ITCH E3 UBIQUITIN LIGASE REGULATES LATS1 TUMOUR SUPPRESSOR STABILITY

by

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ABSTRACT

The Large Tumor Suppressor 1 (LATS1) is a serine/threonine kinase and tumor suppressor found down-regulated in a broad spectrum of human cancers. It is a central player of the emerging Hippo-LATS tumour suppressor pathway, which plays important roles in cell proliferation, apoptosis, and stem cell differentiation. Despite the ample data supporting a role of LATS1 in tumour suppression, how LATS1 is regulated at the molecular level remains largely unknown. In this study, we have identified Itch, a HECT class E3 ubiquitin ligase, as a novel binding partner of LATS1. Itch can complex with LATS1 both in vitro and in vivo through the PPxY motifs of LATS1 and the WW domains of Itch. Significantly, we found that over-expression of Itch promoted LATS1 degradation by polyubiquitination through the 26S proteasome pathway. On the other hand, knockdown of endogenous Itch by shRNAs provoked stabilization of endogenous LATS1 proteins. Finally, through several functional assays, we also revealed that change of Itch abundance alone is sufficient for altering LATS1-mediated downstream signaling, negative regulation of cell proliferation, and induction of apoptosis. Together, our study identifies E3 ubiquitin ligase Itch as the first negative regulator of LATS1 and presents for the first time a possibility of targeting LATS1/Itch interaction as a therapeutic strategy in cancer.
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<th>Description</th>
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<tbody>
<tr>
<td>AF</td>
<td>Alex Fluor</td>
</tr>
<tr>
<td>AIP4</td>
<td>Atrophin-1-Interacting Protein 4</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>ATC</td>
<td>Anaplastic thyroid carcinoma</td>
</tr>
<tr>
<td>Baf A1</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer gene 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>Cyr61</td>
<td>Cysteine-rich 61</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC chemokine Receptor 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMEM-F12</td>
<td>Dulbecco's Modified Eagle's Medium Nutrient Mixture F12 Ham</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Flourescent Protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-Transferase</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to E6 Carboxy Terminus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-1-thio-β-D-galactopyranoside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LATS1</td>
<td>Large Tumour Suppressor 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MHL1</td>
<td>MutL Homolog 1</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLC-γ1</td>
<td>Phospholipase C-γ1</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
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<td>Retionblastoma</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope Labelling With Amino Acids In Cell Culture</td>
</tr>
<tr>
<td>TAZ</td>
<td>Transcriptional co-activator with PDZ-binding motif</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline Tween-20</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel Lindau</td>
</tr>
<tr>
<td>WWP1</td>
<td>WW domain-containing Protein 1</td>
</tr>
<tr>
<td>XIAP</td>
<td>X chromosome-linked IAP</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-Associated Protein</td>
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CHAPTER 1

INTRODUCTION

1.1 Tumour Suppressor Genes

1.1.1 Genetic alterations in cancer

Cancer is a highly adaptable and profoundly complex genetic disease. In any given malignant cell, there are often at least tens, if not hundreds of genetic alterations (1-3). Unlike diseases such as cystic fibrosis or muscular dystrophy, wherein a single gene mutation results in the development of the disease, no one genetic defect can cause cancer (1). Instead, accumulation of at least 3 critical gene defects are required to produce invasive cancers (4, 5). A major reason is that, within the mammalian cells, there exist a number of tumour suppressive devices that share functional homology and redundancy (6, 7). Even if one safeguard mechanism fails, another safeguard mechanism may still function to protect against tumour formation. Interestingly, many tumour suppressive mechanisms identified to date remain highly conserved across different tissue types (6, 7), suggesting that a good understanding of tumour suppressive mechanisms may not only be vital for our understanding of cancer pathology, but may also be useful for developing novel therapeutics that could be effective across different tumour types. The present study, in particular, focuses on how a particular tumour suppressor, named Large Tumour Suppressor 1 (LATS1), is regulated at the molecular level.

