dual Luciferase kit. The fold change in luciferase activity was calculated by normalizing to p21$^{\text{Cip1-luc}}$ alone. Data is mean ± SD of 3 samples. * denotes p-value ≤ 0.05 using Students t-test.
Figure 4.9: LATS activation of \textit{p21}^{Cip1} promoter activity is independent of p53.

Either wildtype HCT116 cells or HCT116 cells with p53 knocked-out were transfected with luciferase reporter plasmid expressing \textit{p21}^{Cip1} promoter (\textit{p21}^{Cip1}-luc) together with vector, LATS1, or LATS2. Luciferase assays were carried out using a Dual Luciferase kit. The fold change in luciferase activity was calculated by normalizing to \textit{p21}^{Cip1}-luc alone. Data is mean ± SD of 3 samples. * denotes p-value ≤ 0.05 using Students t-test.
through the identification of numerous genes differentially expressed upon LATS knockdown.

Previous studies on the role of LATS in cell proliferation have primarily focused on overexpression of either LATS1 (125, 129, 155) or LATS2 (122, 130), showing that LATS1 and LATS2 can inhibit cell proliferation in a variety of cell lines. However, since tumor suppressors are characterized by their loss of function, it was necessary to study how loss of LATS1 and LATS2 affects cell proliferation. In this study, we show that loss of both LATS1 and LATS2 increases cell proliferation not only in the HeLa carcinoma cell line but also in the immortalized epithelial breast cell line MCF10A (Fig. 4.1).

Ultimately cell proliferation is controlled at the level of the cell cycle. Both LATS1 and LATS2 have been shown to inhibit progression through the cell cycle by blocking progression through either the G2/M or G1/S transitions (122, 129, 130, 155). Proposed mechanisms include inhibition of the key cell cycle regulators such as Cdc25C, Cdc2/Cyclin A/B and Cdk2/Cyclin E. Importantly, the effect of LATS on cell cycle progression requires its kinase domain. However, LATS cannot phosphorylate any of the cell cycle proteins listed above (Yang, unpublished). Thus, more precise mechanisms mediating LATS inhibition of cell cycle progression and cell proliferation need to be determined. Using microarray analysis, we identified several genes differentially expressed upon LATS knockdown that are involved in cell proliferation (Table 4.3). Significantly, we identified the CKI p21\textsuperscript{Cip1} (CDKN1A) in our microarray screen. p21\textsuperscript{Cip1} is significantly downregulated when LATS is knocked down (Fig. 4.6) and upregulated when LATS1 is overexpressed (Fig.4.7) in HeLa cells.
p2¹⁷⁵⁴ was originally identified as a gene whose expression is activated by the tumor suppressor p53 (22), and later studies proved p2¹⁷⁵⁴ to be an essential mediator of p53-induced cell cycle arrest (200). Among numerous interacting proteins and functions, the major function of p2¹⁷⁵⁴ is as a pleiotropic cyclin kinase inhibitor, decreasing activity of Cyclin D/CDK4/6, Cyclin E/CDK2, CDK1/2/Cyclin A, and CDK1/Cyclin B complexes and participating in all phases of the cell cycle to induce cell cycle arrest (reviewed in (199)). Although p2¹⁷⁵⁴ is a transcriptional target of p53, we show that LATS is able to activate p2¹⁷⁵⁴ transcription independently of p53 (Fig. 4.9). The critical functions played by p2¹⁷⁵⁴ in maintaining cellular homeostasis suggest that the regulation of p2¹⁷⁵⁴ expression is tightly controlled. As such, p2¹⁷⁵⁴ can be activated or repressed at the transcriptional level by several different transcription factor complexes (201). One intriguing possibility is that LATS may regulate p2¹⁷⁵⁴ expression through its kinase substrate YAP. Recently, YAP was shown to decrease p2¹⁷⁵⁴ expression in esophageal carcinoma cell lines (100), and a YAP-S127A mutant unable to be phosphorylated by LATS had an even greater decrease in p2¹⁷⁵⁴ expression compared to wildtype YAP (202). This provides a novel mechanism whereby LATS phosphorylates YAP, sequestering it in the cytoplasm so that YAP can no longer repress p2¹⁷⁵⁴ transcription, thereby activating p2¹⁷⁵⁴ and inhibiting cell cycle progression and cell proliferation.

Like cell proliferation, there is a clear role for LATS overexpression in inducing apoptosis (122, 129, 155, 159). Since many chemotherapeutics utilize a cell’s inherent apoptotic pathways to induce cell death, when these pathways become deregulated during tumorigenesis, cells can become resistant to these drug treatments (198). This study
suggests that disruption of LATS leads to Taxol resistance. We show that loss of LATS1 in the non-invasive breast carcinoma MCF7 cells leads to less cell death after treatment with 10 nM and 40 nM Taxol, and more dramatically, loss of both LATS1 and LATS2 in Hela cells causes a greater than 2-fold reduction in cell death after Taxol treatment (Fig. 4.2). Since this work, others have also published that LATS plays a role in resistance of leukemia cells to other chemotherapeutics such as doxorubicin (112). Together, it is apparent that LATS plays a critical role in mediating a cell death response.

As further evidence for the role of LATS in a Taxol response, we show that LATS1 is specifically phosphorylated upon Taxol treatment and this phosphorylation correlates with sensitivity to this drug treatment (Fig. 4.3). This indicates that LATS1 is activated in response to Taxol treatment, and this activation leads to the induction of apoptosis. However, we also show that LATS1 is not phosphorylated by MST, the only kinase known to activate LATS and that YAP is not phosphorylated on S127, the primary phosphorylation site of LATS (Fig. 4.4). Thus we propose that the role of LATS in response to Taxol is independent of the Hippo-LATS pathway.

The identification of the kinase responsible for phosphorylating LATS1 and/or YAP requires an understanding of the mechanism behind Taxol-induced cell death. Because Taxol affects microtubule dynamics during mitosis, kinases implicated in monitoring proper spindle assembly would be attractive potential kinases mediating activation of the apoptotic machinery, including LATS. Because LATS is associated with the centrosome during mitosis (113, 119, 121, 124, 125, 127, 128), and because LATS has been shown to mediate the spindle assembly checkpoint (SAC) (125), it is plausible
that LATS is phosphorylated and activated by a mitotic kinase upon initiation of the SAC.

An added layer of complexity arises due to the combined specific phosphorylation of YAP in response to Taxol treatment. At this point, it is not clear if the phosphorylation of YAP is due to LATS and if YAP mediates the apoptotic response of LATS in response to Taxol treatment. If YAP were phosphorylated independently of LATS, it would be interesting to speculate that both LATS and YAP are phosphorylated by the same mitotic kinases. Future work is aimed at delineating the precise phosphorylation events of LATS and YAP after Taxol treatment.

The microarray analysis presented in this chapter provides preliminary insight into potential mechanisms of LATS-induced apoptosis and drug resistance. For example, several genes differentially expressed in LATS-downregulated HeLa cells such as \textit{TP53INP1} and \textit{BIRC4BP} are both important mediators of the apoptotic response. Again, the precise mechanisms and transcription factors mediating their expression remain to be determined. More importantly, \textit{CYR61} was recently identified to function, along with its related gene \textit{CTGF} downstream of TAZ to mediate Taxol resistance (80). Since TAZ is a kinase substrate of LATS, this provides a compelling signaling pathway through which LATS phosphorylates and inactivates TAZ, leading to reduced expression of \textit{CYR61/CTGF} and reduced cell death in response to drug treatment.

In addition to providing insight into the mechanisms of LATS-regulated phenotypes such as cell proliferation and drug resistance, the genes identified in this investigation link LATS to a number of signaling pathways previously implicated in both the tumorigenic process and clinical cancer settings (Fig. 4-10), validating the
Figure 4.10: Novel signaling pathways regulated by LATS. Identification of downstream targets regulated by LATS suggests that LATS may function within well-known signaling pathways including p53, Ras-ERK, WNT, and Hippo-LATS signaling networks.
significance of LATS in regulating tumorigenesis. Importantly, we show that LATS is able to modulate genes downstream of the Hippo-LATS signaling pathway, such as *Cyr61* and *CDKN1A/p21^cip1^* (80, 100, 202). Therefore, the present study confirms that one mechanism of LATS function is through the Hippo-LATS signaling pathway.

Most interesting, however, is the identification of novel pathways in which LATS might function. As a kinase, LATS must possess multiple substrates, and thus, function through multiple signaling pathways. The current study identifies several new pathways that LATS can modify. For example, LATS may function within the WNT/β-catenin pathway. This pathway functions within a wide array of cellular processes during both development and maintenance of adult tissue homeostasis. As such an essential pathway, the WNT/β-catenin pathway has been implicated in tumor development, most classically in colon cancer, but also in other tumor types including melanoma, ovarian, and lung cancer (203). In the current study, we identified several components or downstream targets of this pathway that were differentially expressed when LATS was downregulated including *Kremen2, Prickle1, MMP7, SPRY4* and *WISP2*. Previous studies have shown that upstream components of the Hippo-LATS signaling network, Expanded and Merlin/Nf2 modulate Wnt/Wingless signaling in both *Drosophila* (193) and in human glioma cell lines (83). A recent proposal suggests that cytoplasmic, LATS-phosphorylated TAZ inhibits Wnt signaling by binding and inhibiting DVL phosphorylation (204). In addition, the present study shows that LATS may also directly regulate the transcription of key signaling components within this pathway.

Another potential pathway in which LATS functions is the Ras-MAPK-ERK pathway (Fig. 4-10), which regulates cell proliferation, apoptosis, and migration (205).
Importantly, the current study identified and validated three genes that are both transcriptional targets and act as negative regulators of the Ras-ERK pathway: \textit{SPRED1}, \textit{SPRY2}, and \textit{SPRY4} (206, 207). As part of the Sprouty (SPRY) proteins, these three proteins antagonize receptor-tyrosine kinase-dependent signaling, notably the epidermal growth factor (EGF) or fibroblast growth factor signaling (FGF) by inhibiting activation of Ras (208). Furthermore, these proteins have been suggested to act as tumor suppressors, since their expression is downregulated in several tumors, including hepatocellular carcinoma or gastrointestinal tumors (206). Since LATS regulates SPRED1, SPRY2, and SPRY4 expression, it can be suggested that LATS also modulates receptor tyrosine kinase signaling.

Finally, LATS may function within the p53 tumor suppressor network to regulate cell proliferation and apoptosis, as several genes identified in the current microarray analysis are regulated by p53, including \textit{CDKN1A/p21^{Cip1}} (22) and \textit{TP53INP1} (209) (Fig. 4.10). Although our results suggest that LATS induces p21^{Cip1} expression independently of p53, it is possible that in certain contexts, LATS functions with p53 to induce p21^{Cip1} expression. In fact, previous studies have implicated LATS in the p53 pathway of tumor suppression. Firstly, induction of apoptosis by overexpression of LATS1 was shown to be p53-dependent (155). Secondly, loss of kinase-active LATS1 and subsequent abrogation of G1 tetraploidy checkpoint was shown to result from a defect in induction of p53 expression (125). Finally and most importantly, a study by Aylon et al showed that LATS2 was able to bind and inhibit Mdm2, a E3 ubiquitin ligase regulating p53 activity in response to microtubule damage, thereby stabilizing p53 and inducing the transcription of p53 target genes (128). Together with the current microarray investigation, these
studies show that LATS may cooperate with p53 to regulate cell proliferation, apoptosis and genetic stability.