1.1.2 Concept of tumour suppressor genes

As early as the 1970s, scientists discovered that there exist two classes of genes directly related to cancer (8-10). The first class are oncogenes, genes whose up-regulation
or gain of function contributes to malignancy formation (10). The second class are
tumour suppressor genes, genes whose down-regulation or loss of function contributes to
malignancy formation (10). In particular, tumour suppressors have become a prime focus
of many research studies, as they represent a class of molecular antagonists against
tumourigenesis.

The first clue to the presence and function of tumour suppressor genes came from
a study by Harris and his colleagues when they tested the effect of making cell hybrids
between normal and tumour cells (11). As revealed in their study, fusion of a tumour cell
to a normal cell completely reverted the tumourigenic features, indicating that cancer
may arise through loss of function of certain cellular components (11). Later in the 1980s,
Weissman and his colleagues also found that reintroduction of chromosome 11 alone into
a Wilms’ tumour cell line reverted the associated oncogenic phenotypes (12), suggesting
that genomic defects may be a primary mechanism leading to cancer development (12).

With extensive research efforts, many specific genes and chromosome regions
responsible for suppressing tumours in humans were later identified (1, 13). One
particularly prominent finding is the discovery of the \textit{RB1} gene (9). The \textit{RB1} gene, first
identified in the 1986, is a gene found related to retinoblastoma (9). With more studies on
retinoblastoma cases, Knudson later showed that, induction of tumour development
requires the inactivation of both alleles, known as two “hits”, of a tumour suppressor
gene (14). His finding led to the construction of the two-hit hypothesis, which is later
found applicable to \textit{RB1} and several other tumour suppressor genes associated with
familial disease (15, 16). This hypothesis, however, cannot fully explain the whole area
of malignancy. In fact, certain tumour suppressors, such as p53, were later found to have
a dominant negative function, such that inactivation of a single gene allele can cause complete functional loss of a tumour suppressor (17, 18). In addition, although some tumour suppressors do not directly cause tumour formation upon functional loss, their loss may contribute to genomic instability, promoting the cell to accumulate other genetic defects that ultimately leads to cancer development (19). Hence, tumour suppressor genes are now more broadly defined as genes whose loss or inactivation can promote cancer phenotypes.

1.1.3 Tumour suppressor genes in cancer

The molecular mechanisms underlying the loss or downregulation of tumour suppressors in cancer can be broadly categorized into three distinct categories, acting at the (1) genetic, (2) epigenetic and (3) protein level. At the genetic level, tumour suppressors may be inactivated through in-frame deletion, missense, nonsense, frame-shift or splicing mutations (20). TP53, for example, is one classic tumour suppressor gene found commonly mutated in human cancers. Over 50% of cancer cases analyzed to date involve mutations and/or deletions of the TP53 gene (21), suggesting the critical role of genetic alterations in cancer development. Other tumour suppressor genes, including RB1 (22), PTEN (23), and APC (24), are also frequently mutated in human cancers and their loss-of-functions are linked to aggressive tumour phenotypes and poor prognosis (25, 26).

At the epigenetic level, tumour suppressors may also be transcriptionally silenced through promoter hypermethylation or histone deacetylation (27). Promoter hypermethylation, which silences gene expression through recruitment of repressor
complexes to the promoter of a gene, is a common hallmark of cancers (27, 28). Various tumour suppressor genes are silenced in cancers by promoter hypermethylation, including RASSF1A (29), \textit{RB1} (30), \textit{p16} (30, 31), \textit{MLH1}(32), \textit{BRCA1}(33) and \textit{VHL}(34). For example, although mutations of the RASSF1A gene are rarely found in cancers, downregulation of RASSF1A by promoter hypermethylation is frequently detected (29). Histone deacetylation, which modifies chromatins to a structure unfavorable for transcription, is another common mechanism for repressing the expression of tumour suppressors (27). For instance, although mutation of the maspin tumour suppressor has not been previously reported, maspin is frequently downregulated in breast cancer cells through promoter hypermethylation and histone deacetylation (35), suggesting of the critical role of epigenetic regulation in tumour suppressor function.

Finally, in addition to the mechanisms mentioned above, tumour suppressors may also be downregulated at the protein level through post-translational modifications. Current advances in proteomic profiling have revealed that downregulation of numerous tumour suppressors may be linked to human cancers. In ependymomas, for instance, a reduction in p53 proteins is found correlated with an increased tumour grades and poorer patient survival (36). Additionally, in bladder transitional cell carcinomas, reduced expression of the 14-3-3σ tumour suppressor protein is also correlated with the invasiveness of a tumour (37), suggesting that tumour suppressors may be used as biomarkers in the clinical setting.

1.1.4 Functions of tumour suppressor genes

Although the link between tumour suppressor genes and cancer was long recognized, only recently has their mechanism of action at the molecular level been
revealed. In particular, most, if not all, tumour suppressors identified to date possess a regulatory function in tissue homeostasis, a process requiring the delicate balance between cell proliferation and cell death (38, 39). To better understand the function of tumour suppressors in the regulation of cell proliferation and cell death, we provide here with a brief overview.

1.1.4.1 Regulation of cell proliferation

For cells to proliferate, they must receive and process growth-promoting signals from their extracellular environment (1, 40-44). The types of growth promoting signals received by a cell can range from growth factors, hormones, neurotransmitters to cytokines (1, 40-44). Depending on the type of growth factor received, different cell surface receptors may be activated, which in turn promote the recruitment of intracellular signaling molecules to transmit the proliferative signals from the outside to the inside of a cell (1, 40-44). Ultimately, growth signals delivered to the nucleus can stimulate transcription of cell cycle regulatory proteins, which in turn advances quiescent cells through the cell cycle for division (1, 40-44).

Tumour suppressors, in particular, are capable of suppressing cell proliferation process through multiple layers of control. Firstly, tumour suppressors may render cells less responsive to growth-promoting signals by controlling the expression patterns of cell surface receptors and secretory growth factors. For instance, pVHL, a tumour suppressor found inactivated in over 50% of retinal cell carcinomas (45), is well known for its ability to inhibit the expression of the CXCR4 chemokine receptor (46) and VEGF growth factor (47), both of which are growth inducers with profound oncogenic functions. Secondly,
within the cytoplasmic compartment, tumour suppressors may also inhibit a variety of signal transduction molecules responsible for transmitting a proliferative signal. For example, PTEN, a tumour suppressor commonly downregulated or mutated in virtually all human cancers, plays a critical role in the dephosphorylating PtdIns(3,4,5)P$_3$ (48) and IRS1 (49), both of which are mediators of the AKT and MAPK growth-promoting pathways. Finally, in the nucleus, tumour suppressors may also directly restrict cell cycle progression by arresting the cell at the G1/S or G2/M cell cycle checkpoints, preventing the division of a cell (50).

At the G1/S checkpoint, tumour suppressors ensure the fidelity of the genetic information that is replicated in S phase (50, 51). Many tumour suppressors that function at this checkpoint are capable of sensing DNA damage and promoting cell cycle arrest to facilitate repair of the damaged DNA (50, 51). RB, for example, is a tumour suppressor functioning at this checkpoint and it promotes stress-induced G1/S arrest by repressing the cell-cycle-driving function of the E2F family of transcription factors (52). The p16 tumour suppressor, on the other hand, can also block G1/S progression by binding to Cdk4 and preventing its activation by Cyclin D (53).

In addition to the G1/S checkpoint, the G2/M checkpoint is another regulatory event essential for ensuring accurate replication of the DNA genome prior to division in the M phase (50, 51). p53, for example, is a classic stress-induced tumour suppressor functioning at this checkpoint (54). Upon activation by genotoxic stress, p53 promotes transcriptional upregulation of two Cdk2 inhibitors, p21 and 14-3-3 σ (54). While p21 represses Cdk1 activity by dissociating it from its activator Cyclin B, 14-3-3 σ prevents Cdk1 nuclear action by sequestering it in the cytoplasm (54). Together, these mechanisms
effectively halt the cell cycle, allowing the cell to repair any genomic damage prior to division. In addition to cell cycle arrest, various tumour suppressors, such as BRCA1 and BRCA2, are also known for their abilities to localize at the DNA damage sites and facilitate its repair (55). Together, these findings support a critical role for tumour suppressors in the regulation of cell proliferation.