These pathways highlight some of the mechanisms through which LATS may function. In addition to the genes identified that modulate cell proliferation and apoptotic responses, new functions of LATS were identified, including regulation of the cytoskeleton and cell migration (discussed in Chapter 5). The number of genes identified in the current study suggests that LATS is an essential signaling molecule within the cell, regulating a multitude of functions though a multitude of genes. Future work should aim at examining the relationship between LATS, its differentially expressed genes, and key signaling molecules such as WNT, Ras and p53, and how these relationships affects tumorigenesis.
CHAPTER 5: THE ROLE OF THE HIPPO-LATS PATHWAY IN MEDIATING CYTOSKELETAL DYNAMICS AND CELL MIGRATION

5.1 Contributions

I would like to acknowledge Zhonghua Zhou, a postdoctoral fellow in our lab, who performed the SILAC proteomic screen and identified β-actin as a potential binding partner of LATS1 (Fig. 5.10A,B). Parts of this chapter have been published in Cell Research (2011 Aug 2. doi: 10.1038/cr.2011.122.).

5.2 Introduction

Metastasis is a multifaceted process involving localized invasion of the surrounding environment, intravasation into the bloodstream or lymphatic system, followed by extravasation and subsequent colonization into a new tumor mass (210). The fundamental cellular process associated with metastasis is cell migration. Cell migration itself involves a complex series of events coordinated in both time and space. Importantly, the actin cytoskeleton is dynamically remodeled during cell migration, and serves as the driving force for cell migration. At the leading edge of the cell, actin polymerization drives the extension of filopodia or lamellipodia as well as the formation of new adhesion complexes that attach to the substratum. Meanwhile, in the rear of the cell, adhesions detach, and the cell body is pulled into the newly formed protrusions (211). As such an essential process, cell migration is tightly controlled by numerous regulatory proteins and cytoskeletal-associated proteins.
Increasingly, tumor suppressors are added to the group of cell migration regulatory proteins. Although preliminary evidence suggests that LATS may regulate cytoskeletal dynamics and cell migration, a clear role for LATS in this process has not yet been established. For example, homologs of human LATS1 have been shown to influence actin organization. Mutations in either *dlats* or *trc*, the Ndr homolog in *Drosophila*, result in defects in splitting or branching of many cytoskeletal structures including hairs, bristles, and denticles (165, 166), whereas the *C. elegans* homolog, *sax-1*, has been shown to modulate actin polymerization with effects on neurite initiation, spreading, branching, and tiling (212). In addition, both LATS1 or LATS2 have been shown to interact with various cytoskeletal proteins including Zyxin (121), LIMK1 (119), or Ajuba (127) within a cell division context, suggesting that LATS may also interact with cytoskeletal proteins within a migratory context. Finally, our mRNA microarray analysis (discussed in Chapter 4) identified several genes involved in cell adhesion and migration such as *AJAP1*, *SLIT2*, and *MYLK* (Table 4-2). This prompted us to further explore how LATS and the Hippo pathway may regulate cell migration.

Of the Hippo-LATS pathway components only YAP, TAZ, and NF2 have been implicated in regulating cell migration. Overexpression of either YAP or TAZ enhances cell migration (74, 98, 104) whereas overexpression of NF2 inhibits cell migration (34). This chapter investigates the role of LATS1 and LATS2 in cell migration as part of the Hippo-LATS pathway as well as through distinct mechanisms.
5.3 Materials and Methods

5.3.1 Cell lines and antibodies. MCF7, HeLa, MDA-MB-231, NIH3T3, and MEFs (Table 4.1) were maintained in DMEM supplemented with 10% FBS (Sigma) and 1% Penicillin/Streptomycin (Invitrogen). MCF7 cells with stably downregulated LATS1 or LATS2 expression were generated by infecting cells with lentivirus containing shRNA against the non-mammalian gene GFP (sheGFP) or shRNA targeting LATS1 or LATS2 (Table 4.2). HeLa cells with siRNA-mediated downregulation of LATS1 and LATS2 were created as described in section 4.3.2. To overexpress wildtype LATS1 or kinase-dead LATS1 (S909A/T1079A), lentivirus encoding the respecting genes were infected in LATS1−/− MEFs or MDA-MB-231 cells along with empty vector control (WPI) at 100 percent infection efficiency. siRNA-mediated knockdown of FAT4, MST1/2, YAP or TAZ were as described in section 4.3.2 using specific targeting sequences (Table 4.2) (Integrated DNA Technologies).

Antibodies used in this chapter are as follows: LATS1 rabbit polyclonal (Y03), LATS2 rabbit polyclonal (Bethyl), MST1 rabbit polyclonal (Cell Signaling), MST2 rabbit polyclonal (Cell signaling), YAP rabbit polyclonal (Santa Cruz), TAZ rabbit polyclonal (Cell Signaling), and β-actin mouse monoclonal (Sigma).

5.3.2 Cell migration and cell spreading assays. Cells were grown to 60% confluency and serum starved in 1% FBS overnight. Wounds were introduced using a P10 pipet tip and migration into the wound was monitored using a Nikon TE-2000 inverted microscope at 10x magnification over a 24hr period. To quantify the wound-healing assay, the average wound width was measured and relative distance of cell migration (pixels) or percent wound closure calculated using Q-Capture Pro software.
For cell spreading, cells were plated into a 24-well plate coated with 10 µg/ml fibronectin. 30 minutes after plating, adherent cells were fixed by 4% formaldehyde and stained with Texas Red phalloidin.

5.3.3 SILAC and co-immunoprecipitations (Co-IP). LATS1-KO MEFs (LATS1 knockout mouse embryonic fibroblasts) were supplemented with light lysine and arginine (L-Lysine-2HCl and L-Arginine-HCl) and LATS1-WT-myc MEFs (LATS1-KO MEFs infected with wild-type LATS1 lentivirus) were supplemented with heavy lysine and arginine (13C6-Lysine-2HCl and 13C6-Arginine-HCl) media (Thermo Scientific) for six cell divisions prior to cell lysis. 25mg of cell lysate were immunoprecipitated with anti-myc antibody 9E10 (Roche, Mannheim, Germany) cross-linked by DMP to protein G agarose beads. The beads were washed four times with NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1.0% Nonidet P-40). Bound proteins were pooled before running on SDS-PAGE, followed by in-gel trypsin digestion. Tryptic peptides were analyzed by nanoLC-MS/MS. The ratio of heavy (H) and light (L) peptides for specific proteins was calculated. A ratio higher than 1.5 signifies a specific interaction.

For Co-IP of LATS1 and β-actin, 1 mg of protein lysate from LATS1 K̶/ K̶ MEFs or LATS1-WT-myc MEFs were immunoprecipitated with 2 µg of anti-LATS1 polyclonal antibody (Y03) followed by western blot with anti-LATS1 and anti-β-actin antibodies.

5.3.4 Purification of LATS1 fusion protein and co-sedimentation assays.
Production of GST fusion proteins was as described (119). To produce F-actin, 40 μM G-actin (Molecular Probes) and 2x actin polymerization buffer (100 mM PIPES, PH 7.2, 150 mM KCl, 5 mM MgCl2, 2 mM EGTA, 2 mM DTT, and 2 mM ATP) were mixed in
equal volumes and left at RT for 1 hr. F-actin was stabilized by addition of phalloidin (Sigma). For co-sedimentation assays, 1 µM of F-actin was incubated with increasing concentrations of LATS1-GST for 30 min at RT in a buffer containing 25 mM PIPES, 100 mM KCl, 2 mM MgCl₂, 0.005% Nonidet P-40 and 1 mM ATP. The mixtures were centrifuged at 100,000 g for 30 min at 4°C. Equal amounts of supernatant and pellet proteins were separated on a SDS/PAGE gel and stained with Coomassie blue.

5.3.5 In vitro actin polymerization assays. 40 µl 10% pyrene-labeled G-actin (10 µM) (Moleculare Probes) was mixed with 40 µl of 1×PBS, GST, LATS1-GST, LATS1-N (aa 1-585), or LATS1-C (aa 588-1130) and incubated on ice for 30 min. The polymerization was started by adding 120 µl distilled H₂O and 200 µl 2 × actin polymerization buffer (20 mM Imidazole, pH7.0, 150 mM KCl, 5 mM MgCl₂, and 2 mM EGTA). Fluorescence was monitored for 5 min at 365 nm emission and 407 nm excitation using a HITACHI Model F-2000 Fluorescence Spectrophotometer.

5.3.6 Cell transfection, Cytochalasin D treatment and Immunofluorescence. Cell transfection and immunofluorescence were as described (119). Cells were treated with 2 µM of Cyto D for 30 min prior to fixation and immunostaining. Images were obtained with an MRC-1024 Laser Scanning Confocal Imaging System (Bio-Rad) or Nikon Eclipse TE-2000U Inverted Fluorescent Microscope (Nikon).

5.3.7 Cytoskeletal protein fractionation. For extraction of total protein, cells were lysed in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% Deoxycholate, 0.1% SDS, 1% NP40, 10 mM EDTA, pH7.4, proteinase inhibitor). For extraction of cytosolic proteins, cells were extracted for 1 min in ice-cold cytoskeletal extraction (Csk) buffer (50 mM MES, 3 mM EGTA, 5 mM MgCl₂, 0.5% Triton X-100, pH6.4, proteinase
inhibitors). The remaining cellular fraction containing cytoskeletal proteins was rinsed in Csk buffer and extracted with 500 µl RIPA buffer. The levels of LATS1 and actin in each fraction were analyzed by western blot using anti-LATS1 anti-β-actin antibody.

5.4 Results

5.4.1 LATS is an inhibitor of cell migration and cell spreading.

Using both overexpression and knockdown experiments, the role of both LATS1 and LATS2 in cell migration was assessed. The classical wound-healing assay provides a standard measure of the ability of cells to migrate and fill a defined wound area over a given time period. To eliminate any proliferation effects, cells are serum starved prior to the initial wound and maintained in low serum media throughout the assay. First of all, using the non-invasive MCF7 cells previously generated with stably downregulated LATS1 expression (Fig. 4.2B), we show that although the control cells infected with non-targeting sheGFP lentivirus migrate slowly, only closing the wound by 26 ± 5.4 percent, cells with downregulated LATS1 expression closed the wound by 41 ± 9.4 and 34 ± 9.1 percent in shRNA-LATS1-A and shRNA-LATS1-B, respectively after 20 hrs (Fig. 5.1). In the same way, LATS2 was also knocked down in MCF7 cells and assessed for its ability to enhance migration. As shown in Figure 5.2A, LATS2 is efficiently knocked down using two different shRNA constructs (shRNA-LATS2-A and shRNA-LATS2-B). And as with reduced expression of LATS1, loss of LATS2 enhances cell migration, closing the wound by 43 ± 14.0 or 38 ± 8.8 percent for shRNA-LATS2-A or shRNA-LATS2-B, respectively compared to 26 ± 5.4 percent in the control cell line (Fig. 5.2B,C).
Figure 5.1: Loss of LATS1 enhances cell migration in MCF7 cells. MCF7 cells stably infected with control lentivirus (sheGFP) or lentivirus containing shRNA targeting LATS1 (shRNA-LATS1-1, shRNA-LATS1-2) were plated in 6-well plates and grown to 60% confluency prior to serum starving in 1% FBS overnight. Cell monolayers were wounded using a P10 pipet tip. (A) Migration into the wound area was monitored over a 20 hr time period using a Nikon TE-2000 microscope. (B) Percent wound closure was calculated. Data is mean ± SD of 16 wound areas. * denotes p-value ≤ 0.05 using Students t-test.
A. 