1.1.4.2 Regulation of apoptosis

Apart from their roles in cell proliferation, tumour suppressors may also combat tumour growth by regulating cell death pathways, specifically the apoptotic pathway (7). Apoptosis, or programmed cell death, is a multi-step biochemical program first discovered in the 1970s by Currie and his colleagues (56). Unlike necrotic cell death, which involves the dying of cells upon acute cell injury, apoptosis involves the death of a single cell essential for normal physiological development and tissue maintenance (57). Cells undergoing apoptosis are characterized by unique morphological changes that include cell shrinkage, nuclear fragmentation, chromatin condensation, cytoplasmic and nuclear blebbing, and the eventual digestion of the cell by macrophages (57). In the context of cancer biology, apoptosis is widely regarded as the primary barrier to tumourigenesis, as it is the central mechanism for disposing of hyperproliferative or genetically damaged cells in our body (38, 39).

At the molecular level, apoptosis can be carried out through two major signaling pathways, namely (1) the extrinsic pathway and (2) the intrinsic pathway (58). In the extrinsic pathway, apoptosis is initiated by the binding of extracellular cytotoxic agents to specific cell surface receptors known as the death receptors (58). Activation of these
receptors promotes the activation of a unique class of proteases known as caspases (58). These caspases can then promote subsequent cleavage of various cellular contents, such as DNA and proteins, leading to the ultimate destruction of the cell (58). In addition, cells can also undergo apoptosis through the intrinsic pathway, which is triggered by internal cell stress (58). Cellular stress, which includes DNA damage, serum starvation, γ-irradiation and heat shock, is detected by two classes of proteins residing at the mitochondrial membrane (58). The first class is the anti-apoptotic Bcl-2 members, which includes Bcl-2 and Bcl-X\textsubscript{L}, Mcl-1, Bcl-w and Bfl-1, whereas the second class is the pro-apoptotic Bcl-2 members and it is comprised of Bad, Bid, Blk, Bok, Bcl-X\textsubscript{s}, Bik, Bax, Bim and Nik (58). While the anti-apoptotic Bcl-2 members inhibit mitochondrial membrane permeability, pro-apoptotic Bcl-2 members increases this permeability, allowing the release of pro-apoptotic mitochondrial contents to the cytoplasm and promoting the subsequent activation of caspases (58).

Current efforts to understand tumour suppressor functions revealed that they can induce apoptosis in tumours through several mechanisms. Firstly, at the cell surface, tumour suppressors sensitize cells to undergo apoptosis through the extrinsic pathway by modulating the expression of death receptors. For instance, both p53 and FHIT tumour suppressors induce the transcription of various death receptors upon stimulation by cytotoxic agents (59-61). Secondly, upon detection of cell stress, tumour suppressors, such as p53 and maspin, can also function through upregulating the expression of the pro-apoptotic Bcl-2 members, thereby promoting destruction of the cell through the intrinsic apoptotic pathway (62, 63). Thirdly, in addition to the mechanisms mentioned above, some tumour suppressors, such as APC, can also directly stimulate the transcription of
caspases, leading to the execution of independent apoptosis (64). Finally, apart from upregulating an apoptotic signal, various tumour suppressors also demonstrated a role in downregulating or inhibiting the transmission of an anti-apoptotic signal. For instance, while the Runx3 tumour suppressor induces apoptosis by downregulating the expression of the anti-apoptotic Bcl-2 protein (65), the PTEN tumour suppressor triggers apoptosis by downregulating the activity of the survival-driving AKT pathway (66). Together, through these mechanisms, tumour suppressors effectively reduce the capacity of cancer cells to thrive, protecting against the initiation and metastatic progression of tumours (7).

1.2 Hippo-LATS tumour suppressive pathway

1.2.1 Hippo-LATS tumour suppressive pathway in Drosophila

In order to understand the molecular mechanism of tumourigenesis and to develop effective therapeutics against cancer, many scientists strive to identify novel tumour suppressor genes whose loss of function may contribute to cancer. Among the many approaches used by geneticists, genetic screening using the fruitfly, Drosophila melanogaster, is one of the most popular. This is because not only are flies simple and inexpensive to rear in laboratories, but they are also highly conserved when compared to the vertebrate system (67). Among the many important pathways identified using the Drosophila system (67), the Hippo-LATS tumour suppressive pathway, which is found involved in stem cell differentiation, apoptosis, cell proliferation and organ size control, is one that has been extensively studied (68-70).

In Drosophila, the pathway is comprised of seven core players, namely fat, merlin, expanded, hippo, sav, lats and mats, all of which were identified as negative regulators of
tissue growth from genetic screens in *Drosophila* (71-79). Interestingly, a dramatic tissue overgrowth phenotype was produced in the developing wing or eye of adult flies upon inactivation of any of these Hippo-LATS genes (71-78, 80). Remarkably, reintroduction of these genes readily rescue the overgrowth phenotypes. The striking similarity in the seven genes’ loss-of-function phenotypes was one of the very first pieces of evidence suggesting that they may together function in a common signal transduction pathway regulating tissue homeostasis.

In the following years, the functions of these genes and how they together participate in a molecular pathway to suppress cell growth has been deciphered (70) (Figure 1). The pathway begins with *fat, merlin* and *expanded*. While *fat* encodes for a cell surface cadherin receptor, *merlin* and *expanded* encode adaptor proteins (70). Activation of either fat, merlin or expanded has been shown to stimulate the activity of Hippo, a member of the Ste20 family of serine/threonine protein kinases (70, 81, 82). Active Hippo phosphorylates Salvador, which is a scaffold protein encoded by *sav* (77, 83).

Phosphorylated Salvador then binds to *dLATS*, encoded by *lats*, facilitating a close association between Hippo and *dLATS* (77, 83). Once Hippo comes in close proximity with *dLATS*, Hippo phosphorylates *dLATS*, leading to its partial activation (77, 83). Additionally, Hippo also phosphorylates the adaptor protein Mats, which subsequently associates and further coactivates *dLATS* (84, 85). Hence, an association with the upstream players Hippo, Savaldor and Mats is essential for full activation of *dLATS*.

Although both Hippo and *dLATS* are classified as serine/threonine kinases, *dLATS* belongs to a distinct kinase family, known as the nuclear DBF2-related (NDR) family.
Figure 1. Hippo-LATS signaling pathway in *Drosophila* and mammals. Hippo-LATS tumour suppressive pathway is evolutionally conserved from *Drosophila* to mammals. Mammalian orthologs of *Drosophila* Fat, Merlin, Expanded, Hippo, dLATS, Sav, Mats and Yorkie were identified as Fat, Merlin, Expanded, MST2 (Mammalian Sterile-20 like kinase 2), LATS1/2 (Large Tumour Suppressor 1/2), WW45, MOB1 and YAP/TAZ respectively.
(86). Members from this family are functionally diverse and have poorly defined target specificity (86). However, a study using a yeast two-hybrid system recently revealed that a primary downstream target of \(dLATS\) is a transcriptional coactivator named Yorkie (87). Yorkie is an oncogenic transcriptional coactivator responsible for transactivating genes involved in cell proliferation and survival (87). By phosphorylating Yorkie, \(dLATS\) defines a binding site on Yorkie for the 14-3-3 cytoplasmic proteins, which in turn sequesters Yorkie in the cytoplasm and inhibits its function as a transcriptional coactivator (Figure 1) (88). Hence, it is hypothesized that \(dLATS\) plays a critical role in the negative regulation of oncoproteins encoded by gene targets of Yorkie.