![Bar chart showing percent wound closure](chart.png)

B. 

![Cell images showing 0hr and 20hr comparisons](images.png)
Figure 5.2: Loss of LATS2 enhances cell migration in MCF7 cells. MCF7 cells were stably infected with control lentivirus (sheGFP) or lentivirus containing shRNA targeting LATS2 (shRNA-LATS2-1, shRNA-LATS2-2). (A) Knockdown of LATS2 was confirmed by Western blot using anti-LATS2 and anti-β-actin as internal loading control. (B) Cells were plated in 6-well plates and migration was monitored over 20hrs as described in Fig. 5.1 (B) Percent wound closure was calculated. Data is mean ± SD of 16 wound areas. * denotes p-value ≤ 0.05 using Students t-test.
A more dramatic difference in wound closure is seen by downregulating both LATS1 and LATS2 in another non-invasive carcinoma cell line, HeLa. Using siRNA (as described and shown in Fig. 4.1) to simultaneously knockdown both LATS1 and LATS2, we show that cells with reduced LATS expression migrate significantly faster than control cells. In this case, wound closure was quantified by measuring the distance cells traveled into the wound area. After 16hrs, cells with downregulated LATS expression migrated into the wound, respectively migrating 90.9 ± 18.3 µm and 92.3 ± 15.8 µm for siRNA-LATS-A and siRNA-LATS-B, accounting for approximately 20% of the wound area. In contrast, very few siRNA-Control cells entered into the wound, traveling only 41.3 ± 17.6 µm into the wound, which accounts for less than 10% of the wound area (Fig. 5.3). Therefore, whereas knockdown of either LATS1 or LATS2 independently enhances cell migration by approximately 1.5-fold, downregulation of both LATS1 and LATS2 enhances migration by more than 2-fold.

Since LATS functions as a Ser/Thr kinase, we assessed whether the kinase activity of LATS is essential for its effects on cell migration. We expressed either wildtype or kinase-dead LATS1 in two different cell lines. LATS1 kinase activity is dependent on phosphorylation on both S909 and T1019 (68). Mutation of these two residues to alanine renders LATS1 inactive. First of all, whereas LATS1−/− MEFs infected with control lentivirus (WPI) migrate relatively quickly, closing the wound by 77.13 ± 7.61 percent after 12 hrs, when wildtype LATS1 (LATS1-WT) is re-expressed, migration is severely inhibited with cells covering only 45.28 ± 5.89 percent of the wound area. Importantly, expression of the kinase-dead isoform of LATS1 (LATS1-KD), unlike its wildtype counterpart, has no dramatic effect on cell migration with a
Figure 5.3: Loss of both LATS1 and LATS2 enhances cell migration in HeLa cells. LATS1 and LATS2 were knocked-down in HeLa cells using siRNA for LATS1 and LATS2 or control siRNA. (A) Wound healing assay as described in Fig. 5.1. (B) Distance of cell migration was calculated by measuring the average wound width at the indicated time points. Data is mean ± SD of 16 wound areas. * denotes p-value ≤ 0.05 using Students t-test.
similar 71.00 ± 7.41 percent wound closure (Fig. 5.4). The same trend occurs when LATS1-WT or LATS1-KD are overexpressed in the aggressive breast cancer cell line MDA-MB-231 which normally migrate relatively quickly. Cells expressing wildtype LATS1 only just begin to enter the wound after 16hrs whereas both control cells (WPI) and cells expressing kinase-dead LATS1 have significantly closed the wound area (Fig. 5.5). Together, this data demonstrates that LATS1 is a potent regulator of cell migration, an effect dependent on its kinase activity.

Finally, since cell spreading is integrally linked to the mechanism of cell migration, we assessed how expression of LATS1 affects cell spreading in LATS1−/− MEFs. To this end, we compared the ability of LATS1−/− MEFs and LATS1-WT MEFs to adhere and spread on fibronectin-coated plates. As shown in Figure 5.6A, cells expressing LATS1 have formed less stable actin structures such as filopodia compared to LATS1-KO cells. In addition, the percentage of cells expressing LATS1 (LATS1-WT MEFs) that spread was significantly less compared to LATS1−/− MEFs (Fig. 5.6B). Together, these results illustrate how LATS is involved in mediating both cell spreading and cell migration.

5.4.2 Role of the Hippo pathway in cell migration

To understand the mechanisms mediating LATS regulation of cell migration and to determine if the Hippo-LATS pathway is responsible for mediating this effect, we analyzed several components of the Hippo pathway for their ability, like LATS, to modulate the ability of cells to migrate into wound areas. Because FAT4, an upstream component of the Hippo-LATS pathway (Fig. 1.1B) belongs to the cadherin superfamily...
Figure 5.4: LATS1 inhibits cell migration in LATS1−/− MEFS. LATS1−/− MEFs were infected with control lentivirus (WPI), wildtype LATS1 (LATS1-WT) or kinase-dead LATS1 (LATS1-KD). (A) Western blot analysis shows expression of LATS1 using anti-LATS1 antibody and anti-β-actin as internal loading control. (B) Wound assay as described in Fig. 5.1 (C) Percent wound closure was calculated. Data is mean ± SD of 9 wound areas. * denotes p-value ≤ 0.05 using Students t-test. * denotes p-value ≤ 0.05 using Students t-test.
Figure 5.5: LATS1 inhibits cell migration in MDA-MB-231 cells. MDA-MB-231 cells were infected with control lentivirus (WPI), wildtype LATS1 (LATS1-WT) or kinase-dead LATS1 (LATS1-KD). (A) Procedure for cell migration assay as described in Fig. 5.1 (B) Percent wound closure was calculated. Data is mean ± SD of 9 wound areas. * denotes p-value ≤ 0.05 using Students t-test.
A. 

0hr

WPI  LATS1-WT  LATS1-KD

16hr

B.

Percent Wound Closure

WPI  LATS1-WT  LATS1-KD

*  *

109
Figure 5.6: LATS1 inhibits cell spreading. (A) LATS1-WT or LATS1<sup>−/−</sup> MEFs were plated onto coverslips coated with 10 µg/ml of fibronectin. Thirty minutes after plating, adherent cells were fixed by 4% formaldehyde and stained with Texas Red phalloidin. (B) Cell spreading was determined by quantifying the number of cells possessing filopodia. Of total number of cells examined, percentage of spread cells is shown.
(213) and therefore has the potential to mediate cell adhesive properties, we hypothesized that it would also play a role in cell migration. As for LATS, we downregulated human FAT4 using three different specific siRNA targeting sequences. Because FAT4 is not a well-studied protein in mammalian systems and a reliable antibody is not yet available, we assessed expression levels of FAT4 by measuring mRNA levels using qRT-PCR. Although FAT4 is downregulated by more than 50 percent in each of the MCF7 cell lines transfected with siRNA targeting FAT4 compared to non-silencing siRNA (Fig. 5.7A), there is no significant difference on the percent closure of wounds after 24hrs (Fig. 5.7B). Thus, unlike LATS1 or LATS2, loss of FAT4 in MCF7 cells does not enhance cell migration.

More directly linked to LATS, MST1 and MST2 are the key activators of LATS kinase activity by phosphorylating LATS1 or LATS2 (68). To determine if MST1 and MST2 are involved in cell migration and thus, may be responsible for inducing LATS inhibition of cell migration, we knocked-down MST1, MST2 or both MST1 and MST2 using siRNA specifically targeting either MST1 or MST2 in MDA-MB-231 cells which express high levels of MST1 and MST2. Western blot analysis shows more than 90 percent knockdown of both MST1 and MST2 (Fig. 5.8A). Compared to control, cells with loss of MST1 or MST2 expression had no significant effect on the rate of wound closure after 24 hrs. Combined loss of both MST1 and MST2 (MST) actually migrated slightly slower, although not statistically significant, which is in contrast to the enhanced migration in cells with reduced LATS expression. Altogether, this suggests that upstream components of the Hippo pathway, namely FAT4 and MST, are not involved in cell migration and therefore, are not responsible for the effects of LATS on cell migration.
Figure 5.7: The role of the Hippo pathway component FAT4 in cell migration. FAT4 was knocked down in MCF7 cells by transfection with 3 different siRNA constructs. As a negative control, a non-targeting siRNA was transfected. (A) qRT-PCR analysis was performed to determine relative mRNA expression of FAT4. SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) was used according to manufacturer’s instructions and run using ABI PRISM 7700 Sequence Detection System. 18S rRNA was used an internal control. Relative mRNA expression was calculated according to the $2^{-ΔΔT}$ method. Data is mean of 3 samples ± SD. (B) Cells were grown to 60% confluency, serum starved overnight, wounded, and migration monitored at 0hr and 16hr time points. Percent wound closure was calculated. Data is mean ± SD of 9 wound areas.
Figure 5.8: The role of the Hippo pathway components MST1/2 in cell migration (A) MDA-MB-231 cells were transfected with negative control siRNA, siRNA targeting MST1, MST2, or a combination of siRNA targeting MST1 and MST2. Western blot analysis shows expression of MST1 and MST2. (B) Wound healing assays were performed as described in Fig. 5.7, and percent closure calculated. Data is mean ± SD of 9 wound areas.
Despite the inability of MST to modulate cell migration, the kinase activity of LATS1 is still essential for inhibition of cell motility. Since YAP and TAZ are the two main substrates of LATS, we analyzed their effect on LATS1 mediated cell migration. Using the MCF7 cell line with stably downregulated LATS1 expression (Fig. 4.2B), YAP, TAZ, or both YAP and TAZ were also knocked down using specific siRNA targeting sequences. Western blot analysis shows that YAP can be effectively downregulated, and although TAZ levels are relatively low in the MCF7 cell line, it can also be knocked down (Fig. 5.9A). Once transfected cell lines reached confluence, they were wounded and analyzed for their ability to close the wound. As shown in Figure 5.9B, untransfected shRNA-LATS1 cells migrated significantly faster than control sheGFP cells after 16 hrs. Importantly, shRNA-LATS1 cells transfected with the non-targeting siRNA also migrate significantly faster than control sheGFP cells, proving that transfection does not affect the rate of cell migration. However, loss of YAP in shRNA-LATS1 cells almost completely abolished the ability of cells to enter the wound area and loss of TAZ alone reversed the enhanced cell migration phenotype in shLATS1 MCF7 cells back to control (sheGFP) levels. The combined loss of both YAP and TAZ also severely inhibited the ability of cells to migrate into the wound, although the effect was not more dramatic than loss of either YAP or TAZ alone. These results provide evidence that YAP and TAZ function downstream of LATS1 to regulate cell migration.

5.4.3 Identification of actin as a novel binding partner of LATS1

Intriguingly, through a proteomic screen, we identified β-actin as a LATS1 binding partner. Combined with mass spectrometry, SILAC (Stable Isotope Labelled by Amino
Figure 5.9: Enhanced cell migration in shRNA-LATS1 cells is dependent on YAP and TAZ expression. MCF7 cells with stably downregulated LATS1 were transiently transfected with siRNA targeting YAP, TAZ or a combination of YAP and TAZ. (A) Western blot analysis shows downregulation of YAP and TAZ. (B) Wound healing assay. Transfected cells plated for wound assay as described in Fig 5.1. Percent wound closure was calculated. Data is mean ± SD of 9 wound areas. * denotes p-value ≤ 0.05 using Students t-test.
acids in Cell culture) is a powerful tool for the identification of specific protein-protein interactions. Applying this approach to identify novel LATS1 interaction partners, LATS1-KO MEFs and LATS1-WT-myc MEFs (LATS1 knockout MEFs infected with wild-type myc-tagged LATS1 lentivirus) were labeled with “light” (L) or “heavy” (H) amino acids, respectively, and cell lysate was extracted, immunoprecipitated for LATS1 and mixed elutes subjected to mass spectrometry analysis. From this screen, several novel LATS1-interacting partners were identified including β-actin (Fig. 5.10A).

Compared to the 40S ribosomal protein S3, a housekeeping gene, with a relative abundance ratio of 1.02, β-actin has a H/L ratio of 1.91, suggesting that β-actin is a novel LATS1 binding partner.

To confirm that LATS1 indeed binds β-actin, we immunoprecipitated LATS1 as before and show that β-actin is also pulled down (Fig. 5.10B). Furthermore, co-sedimentation assays using 1.0 µM of F-actin demonstrate that with increasing amounts of LATS1-GST (0.125, 0.25, 0.5, and 1.0 µM), the LATS1/actin ratios in the pellet (P) after ultracentrifugation gradually increased (Fig. 5.10C). These results indicate that full-length LATS1 directly and specifically interacts with F-actin in a concentration-dependent manner.

We next examined whether LATS1 co-localizes with F-actin in NIH3T3 fibroblasts using immunofluorescent analysis. Importantly, endogenous LATS1 is found on both stress fibers and the leading edge of migrating cells, regions where F-actin is also localized (Fig. 5.11A). Dramatically, after treatment of cells with Cytochalasin D, an actin-depolymerization reagent which severs F-actin into small F-actin fragments and causes actin foci in cells, LATS1 aggregates and co-localizes with these F-actin
**Figure 5.10: Identification of actin as a novel LATS1 interaction partner.** (A) LFQ-FT MS spectra of the doubly charged ion pairs of SILAC labeled peptides. On the right, β-actin (gi: 187951999, peptide AVFPSIVGR); on the left, 40S ribosomal protein S3, a housekeeping gene (gi: 6755372, peptide AELNEFLTR) (B) LATS1 was immunoprecipitated from LATS1−/− MEFs and LATS1-WT-myc MEFs with anti-LATS1 antibody. Lysates were subjected to Western blot using LATS1 and β-actin antibodies. (C) Co-sedimentation analysis of LATS1-actin interaction *in vitro*. A fixed concentration of F-actin (1 µM) was mixed with increasing concentrations of LATS1-GST (0.125 to 1 µM). The molar ratios of LATS1 and F-actin are shown.
foci (Fig. 5-11B). Together, these findings strongly suggest that LATS1 is a novel actin-binding protein.

5.4.4 LATS1 inhibits actin polymerization

The identification of actin as a LATS1-binding partner poses the possibility that LATS1 may also regulate actin dynamics. Complex actin dynamics are important for many cellular functions including cell structure, division, adhesion, and motility. At the core of actin dynamics is its polymerization, which itself can involve multiple processes regulated by various actin-binding proteins. Because LATS1 binds actin, we examined how LATS1 may also affect F-actin polymerization and thus be added to the growing list of regulators. We first examined actin polymerization in vitro using pyrene-actin mixed with 1× PBS (control), 10 µM GST, or 10 or 25 µM LATS1-GST. Compared to the control, while addition of GST had no effect on actin polymerization, addition of increasing concentrations of LATS1-GST (from 10 µM to 25 µM) significantly inhibited actin polymerization (Fig. 5.12A). Because LATS1 is a Ser/Thr kinase, we assessed how N-terminal LATS1, which lacks the kinase domain, or how the C-terminal kinase domain LATS1 modulates actin polymerization. Whereas 25 µM N-terminal LATS1-GST inhibits actin polymerization in a similar manner to wildtype LATS1, 25 µM C-terminal LATS1-GST has no effect on polymerization (Fig. 5.12A). Together, this shows that LATS1 inhibits actin polymerization independently of its C-terminal kinase activity.

Since actin polymerization usually leads to decreased levels of G-actin (soluble cytosolic fraction) with a concomitant increase in F-actin levels (insoluble cytoskeletal fraction), assessing the relative levels of G-actin and F-actin serves as an appropriate
Figure 5.11: Co-localization of LATS1 and F-actin (A) Migrating NIH3T3 cells were stained for LATS1 (green) using anti-LATS1 and AF488 anti-rabbit IgG antibodies and F-actin (Red) using Texas Red phalloidin. Data representative of more than 60 percent of cells in the field (B) LATS1-transfected HeLa cells were treated with Cyto D for 30 min before staining for LATS1 (green) and F-actin (red). Data is representative of more than 70 percent of cells examined.
assay to measure *in vivo* actin polymerization. As shown in Figure 5.12B, overexpression of LATS1 (LATS1-Ad) through adenovirus-mediated infection in MDA-MB-231 breast carcinoma cells, compared to empty vector (Ad), causes a minor increase in the G-actin level (soluble fraction) but a significant decrease in F-actin (insoluble cytoskeletal fraction). In addition, consistent with our above experimental findings that LATS1 is a cytoskeletal protein due to its interaction with F-actin, LATS1 was mostly found in the insoluble cytoskeletal fraction. Finally, decreased levels of F-actin are visualized in actin-stained LATS1-WT-MEFs compared to LATS1-KO MEFs (Fig. 5.12C). These results provide convincing evidence that LATS1 is negative regulator of actin polymerization both *in vitro* and *in vivo*.

### 5.5 Discussion

The above results implicate LATS in the regulation of cell migration and suggest two possible mechanisms for these effects. Not only did loss of either LATS1 or LATS2 in non-invasive MCF7 breast cancer cells enhance cell migration, but loss of both LATS1 and LATS2 had a greater effect on migration in another non-invasive carcinoma cell line, HeLa (Fig. 5.1, 5.2, 5.3). In addition, overexpression of wildtype LATS1, but not kinase-dead LATS1 inhibited migration in the high migratory LATS1+/− MEFs and MDA-MB-231 breast carcinoma cell lines (Fig. 5.4, Fig. 5.5). Although not analyzed, overexpression of LATS2 most likely also inhibits cell migration in a similar manner as cells with overexpressed LATS1. Not only does knockdown of LATS2 alone affect cell migration, but the conserved kinase domain between LATS1 and LATS2 also appears to mediate the migratory effect. Importantly, since the work of this thesis was completed,
Figure 5-12: LATS1 inhibits actin polymerization. (A) Regulation of actin polymerization in vitro. 10% pyrene-actin (10 µM) was mixed with equal volumes of 1×PBS (control), 10 µM GST, and 10, 25 µM LATS1-GST, 25 µM LATS-1-N-GST (amino acids 1-585), or 25 µM LATS1-C-GST (amino acids 588-1130). Polymerization was monitored by measuring fluorescent intensity for 5 min at 365 nm emission and 407 nm excitation. (B) Regulation of actin polymerization in vivo. MDA-MB-231 cells infected with Ad or LATS1-Ad were lysed and separated into soluble (Sol) and insoluble cytoskeletal fractions (Insol) and used for Western blot analysis. (C) MEFs with LATS1 knockout or expressed were subjected to protein extraction and western blot analysis (top panel) or stained for F-actin with TR phalloidin (bottom panel).
several other groups have also shown that LATS1 or LATS2 can regulate cell migration. Zhang et al. show that overexpression of LATS1 or LATS2 inhibits cell migration in the immortalized breast epithelial cell line MCF10A (156) while another group showed that loss of LATS2 in an oncogenic H-RasV12 background further enhanced migration (138). The variety of cell lines used and the diversity of techniques used to manipulate LATS expression show that these effects are not cell line or context dependent, but instead suggest a universal role for LATS in controlling cell migration.

We extended the role of LATS in the regulation of cell migration by showing that LATS1 also affects the ability of cells to spread on substratum. Based on the canonical cell migration process, the ability of cells to extend lamellipodia and attach to new substratum is essential for forward movement (211). We show that expression of LATS1 in LATS1−/− MEFS inhibits their ability to attach and spread onto fibronectin-coated coverslips (Fig. 5.6), implying that LATS1 functions at the leading edge during cell movement.

Not only does this work provide convincing evidence that LATS is a critical player in cell migration, but it also proposes potential mechanisms through which LATS may function. As a central player within the Hippo-LATS pathway, the effects of LATS on cell migration may be regulated and/or mediated by the upstream or downstream signaling components of this pathway, respectively. As an atypical cadherin (213), the membrane associated activator of the Hippo-LATS pathway, FAT4, may also possess the capability to mediate adhesion and migration. However, in our system, when we knockdown FAT4 expression in MCF7 cells, there was no change in the relative rate of cell migration, suggesting that FAT4 does not regulate cell motility. Similarly, loss of
both MST1 and MST2, which are key activators of LATS1, in MDA-MB-231 cells has no effect on cell migration (Fig. 5.7, Fig. 5.8). The role of these proteins in cell migration in other cellular contexts cannot be excluded. However, MST2 alone was downregulated in several breast cancer cell lines including MCF7, BT20, and MDA-MB-231 cells with no dramatic effect on cell migration observed (data not shown). Thus, these upstream regulators of the Hippo-LATS pathway are not responsible for activating LATS to inhibit cell migration.

Alternatively, other upstream regulators, such as NF2 may function through LATS to inhibit cell migration. NF2 (Merlin) has previously been shown to both inhibit cell migration (34) and enhance activation of LATS and phosphorylation of YAP (83). In addition, Crumbs3, a mammalian homolog to Crbs, the recently added player to the Hippo-LATS pathway in Drosophila, has also been shown to inhibit cell migration (214). Specific studies on the role of these proteins in cell migration with respect to LATS should be the focus of future studies.