1.2.2 Hippo-LATS tumour suppressive pathway in Mammals

The discovery of the Hippo-LATS pathway in \emph{Drosophila} is a major breakthrough in the cancer field, as it not only provides further insight to the mechanism of tissue growth homeostasis, but it also serves as a potential molecular target for cancer drug discovery. Most interestingly, the \emph{Drosophila} Hippo-LATS pathway is recently found evolutionally conserved in mammals (70). Through analysis of sequence homology, the homologs of \emph{Drosophila} Fat, Merlin, Expanded, Hippo, \(dLATS\), Savaualdor, Mats and Yorkie were identified in mammals as FAT, Merlin, Expanded, MST2 (Mammalian Sterile-20 like kinase 2), LATS1/2 (Large Tumour Suppressor 1/2), WW45, MOB1 and YAP/TAZ respectively (68-70) (Figure 1). Further research on their biological functions also revealed that the interactions between MST1/2, LATS1/2, WW45, MOB1, YAP and TAZ closely resembled those between their \emph{Drosophila} counterparts (89-94). Most interestingly, human MST2, MOB1 and LATS1 also readily rescued all developmental
defects in the corresponding *Drosophila* gene mutants, suggesting that there exists a high degree of functional conservation between the human and *Drosophila* system (84, 95, 96).

1.3 Large Tumour Suppressor 1 (LATS1)

1.3.1 Introduction to LATS1

The finding that the *Drosophila* and human Hippo-LATS pathways shared strong functional homology suggests that components of this pathway may help in suppression of human tumours. In particular, among the multiple core components identified, the *Drosophila lats* gene has received the most research attention in the recent years for it is the central player of the Hippo-LATS pathway (68-70, 97).

The *lats* gene, also known as *warts*, was a gene first identified in 1995 for its tumour suppressive ability in flies. Through two independent studies, it was found that loss of *Drosophila lats* resulted in profound developmental defects, including over-proliferation in the head, broadened wing blades and malformed bristles, all of which indicates a defect in cell proliferation and differentiation pathways (75, 76). Using the *Drosophila lats* gene as a probe, several follow-up studies isolated two human homologs of *lats*, namely *LATS1* and *LATS2* (96, 98). Like *Drosophila lats*, human *LATS1* and *LATS2* encode putative serine/threonine kinases. Although they share high (84.6%) sequence identity in their C-terminal kinase domains, they are less conserved in other regions, thus making them distinct tumour suppressors with potentially redundant functions (97).
Our laboratory is particularly interested in the study of LATS1 due to its well-established role in mammalian tumourigenesis. Firstly, *LATS1*-deficient mice spontaneously develop soft-tissue sarcomas and ovarian cancers (99). Secondly, like many tumour suppressor genes, the *LATS1* gene, located at chromosome 6q25.1, is commonly lost or downregulated in cancers (100-104). Furthermore, many human cancers have been identified with deficient LATS1 expression, including soft-tissue sarcomas, ovarian cancers, breast cancers, lymphoblastic leukemia, astrocytoma and lung cancers, suggesting that loss of *LATS1* may be a critical mechanism leading to cancer development in humans (103-108). Finally, although no mutation of the *LATS1* gene has been reported, the promoter of *LATS1* is frequently hypermethylated in various cancers, suggesting that LATS1 may be downregulated in cancers through epigenetic mechanisms (107-110). Collectively, these data indicate a role for LATS1 in the development of human cancers, further highlighting the importance for a comprehensive understanding of LATS1 function at the molecular level.