Although our work has shown that some of the upstream components of the Hippo-LATS pathway are not essential for cell migration, the downstream components YAP and TAZ are able to function downstream of LATS1 in cell migration. Previous studies have shown that overexpression of either YAP (104) or TAZ (74, 98) enhances cell migration. Since YAP and TAZ can be inhibited by LATS phosphorylation (72, 74) and since our results show that kinase-dead LATS1 is unable to inhibit cell migration (Fig. 5.4, 5.5), we hypothesized that LATS may function through YAP and/or TAZ. Indeed, siRNA-mediated knockdown of YAP, TAZ or both YAP and TAZ abrogates the enhanced cell migration in MCF7 cells with reduced LATS1 expression (Fig. 5.9). A
second group confirmed these results by showing that overexpression of LATS1 inhibits YAP-induced cell migration in another cell line (156).

Besides functioning through YAP and TAZ, we also propose a second mechanism of LATS-mediated cytoskeletal changes and cell migration. This mechanism involves the direct effect of LATS on actin and actin polymerization. Several tumor suppressors also bind directly to actin and modulate different aspects of actin dynamics, thereby regulating cell migration. For example, p53 was found to bind to F-actin in a calcium-dependent manner (215), the tumor suppressor NF2 binds and stabilizes F-actin filaments through a lateral association (216), and lastly, APC directly interacts with and bundles F-actin (33). In a similar way, our results show that LATS1 binds directly to F-actin and inhibits actin polymerization, and therefore, LATS1 can be added to this subgroup of tumor suppressors that directly modulates cytoskeletal dynamics and cell migration.

Convincingly, we show that LATS1 interacts directly with actin. First of all, in a non-biased proteomic screen, β-actin was identified as a novel LATS1-binding partner. This was further confirmed by co-immunoprecipitations of endogenous LATS1 where β-actin also precipitated and \textit{in vitro} co-sedimentation assays showing that with increasing amounts of LATS1, the ratio of LATS/F-actin also increased (Fig. 5.10). In addition, LATS1 and F-actin co-localize in migratory NIH3T3 fibroblasts. Finally, disruption of F-actin into small F-actin fragments known as actin foci by the Cyto D reagent caused LATS1 to perfectly co-localize with the actin foci (Fig. 5.11).

Importantly, we also show that LATS1 inhibits actin polymerization both \textit{in vitro} and \textit{in vivo}. This inhibition of actin polymerization is independent of LATS1 kinase activity since N-terminal LATS1 (lacking the C-terminal kinase domain) has the same
effect on actin polymerization as full-length LATS1. Conversely, C-terminal LATS1 has no effect on actin polymerization (Fig. 5.12A), suggesting that the region responsible for inhibiting actin polymerization is located within the first 585 amino acids. Since the kinase domain of LATS1 is not responsible for modulating actin polymerization, the precise mechanism by which LATS1 inhibits actin dynamics remains to be elucidated. In addition, although we show that LATS1 binds F-actin and that LATS1 can inhibit actin polymerization, thereby raising the possibility that LATS1 directly affects actin dynamics, we have not excluded the possibility that LATS1 may indirectly affect actin polymerization through its interaction with other cytoskeletal regulatory proteins such as Zyxin (121) and LIMK1 (119).

Recently, the Hippo-LATS pathway in *Drosophila* was also linked to F-actin dynamics. When certain Hippo-LATS components were abolished, including *dlats*, but not *yki*, F-actin accumulated (217), suggesting that the Hippo-LATS pathway can inhibit F-actin polymerization independently of Yki. This group suggests that Hpo signaling may inhibit an actin-nucleating factor. Interestingly, a related study also showed that F-actin accumulation inhibited the Hippo-LATS pathway, primarily through *dlats* (218). Therefore, this recent work in *Drosophila* not only confirms our results but also points to an integral relationship between actin dynamics and Hippo-LATS pathway activity.

The fact that LATS can affect cell migration both through its kinase activity and substrates YAP and TAZ as well as by modulating actin polymerization independently of its kinase domain does not necessarily lead to mutually exclusive mechanisms of LATS-mediated inhibition of cell migration. In light of the recent studies in *Drosophila* whereby F-actin accumulation can both modulate the Hippo-LATS pathway and be
modulated by this same pathway, a integrated signaling program can be proposed. In particular, when F-actin accumulation is altered due to growth or migratory signals, interaction between F-actin and LATS1 could stimulate the kinase activity of LATS1 to thereby phosphorylate and inhibit the pro-migratory effect of YAP and TAZ. At the same time, the interaction of LATS1 with F-actin could inhibit actin polymerization at the cell membrane and directly affect cell migration at the leading edge. This provides an attractive model of LATS-mediated inhibition of cell migration.

Because cell migration is the fundamental process involved in metastasis and because we show that LATS is a critical regulator of cell migration, it can be suggested that LATS also plays a role in metastasis. Significantly, a recent study showed that downregulation of LATS2 is associated with high lymph node metastasis in breast cancer patients (111). Therefore, examining the role of LATS in metastasis using mouse models will be essential to our understanding of the role of LATS-mediated tumorigenesis and metastasis.
6.1 Contributions

This work was primarily performed by myself. I would like to acknowledge Matt Gordon at the Queen’s Cancer Research Institute for his help with the FACS analysis (Fig. 6.8) and Yawei Hao, our research associate, who helped me with technical aspect of the soft agar assays (Fig. 6.5) and the mouse xenograph tumorigenesis assays (Fig. 6.6). This chapter have been published in *Oncogene* (2011 Jul 25. doi: 10.1038/onc.2011.318).

6.2 Introduction

The FERM domain superfamily includes proteins with membrane stabilizing, kinase, phosphatase and other less characterized functions (170). Due to this diversity of functions, FERM domain proteins can coordinate various signaling events.

Importantly, two FERM domain proteins belong to the Hippo-LATS tumor suppressor network in *Drosophila*: Merlin and Expanded (ex). In *Drosophila*, activation of this pathway involves the WW and C2-domain containing protein Kibra and two FERM domain proteins ex and mer, all of which localize to the apical membrane and cooperatively promote dlats phosphorylation and activation. Connecting these membrane-associated proteins with external growth cues is the atypical cadherin fat or the apical transmembrane protein crumbs, both of which activate ex (reviewed in (37,
In this system, both mer and ex function as tumor suppressors since loss of their expression leads to overgrowth in the eyes or wings of flies (50, 51).

In mammalian systems, this upstream regulation of the Hippo pathway is less understood. Homologs exist for each membrane-associated protein: Merlin/NF2 for mer, FRMD6/Willin/human Expanded (hEx) for ex, KIBRA for Kibra, FAT4 for fat, and Crumbs1-3 for Crumbs. Although Merlin/NF2 is a well-characterized tumor suppressor in mammalian systems, primarily downregulated in tumors of the nervous system, and has been shown to enhance LATS1/2 phosphorylation (52, 83), and human KIBRA has also been found to bind LATS1 and LATS2 enhancing their phosphorylation and kinase activity (52, 84), thereby functioning in parallel to their Drosophila homologs, little is known about hEx in human systems.

Human Expanded is a FERM domain protein similar to Merlin and the ERM (Ezrin, Radixin, Moeisin) family of cytoskeletal crosslinkers and is localized throughout the cytoplasm or along the plasma membrane (194). In this chapter we outline the key cellular functions of hEx and suggest that hEx possesses tumor suppressor properties. By analyzing individual components of the Hippo pathway, we provide the first evidence that hEx does not activate or function through this pathway.

6.3 Materials and Methods

6.3.1 Cell lines and antibodies. MDA-MB-231 and MDA-MB-436 cells were maintained in DMEM supplemented with 10% FBS (Sigma) and 1% Penicillin/Streptomycin (Invitrogen). MCF10A cells were maintained in DMEM-F12 supplemented with 5% horse serum (Sigma), 10 µg/ml insulin (Sigma), 20 ng/ml EGF.
(Sigma), 100 ng/ml cholera toxin (Sigma), 0.5 µg/ml hydrocortisone (Sigma) and antibiotics (Table 4.1). Antibodies used were anti-FLG M2 (Sigma), anti-LATS1 Y03, anti-β-actin (Sigma), anti-YAP (Santa Cruz); from Cell Signaling, anti-S909-LATS1, anti-pYAP, anti-TAZ, anti-MST1, anti-MST2, anti-pMST1/2, anti-ezrin, anti-p21<sup>Cip1</sup>, anti-p27<sup>Kip1</sup>, anti-p15, anti-p18, anti-cyclin A2, anti-cyclin B1, anti-cyclin E1, and anti-pTAZ.

6.3.2 Lentiviral infections. To establish cell lines overexpressing hEx, MDA-MB-231 and MDA-MB-436 cells were infected with lentivirus encoding FLG-tagged hEx or an empty vector control (WPI). To establish hEx-shEx cell lines, cells were infected with lentivirus encoding hEx, next day infected with lentivirus encoding specific shRNA hEx targeting sequences, and selected with 1.0 µg/mL and 0.5 µg/mL puromycin for MDA-MB-231 and MDA-MB-436 cells, respectively. For dose-dependent assays, MDA-MB-436 cells were infected with hEx lentivirus at MOI of 0.5, 1.0, and 2.0. To establish cell lines with downregulated hEx expression, lentivirus encoding specific shRNA targeting sequences (Table 4.2) were infected into MCF10A cells and selected for using 1.0 µg/mL puromycin.

6.3.3 Cell proliferation and cell death assays. For cell proliferation assays, cells were plated in triplicate with equal cell number into 24-well plates and cell numbers were counted on days 1, 2, 3, 4, and 5 days post-plating. For cell death assays, WPI, hEx, and hEx-shEx MDA-MB-231 cell lines were plated in triplicate in 24-well plates at a density of 2 x 10<sup>4</sup> cells/well. Cells were treated with 0, 10, 25, 50, 100, and 200 nM of Taxol (Sigma) for 48 hrs. Percent cell death was quantified by trypan blue analysis. MCF10A cells with decreased hEx expression were plated in triplicate at 3 x 10<sup>4</sup> cells/well of 24-
well plates and treated with 0, 1, 2.5, 5, 10, or 25 nM Taxol for 48 hrs. Cell death was quantified as above.

6.3.4 Colony formation assays. 1 x 10^3 cells were plated in 100 mm plates and incubated at 37°C, 5% CO_2 for 10 days. Colonies were stained with 0.005% crystal violet in 20% methanol and pictures were taken using TE200 Nikon Inverted Fluorescent Microscope (Nikon, Montreal, Canada). Colony number was counted using Bio-Rad Gel Doc System software (Bio-Rad, Mississauga, Canada).

6.4.5 Soft agar assay. 2 x 10^4 cells were mixed with 0.4% agarose in growth media and overlaid on 0.8% agarose in a 35 mm plate. Colony formation was examined by staining colonies with 0.005% crystal violet in 20% methanol. Pictures were taken using TE200 Nikon Inverted Fluorescent Microscope (Nikon, Montreal, Canada). Colony number was counted using Bio-Rad Gel Doc System software (Bio-Rad, Mississauga, Canada).

6.4.6 Xenograph tumor model. About 4 x 10^6 cells from each cell line were injected into each flank (left: MDA-MB-231-WPI; right: MDA-MB-231-hEx) of twelve nude mice. On days 8, 16, and 24 after injection, tumor size of each tumor was measured using a digital caliper and calculated as follows: 0.4 x A x B^2 (A, length; B, wide; in mm).

6.4.7 FACS analysis. About 1 x 10^6 MDA-MB-231 stable cell lines expressing WPI, hEx, or hEx-shEx were harvested, treated with RNase A (200 µg/ml), stained with propidium iodide (50 µg/ml) and analysed with EPICS ALTRA HSS Flow cytometer at the Queen’s Cancer Research Institute. The percentage of each cell cycle phase (G1, S, G2/M) was calculated using Cylchred software.
6.4 Results

6.4.1 hEx inhibits cancer cell proliferation

To examine the cellular functions of hEx in human cancer cell lines, we established hEx-expressing MDA-MB-231 cell lines using lentivirus expressing vector alone (WPI) or FLAG-tagged hEx (Fig. 6.1A). MDA-MB-231 is an aggressive breast cancer cell line but when hEx is overexpressed their growth is severely inhibited (Fig. 6.1B). Importantly, this phenotype is specific to expression of hEx and not due to any nonspecific virus effects since knockdown of hEx in the hEx-overexpressing cells (hEx-shEx) using lentivirus expressing shRNA targeting hEx completely reverses the overgrowth phenotype with a growth rate similar to the WPI control cells. To show that the effects of hEx are not cell line specific, these experiments were repeated using another aggressive breast cancer cell line MDA-MB-436 (Fig. 6.1C). As with the MDA-MB-231 cells, expression of hEx dramatically inhibits MDA-MB-436 cell proliferation whereas the hEx-shEx cell line proliferates at a rate similar to the WPI control cell line (Fig. 6.1D). Moreover, hEx can inhibit cell proliferation in a dose-dependent manner. As shown in Figure 6.1E,F, MDA-MB-436 cells infected with a low level of hEx (MOI 0.5), proliferate at a similar rate compared to control cells infected with vector control (WPI MOI of 2). However, cells infected at an MOI of 1 proliferate significantly slower than control cells, and cells expressing high levels of hEx (MOI of 2) have a negligible rate of proliferation compared to control cells. Together, this shows that rates of cell proliferation correlates with hEx expression in these cell lines.

Traditionally, tumor suppressors are defined by their loss of function in cancer. To recapitulate this in our cell system we down-regulated endogenous hEx in the
Figure 6.1: Overexpression of hEx inhibits cell proliferation. hEx is a regulator of cell proliferation. (A) Western blot analysis of hEx expression levels in MDA-MB-231 cells. Cells were infected with lentivirus expressing vector alone (WPI), hEx-FLG (hEx) or hEx-FLG followed by subsequent infection with lentivirus expressing shRNA against hEx (hEx-shEx). Cell lysate extracted from established cell lines was subjected to western blot using anti-FLG M2 monoclonal antibody (Sigma) and β-actin (Sigma) as internal loading control. (B) Cell proliferation analysis. Cells were plated in triplicate with equal cell number into 24-well plates and cell numbers were counted on days 1, 2, 3, 4, and 5 days post-plating. Experiments were performed at least three times. Data is mean ± SD. (C) Western blot analysis of hEx expression in MDA-MB-436 cells. Cell line establishment and western blot analysis were as described above. (D) Cell proliferation analysis was as described for MDA-MB-231 cells. (E) hEx inhibits cell proliferation in a dose-dependent manner. MDA-MB-436 cells were infected with WPI (MOI=2) and increasing concentrations of hEx-FLG lentivirus (MOI = 0.5, 1.0, 2.0). Western blot showing expression of hEx (anti-FLG) and internal loading control (β-actin). (F) Cell proliferation assay. Each cell line was plated at equal cell density into 24-well plates and total cell number was counted over a period of 4 days. Data is mean ± SD of 3 wells.
MCF10A non-tumorigenic immortalized breast epithelial cell line using lentivirus expressing vector alone (pLKO.1) or one of two shRNAs targeting hEx for degradation (shEx-1 and shEx-2). Due to the understudied nature of hEx, a reliable antibody for hEx does not exist. Therefore, to assess the relative levels of hEx in MCF10A cells qRT-PCR was used to measure mRNA levels and shows that hEx expression is significantly reduced (Fig. 6.2A, B). Significantly, when hEx is downregulated, the rate of proliferation dramatically increases, providing final evidence that hEx is directly involved in regulating cell proliferation (Fig. 6.2C).

6.4.2 hEx mediates sensitivity to the chemotherapeutic drug Taxol

In addition to the importance of cell proliferation in the progression of tumors, the apoptotic machinery also plays a vital role, both in eliminating tumor cells in the early onset of tumorigenesis, as well as later, in the development of drug resistance. A widely used chemotherapeutic in the treatment of breast and ovarian cancer is the microtubule inhibitor Taxol (Paclitaxel) (198). Using the MDA-MB-231 stable cell lines expressing hEx, cells were treated with increasing concentrations of Taxol. As shown in Figure 6.3A, although the control cells are relatively resistant to Taxol with a maximum 28.53 ± 3.46 percent cell death observed using trypan blue analysis after treatment with 200 nM Taxol, cells expressing hEx were significantly more sensitive to this drug treatment with 42.03 ± 2.97 percent cell death. Importantly, knockdown of hEx in hEx-expressing cells are also resistant to Taxol treatment and behave similarly to the WPI control cells with a maximum of 29.29 ± 3.21 percent cell death. Similar results were also obtained when
Figure 6.2: Knockdown of hEx enhances cell proliferation. Quantitative RT-PCR analysis of hEx mRNA levels in MCF10A cells. (A) hEx is overexpressed and knocked down at the mRNA level. RNA was extracted from MDA-MB-231 cells overexpressing WPI, hEx, or hEx followed by shRNA-mediated hEx knockdown (hEx-shEx). SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) was used according to manufacturer’s instructions. qRT-PCR was run using ABI PRISM 7700 Sequence Detection System. 18S rRNA was used as an internal control. Relative mRNA expression was calculated according to the 2^{-ΔΔT} method. Data is mean of 3 samples ± SD. (B) Cells were infected with lentivirus expressing vector alone (pLKO.1) or expressing one of two shRNAs targeting hEx (shEx-1, shEx-2). mRNA was extracted and qRT-PCR was performed and quantified as above. Data is mean of 3 samples ± SD. (C) Cell proliferation analysis as previously described.
cells were treated with lower concentrations of Taxol, showing that sensitivity to Taxol correlates with hEx expression.

A role of hEx in mediating a drug response is further supported by analysis of MCF10A cells with reduced hEx expression. Compared to MDA-MB-231 cells, MCF10A cells are relatively sensitive to Taxol treatment. However, when hEx expression is reduced, the cells become resistant to Taxol treatment (Fig. 6.3B). Unlike control cells (pLKO.1) that respond dramatically to low concentrations of Taxol with significantly increasing amounts of cell death with increasing Taxol concentrations, both shEx-1 and shEx-2 have very little cell death at low drug concentrations. The most dramatic difference is seen at 10 nM Taxol with 39.26 ± 4.60 percent cell death in pLKO-1 expressing MCF10A cells compared to an almost two fold reduction in percent cell death in both shEx-1 and shEx-2 cell lines with 23.04 ± 1.51 and 23.33 ± 1.06 percent cell death respectively.

6.4.3 hEx functions as a putative tumor suppressor

The acquisition of the sustained proliferative and apoptotic resistance properties exhibited by cells with down-regulated hEx levels are reminiscent of key hallmarks of tumor cells (17). This prompted us to verify the tumor suppressor function of hEx in vitro. Initial analysis with a clonogenic assay measuring the ability of single cells to form clones, a property attributed to tumor cells, demonstrated that whereas the control MDA-MB-231 breast cancer cells expressing vector alone (WPI) were able to efficiently form colonies after 10 days with a total number of 325 ± 34 colonies, expression of hEx significantly inhibited this effect with only 192 ± 31 colonies formed (Fig. 6.4A). As
Figure 6.3: hEx expression affects sensitivity to the chemotherapeutic drug Taxol. (A) WPI, hEx, and hEx-shEx MDA-MB-231 cell lines described in Figure 6.1 were plated in triplicate in 24-well plates at a density of 2 x 10^4 cells/well. Cells were treated with 0, 10, 25, 50, 100, and 200 nM of Taxol for 48 hrs. Cell death was quantified by trypan blue analysis. Experiments were performed at least three times. Data is mean ± SD. (B) Loss of hEx renders MCF10A cells resistant to Taxol-induced cell death. MCF10A cells with decreased hEx expression as established in Figure 6.2 were plated in triplicate at 3 x 10^4 cells/well of 24-well plates and treated with 0, 1, 2.5, 5, 10, or 25 nM Taxol for 48 hrs. Cell death was quantified as above.
before, loss of hEx in the hEx overexpressing line rescues the ability of cells to form colonies with 309 ± 49 colonies.

Transformation assays provided further evidence that hEx is a tumor suppressor in breast cancer cell lines. Significantly, expression of hEx severely inhibited the ability of MDA-MB-231 cells to form colonies in soft agar with only 24 ± 16 colonies formed compared to 86 ± 11 formed in the WPI control cells and 77 ± 19 colonies in the hEx-shEx cell line (Fig. 6.4B). On the other hand, loss of hEx in the non-tumorigeneic MCF10A cell line causes transformation of these cells. Where essentially no colonies form in control pLKO.1 cells (12 ± 3), there is a significant increase in anchorage independent growth in both shEx-1 (78 ± 9) and shEx-2 (52 ± 7) cell lines (Fig. 6.4C).

Finally, as in vivo evidence for the tumor suppressor capabilities of hEx, we assessed the ability of hEx to suppress the ability of MDA-MB-231 cells to form tumors in xenograph mouse models. Twelve mice were injected subcutaneously with control cells (WPI) or cells expressing hEx (hEx) into the left or right flank, respectively, and tumor growth was monitored over a period of 24 days. Control cells form large tumors, but hEx cells failed to seed any significant tumor growth (Fig. 6.5A,B).

6.4.4 hEx functions independently of the Hippo pathway

Because expanded is an upstream component of the evolutionarily conserved Drosophila Hippo pathway known to function in cancer progression and because we show that hEx functions as a putative tumor suppressor, we wanted to determine if hEx functions in an evolutionarily conserved manner to regulate cell proliferation by activating the Hippo pathway. In Drosophila, expression of ex leads to enhanced
Figure 6.4: hEx is a putative tumor suppressor in vitro. (A) Colony formation assay. MDA-MB-231 stable cell lines expressing WPI, hEx, or hEx-shEx as described in Figure 6.1 were plated at 1 x 10^3 cells/100mm plate. 10 days after plating, colonies were stained with 0.005% Crystal violet in 20% methanol. Quantization is shown on the right. Data is mean ± standard deviation of 3 samples/cell line. Statistical analysis using a Student’s t-test was performed and * indicates significant difference (p-value ≤ 0.05). (B-C) Soft agar assay. About 2 x 10^4 MDA-MB-231 cells overexpressing hEx (B) or MCF10A cells with reduced hEx expression (C) were mixed with 0.4% agarose in growth media and overlayed on 0.8% agarose in a 6-well plate. 4 weeks after plating, colonies were stained and quantified as above. Quantization is shown on the right. Data is mean ± standard deviation of 3 samples/cell line. Statistical analysis using a Student’s t-test was performed and * indicates a significant difference (p-value ≤ 0.05).
Figure 6-5: hEx is a putative tumor suppressor in vivo. (A) About $4 \times 10^6$ cells were injected into each flank (left: MDA-MB-231-WPI; right: MDA-MB-231-hEx) of twelve nude mice. Tumor size was measured every 8 days using a digital caliper and calculated as follows: $0.4 \times A \times B^2$ ($A$, length; $B$, wide; in mm). Data is mean ± SD of twelve mice. (B) Representative image of tumor growth in mice.
phosphorylation and activation of dlats and subsequent phosphorylation and inhibition of Yorkie (50, 52). Surprisingly, when we assessed the relative phosphorylation levels of the mammalian Hippo pathway components, including MST1/2, LATS1, YAP and TAZ, in hEx-overexpressing MDA-MB-231 cells we found no significant differences (Fig. 6.6A). Upon stimulation, MST1 and MST2 autophosphorylate on conserved threonine residues (220) and once active, phosphorylate LATS1 on T1079 leading to LATS1 autophosphorylation on S909 (68). Once active, LATS1 phosphorylates YAP on S127 (72) and TAZ on S89 (74). Using specific antibodies for these phosphorylation sites, it is evident that hEx does not enhance the phosphorylation and subsequent activity of the canonical Hippo pathway in this cell line. This provides the first evidence the hEx functions in a distinct manner to Drosophila ex. To further confirm that hEx does not function through this pathway, we knocked-down the central kinases LATS1 and LATS2 in hEx-expressing MDA-MB-231 cells (Fig. 6.6B) and show that loss of LATS1 and LATS2 was not able to alter the inhibitory effect of hEx on cell proliferation (Fig. 6.6C). Therefore, unlike in Drosophila, where loss of dlats rescues the ex phenotype (221), hEx tumor suppressor function is independent of the Hippo pathway.

6.4.5 hEx induces p21Cip1 expression and causes S phase cell cycle arrest

Since hEx does not function through the Hippo-LATS pathway in the MDA-MB-231 cell line, an alternative mechanism needs to be determined to explain the tumor suppressor function of hEx. To specifically address the mechanism behind inhibition of cell proliferation in hEx overexpressing cells, we analyzed how hEx alters cell cycle progression. FACS analysis shows that hEx increases the percentage of cells in the S-
Figure 6.6: **hEx functions independently of the Hippo pathway.** (A). Total cell lysate was extracted from MDA-MB-231 cells infected with lentivirus expressing WPI or hEx-FLG. Western blot of using specific phospho-antibodies shows hEx does not activate the Hippo pathway. (B) Downregulation of LATS1 and LATS2 in hEx-expressing MDA-MB-231 cells. Western blot shows expression of hEx (anti-FLG), LATS1, and LATS2 after infection of lentivirus expressing vector alone (WPI), hEx-FLG (hEx) or hEx followed by subsequent infection with lentivirus expressing shRNA targeting LATS1 and lentivirus expressing shRNA targeting LATS2 (hEx-shLATS). (C) Cell proliferation assay as described previously.
phase of the cell cycle with a concomitant decrease in the percentage of cells in the G2 phase (Fig. 6.7A). Therefore, hEx inhibits proliferation by impeding progression through the S phase.

To further assess the mechanism of this S phase arrest, the relative expression of important cell cycle regulatory proteins were analyzed by western blot analysis. Although there is no significant change in CKIs p15, p18, or p27, there is a dramatic increase in the expression of the cyclin kinase inhibitor p21\textsuperscript{Cip1}. Furthermore, there is a decrease in Cyclin A expression whereas there is no significant difference in Cyclin B or Cyclin E (Fig. 6.7B). This upregulation of p21\textsuperscript{Cip1} and downregulated Cyclin A are consistent with our cell cycle analysis. Thus we propose a model whereby hEx suppresses S phase progression by upregulating p21\textsuperscript{Cip1} and downregulating cyclin A.

6.5 Discussion

The FERM domain proteins make up a large superfamily with a multitude of functions. Upstream activation of the Hippo-LATS pathway in \textit{Drosophila} includes two FERM domain proteins, mer and ex. The human homolog of expanded, hEx, was previously uncharacterized both with respect to its cellular functions as well as its ability to activate the mammalian Hippo-LATS pathway. This chapter shows how hEx functions as a tumor suppressor and functions independently of the Hippo-LATS pathway.

The ability of hEx to inhibit cell proliferation and increase sensitivity to the chemotherapeutic Taxol (Fig. 6.1, 6.2, 6.3) are consistent with \textit{Drosophila} models where ex has also been implicated in cell growth and apoptosis as functions of its tumor
Figure 6.7: hEx inhibits progression through the S phase of the cell cycle. (A) About $1 \times 10^6$ MDA-MB-231 stable cell lines expressing WPI, hEx, or hEx-shEx as described in Fig 6.1 were harvested, treated with RNase A (200 µg/ml), stained with propidium iodide (50 µg/ml) and analysed with EPICS ALTRA HSS Flow cytometer. The percentage of each cell cycle phase (G$_1$, S, G$_2$/M) was calculated using Cylchred software. (B) Protein lysate was extracted from MDA-MB-231 stable lines expressing WPI, hEx, or shEx and analyzed by western blot. Antibodies used are as follows: anti-FLAG M2, anti-p15, anti-18, anti-p21, and p27, anti-cyclin A2, anti-cyclin B1, anti-cyclin E2.
suppressor activity. In fact, some of the first studies on ex showed that it regulated cell proliferation in the imaginal discs in the developing flies and when overexpressed led to decreased cell number in the wings (189). In addition, further studies also suggested that where overexpression of ex induces cell death (190), loss of ex along with its FERM domain partner mer suppressed apoptosis in the developing eye (50). Together, the enhanced proliferation and the loss of apoptosis in ex mutant flies leads to tumor overgrowth. In the same way, hEx can function as a tumor suppressor in vitro (Fig. 6.4) and in vivo (Fig. 6.5). This study is the first to characterize the cellular functions of hEx and show that it has the capability to function as a tumor suppressor in mammalian systems. Future work should confirm this nature of hEx through mutational studies and/or clinical cancer sample analysis.

Although the Hippo pathway is more complex in mammalian systems, the proliferation, apoptosis and tumor suppressive functions of hEx are similar to several human Hippo pathway members, including LATS1 and LATS2. In Drosophila, ex expression leads to enhanced phosphorylation and activation of dlats and subsequent phosphorylation and inhibition of yorkie (50, 52). However, in our system, overexpression of hEx does not modulate the phosphorylation and or activity of any of the core Hippo-LATS pathway components, and the ability of hEx to inhibit cell proliferation is not dependent upon LATS expression (Fig. 6.6).

Importantly, although much of the Hippo pathway is conserved from flies to humans, complexity increases moving up the evolutionary tree. Therefore, differences can arise in protein structure, interactions and function. Evidence already exists to support this hypothesis. Although hEx is the closest human homolog to ex, their
structure is considerably different. hEx and *Drosophila* ex share the conserved FERM domain, but hEx lacks the C-terminal region of *Drosophila* ex which contains several PPXY motifs responsible for protein-protein interactions (54, 194). Interestingly, it has been suggested the tumor suppressor function of *Drosophila* ex is mediated primarily via its C-terminus (193), which is further evidence that hEx and *Drosophila* ex, although both tumor suppressors, mediate their effects by distinct mechanisms.

In addition, disparities between *Drosophila* and human systems have been shown in both the downstream transcriptional targets and the upstream regulatory proteins of the Hippo pathway. For example, *cyclin E*, *diap1*, and *bantam* are key genes downstream of Yki in *Drosophila* (2), but have not been shown to be transcriptional targets of its orthologs (77, 78, 80). Additionally, whereas dRassf antagonizes the Hippo pathway in *Drosophila* (63), its closest mammalian homolog RASSF1A activates MST1/2 and the kinase cascade (81). Also upstream, *Drosophila* Kibra interacts with multiple Hippo pathway components including mer, ex, hpo, and dlats. However, its human homolog only binds Merlin but not MST2 or hEx (52, 53). Together, this suggests that although the core Hippo kinase cassette is conserved, both the mediators and regulators of this pathway are more diverse in mammalian systems.

Since hEx does not function through the Hippo-LATS pathway in our system, the identification of a potential mechanism mediating its tumor suppressor function was imperative. Importantly, we propose a model whereby hEx inhibits progression through the S phase of the cell cycle by upregulating p21\(^{Cip1}\) expression and downregulating Cyclin A expression (Fig. 6.7). The p21\(^{Cip1}\) cyclin kinase inhibitor has pleiotrophic effects, affecting all stages of cell cycle progression (199), and the ability of p21\(^{Cip1}\) to
inhibit S phase progression has been linked to the downregulation of cyclin A expression (222). If hEx affects p21^Cip1 and Cyclin A expression at the transcriptional or post-transcriptional level remains to be determined. The identification of hEx binding partners will aid in understanding these precise mechanisms of hEx function.

In summary, this work characterizes the cellular functions of hEx protein in cancer cell lines and shows that it is a potent inhibitor of cell proliferation, transformation, and modulator of drug sensitivity. This suggests that hEx has tumor suppressor properties. Indeed, hEx inhibits anchorage independent growth *in vivo* and tumor growth *in vivo*. Importantly, hEx functions in a Hippo-independent fashion, shedding new light on distinct Hippo signaling pathways between flies and mammals.
PART 4: CONCLUSIONS AND DISCUSSION

CHAPTER 7: OVERALL DISCUSSION AND FUTURE DIRECTIONS

The increasing importance of the Hippo-LATS pathway is demonstrated by the growing number of studies investigating the regulatory machinery, the cellular outcomes, and the implication of this pathway in human disease. Significantly, this thesis adds new facets to the Hippo-LATS pathway including the integration of this pathway with other well-known signaling networks, the addition of cell migration and Taxol resistance as novel cellular outputs, as well as the elucidation of hEx as a Hippo-LATS independent signaling molecule (Fig. 7.1).

Arguably, the key signaling molecules within the Hippo-LATS signaling pathways are LATS1 and LATS2. Ultimately, upstream regulatory signals converge on LATS1 and LATS2, relaying growth inhibitory signals through LATS-mediated phosphorylation of downstream effectors YAP and TAZ. Due to the importance of these signaling kinases, LATS may also mediate its effects through kinase-independent or Hippo-independent mechanisms. This thesis has explored some of these possibilities, providing evidence that LATS is a broad signaling molecule within the cell.

Previous studies on LATS1 and LATS2 focused on how overexpression of either LATS1 or LATS2 inhibited cell proliferation or induced apoptosis (122, 125, 129, 130, 155). The experimental basis of studying the loss of endogenous protein expression provides analysis more consistent with a tumor environment where loss of a particular tumor suppressor can accelerate tumor progression and thus is an essential component when studying tumor suppressor genes. This thesis studied how loss of LATS1, LATS2,
Figure 7.1: Distinct signaling mechanisms of LATS. Distinct signaling pathways are coloured accordingly. In blue is the Hippo-LATS pathway which consists of identified and potential activators NF2, hEx, MOB1, WW45, and MST1/2, as well as downstream targets YAP and TAZ. In red is the LATS-actin pathway which can regulate cell migration. In green is the p53 pathway through which LATS modulates the expression of p53 transcriptional targets. LATS also regulates the expression of proteins in the Ras pathway, shown in purple. Other downstream targets of LATS link LATS to the WNT pathway, shown in black. Upstream of LATS, an unknown kinase is activated by Taxol to phosphorylate LATS leading to the induction of cell death. hEx functions independently of LATS to inhibit cell proliferation and tumorigenesis. ? depicts signaling mechanisms that are currently unknown.
or both LATS1 and LATS2 modulated several tumor phenotypes. As expected, the ability of LATS to control cell proliferation was confirmed (Fig. 4.1).

More importantly, this thesis also described novel functions of LATS including its role in transcriptional regulation, mediating Taxol resistance, and inhibiting cell migration. Firstly, we identified a multitude of genes differentially expressed upon LATS knockdown (Fig 4.6). In addition to genes that are regulated through the Hippo-LATS pathway such as CYR61 (80) and potentially CKDN1A (100, 202), our whole human genome microarray analysis also couples LATS to other important signaling networks in particular the Ras, p53, and WNT signaling pathways (Fig. 7.1). Firstly, LATS regulates several negative regulators of the Ras signaling pathway including SPRY4, SPRY2, and SPRED1 (Table 4.3, Fig. 4.6). Interestingly, constitutively active Ras induces LATS2 expression (138). Combined with our microarray results, a novel negative feedback mechanism involving LATS2 inhibition of Ras upon oncogene activation can be proposed. Secondly, significant evidence links LATS to p53, including its role in mediating LATS effects on the G1 tetraploidy checkpoint (125, 128), as well as the identification of overlapping transcriptional targets between LATS and p53, such as TP53INP1 (209). Finally, LATS may also modulate the WNT signaling pathway by upregulating WNT target genes such as WISP2 (Fig. 4.6). The broad regulatory function of LATS as transcriptional regulator allows it to modulate several cellular functions including proliferation, apoptosis, and cell migration.

A second novel function identified in this thesis is the cooperation of LATS1 and LATS2 for the induction of cell death in response to Taxol. Although loss of LATS1 alone leads to moderate Taxol resistance in MCF7 cells, loss of both LATS1 and LATS2
resulted in a greater than two-fold reduction in cell death after Taxol treatment. The cooperation between LATS1 and LATS2 in mediating cell death in response to Taxol implies that one protein may compensate for loss of the other or alternatively that LATS1 and LATS2 mediate cell death through distinct pathways. The latter scenario may hold true since previous studies on LATS showed that LATS1 upregulates pro-apoptotic proteins p53 and Bax (155, 159) whereas LATS2 downregulates anti-apoptotic proteins Bcl-XL and Bcl-2 (159). As further evidence for a new role of LATS in mediating a response to Taxol, we show that the phosphorylation of LATS1 correlates with Taxol sensitivity in two breast cancer cell lines suggesting that the activation of LATS1 is essential for efficient cell death. Taxol is a widely used chemotherapeutic for the treatment of breast cancer, however its success is limited by the development of cellular resistance which can include multiple mechanisms such as the altered expression of key signaling molecules (198). Importantly, LATS may be one of those key signaling molecules. Previous studies showed that expression of LATS1 and LATS2 is downregulated in human breast cancer and low expression correlates with more aggressive breast tumors (10, 111). Since our study showed that low levels of LATS1 and LATS2 are associated with Taxol resistance, combined with previous studies, our results suggest that LATS1 and/or LATS2 may serve as predictive markers for the use of Taxol in breast cancer treatment. More analysis, particularly the assessment of total protein levels as well as levels of phosphorylated LATS in clinical cancer samples and their correlation to treatment response will provide the necessary information to formally draw this conclusion.
This thesis also described how LATS modulates cell migration. Using multiple cell lines and both overexpression and knockdown experimental techniques, a role for both LATS1 and LATS2 in controlling cell migration was established. Loss of LATS1 or LATS2 alone moderately enhanced cell migration (Fig. 5.1, Fig. 5.2) and overexpression of LATS1 independently inhibited cell migration (Fig. 5.4, Fig. 5.5). Simultaneous loss of both LATS1 and LATS2 increased cell migration greater than two-fold (Fig. 5.3). Thus, as with a Taxol response, LATS1 and LATS2 may cooperate to control cell migration. Additional groups have also shown that either LATS1 (156) or LATS2 (138) can regulate cell migration, further highlighting the importance of the ability of LATS to control cell motility. Cell migration is the fundamental process in mediating metastasis. Therefore, the implication of LATS in cell migration points to the potential role of LATS in mediating the final fatal step of tumorigenesis. Metastasis involves a series of steps including the transition from epithelial to mesenchymal-like cells (EMT). In this way, cells adopt a more migratory phenotype characterized by loss of cell-cell adhesions (210). One study has shown that loss of LATS1 enhances EMT in immortalized breast epithelial cells by increasing E-Cadherin expression and downregulating N-cadherin expression (156). Both YAP (156) and TAZ (74, 80) have also been implicated in the epithelial mesenchymal transition, but whether these proteins also contribute towards metastasis is an area of ongoing research. The use of mouse models will greatly facilitate this research.

In addition to describing new functions of LATS, we have also elucidated novel mechanisms mediating these responses. Some of these mechanisms include kinase-independent and/or Hippo-LATS pathway independent signaling events. For example,
the identification of the numerous genes differentially expressed upon loss of both LATS1 and LATS2 provides clues to new downstream targets mediating LATS phenotypes. Firstly, the proteins encoded by the SLIT2 (223) and MYLK (224) genes have been implicated in mediating cancer cell migration. Additionally, genes such as TP53INP1 (209) may mediate LATS effects on apoptosis. Finally, we identified CDKN1A encoded by p21\textsuperscript{Cip1} as a transcriptional target of LATS1 and LATS2. The regulation of p21\textsuperscript{Cip1} expression may convey the growth inhibitory signals from LATS to the cell cycle (122, 129, 130, 155). Of all the genes identified to be differentially expressed when LATS is downregulated, only CYR61, and potentially CDKN1A are identified as a genes directly downstream of TAZ and/or YAP (80, 100, 156, 202). In addition to YAP and TAZ, LATS most likely functions through distinct transcription factor complexes to control expression of various genes. In Drosophila new transcription factors such as homothorax and teashirt were shown to act downstream of the Hippo-LATS pathway and Yki (225). In mammalian systems, both YAP and TAZ can bind and function through multiple transcription factors (226). Thus, the possibilities for LATS-mediated transcriptional regulator are immense.

This thesis also proposes two mechanisms of LATS regulated cell migration. As expected, we show that expression of TAZ and YAP is important for the enhanced migration observed in cells with downregulated LATS1 expression. Another group has also confirmed this result showing that LATS1 functions through YAP to modulate cell migration (156). In addition to kinase-dependent cell migration, we also propose that LATS controls cell motility independently of the Hippo pathway and its kinase domain. Since neither MST1/2 nor FAT4 have any significant effects on cell migration, the
upstream anti-migratory signals that activate LATS originate from molecules separate from the canonical upstream Hippo signaling molecules. Additionally, we show that LATS1 directly binds actin and inhibits actin polymerization \textit{in vitro} and \textit{in vivo}. Since actin dynamics are integral to directed cell migration (211), the enhanced cell migration observed in cells with downregulated LATS1 and/or LATS2 could be a result of deregulated actin polymerization. Importantly, inhibition of actin polymerization occurs independently of the C-terminal kinase domain of LATS1, providing evidence that downstream kinase substrates YAP and TAZ are not involved in regulating actin dynamics. Kinase-independent functions of LATS have been previously reported. For example, LATS1 inhibits the kinase activity of LIMK1 by binding the cytoskeletal protein (119). Like LATS inhibition of actin, the ability of LATS1 to inhibit LIMK1 does not involve phosphorylation since neither molecule contains the LATS phosphorylation consensus motif. Thus LATS possesses both kinase independent and Hippo pathway independent functions.

Not only does LATS function independently of the Hippo-LATS pathway with respect to its modulation of actin dynamics, but its ability to mediate a response to Taxol also occurs through a distinct mechanism. Neither its phosphorylation site by MST1/2 (T1079) nor the LATS phosphorylation site of YAP (S127) is affected by treatment with Taxol. However, it is clear that LATS plays a role in Taxol induced cell death since loss of LATS leads to Taxol resistance. This suggests that the role of LATS in Taxol resistance is separate from its role within the Hippo-LATS pathway. Multiple studies have shown that LATS1 and LATS2 are located at the spindle apparatus, are specifically phosphorylated during mitosis, and control progression through mitosis (113, 119, 121,
124, 125, 127, 128). However, the kinase responsible for these phosphorylations has not been determined. Whereas the Hippo-LATS activates LATS through binding and phosphorylation by MOB1 and MST2 at the cell membrane (71), these mitotic specific phosphorylation events occur in the nucleus. Although we can hypothesize that a mitotic kinase such as an Aurora family member, Bub1 or Mps1 (227) will phosphorylate LATS in response to Taxol, the specific elucidation of the kinase will require careful biochemical analysis and elucidation of the specific phosphorylation site on LATS1. In addition, since YAP S127 phosphorylation is not enhanced by treatment with Taxol, the downstream signals mediating the cell death response of LATS also need identification. Currently, this work is being continued in our lab. Not only will this work contribute to our understanding of mechanisms behind Taxol action, but will also establish both a novel role for LATS and a novel mechanism of LATS activation.

Another aim of this thesis was to elucidate the regulatory mechanisms controlling the Hippo-LATS pathway. The novel mechanism of LATS activation in response to Taxol treatment is one example of how this work has provided new information on how LATS and the Hippo-LATS pathway are regulated. In addition, based on the conservation between the Hippo-LATS pathway in Drosophila and mammals, we also investigated the role of hEx in modulating the Hippo-LATS pathway. Importantly, we characterized the functions of hEx and show that it possesses the ability to act as a tumor suppressor by inhibiting cell proliferation, sensitizes cancer cells to Taxol treatment, and inhibiting tumor growth in vitro and in vivo. Although these functions are consistent with Drosophila models, in our system, hEx does not activate LATS or function through the
Hippo-LATS pathway. Instead, hEx induces p21<sup>Cip1</sup> expression, downregulates Cyclin A expression and inhibits progression through the S phase of the cell cycle.

In conclusion, the work of this thesis has provided new functions for LATS and new mechanisms of LATS function. In addition to its conserved kinase activity, LATS also possesses kinase-independent and Hippo-LATS pathway independent functions. With the multitude of signaling mechanisms mediated by LATS1 and LATS2 and the numerous different functions that they possess, it is evident that these tumor suppressors as well as the Hippo-LATS pathway, are essential for maintaining a homeostatic state. Disruption of these proteins can lead to tumors in many human tissues. With this in mind, the research within this thesis as well as ongoing studies into the specific mechanisms mediating these functions will hopefully provide new clues for the development of therapeutic targets in our fight against human disease.
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