1.3.2. Structure of LATS1

The LATS1 protein contains 1130 amino acids (aa) and has a molecular weight of 140kDa. Structurally, LATS1 is comprised of four notable conserved regions (97) (Figure 2). The first region, located at aa.110-138, is a UBA domain (97). Although functional significance of this domain in LATS1 has not been clearly defined, it is speculated that the UBA domain is responsible for interaction with ubiquitin since it is also found in many proteins related to the ubiquitination pathway (111). The second region, located at aa. 235-240, is a proline-rich region known as P-stretch (97) and it is
responsible for mediating LATS1 protein-protein interactions. Thirdly, LATS1 also
habours two PPxY (P, Proline; Y, Tyrosine; x, any amino acids) motifs located at aa.
373-376 and aa. 556-559 of the protein (97). These PPxY motifs interact specifically with
Group I WW domains, which are compact 38 residue domains that contain two conserved
tryptophan (W) residues spaced 20-22 amino acids apart (112). Many known binding
partners of LATS1, such as WW45, YAP and TAZ, interact with LATS1 in a WW
domain / PPxY-dependent fashion, suggesting the functional significance of the PPxY
motifs in LATS1. Lastly, at its C terminal (aa. 703-1084), LATS1 has a conserved kinase
domain that is responsible for phosphorylating protein substrates at serine or threonine
residues within the conserved sequence Hx(R/H/K)xx(S/T) (H, Histidine; R, Arginine; K,
Lysine; S, Serine; T, Threonine; x, any amino acids) (113). Both YAP and TAZ, which
are well known downstream kinase substrates of LATS1, contain such conserved
sequences for LATS1-mediated phosphorylation (93, 113).

1.3.3 Function of LATS1

One of the very first pieces of evidence supporting a role for LATS1 in tumour
suppression came from the study by Yang et al. in 2001 (114). In their study, it was
shown that over-expression of LATS1 in six human cancer cell lines completely blocked
cell proliferation (114). Further study of the mechanism leading to LATS1-mediated
growth suppression revealed that LATS1 mediates this function by arresting cells at the
G2/M phase through CDC2 inactivation (114), thereby providing the first compelling
evidence that LATS1 is a key inhibitor of tumour cell growth (114). In addition to the
finding from over-expression studies, a separate report by Visser et al. also recently
Figure 2. Domain structure of LATS1 tumour suppressor. The LATS1 tumour suppressor comprises five notable regions, including an N-terminal UBA domain, a P-P-stretch of PPPPP, two PPxT motifs (P, Proline; Y, Tyrosine; x, any amino acids) at aa.376 and aa.559, and a C-terminal Serine/Threonine kinase domain.
demonstrated that knockdown of endogenous LATS1 and LATS2 enhanced the proliferation of cervical cancer HeLa cells (115), further supporting the physiological significance of LATS1 tumour suppression activity in human cancers.

In addition to its role in growth suppression, LATS1 also possesses a strong pro-apoptotic function in human tumours. As reported by the Yang and Tao groups, over-expression of LATS1 directly induced apoptosis through upregulating two pro-apoptotic proteins, namely p53 and Bax (114, 116). Consistent with this finding, knockdown of LATS1 together with LATS2 in HeLa cells also rendered cell more resistance to Pacitaxel-induced cell death (115), suggesting that LATS1 may also play a role in conferring chemosensitivity.

The ability of LATS1 to inhibit cell proliferation and promote apoptosis suggests a potential role for LATS1 in regulating cell transformation, an event resulting from deregulation of either the cell proliferation or cell death pathways. Through the study by Yang et al., it was demonstrated that over-expression of LATS1 substantially inhibited anchorage-independent growth, an indication of cell transformation, in all the six human cancer cell lines tested, suggesting a direct role for LATS1 in inhibiting cell transformation (114). Moreover, this finding was also later supported by a study by Zhang et al., where they found that knockdown of LATS1 promoted transformation of the non-tumourigenic human immortalized mammary epithelial MCF10A cells using a soft agar assay (117).

Although it is long recognized that LATS1 plays a role in tumour suppression, the molecular mechanism underlying this function has only been recently uncovered upon isolation of YAP and TAZ, two downstream kinase substrates of LATS1 (74, 113). Both
YAP and TAZ are mammalian orthologs of *Drosophila* Yorkie (70). Similar to Yorkie, YAP and TAZ function as transcriptional coactivators and are responsible for inducing the expression of growth-promoting genes (70). Upon activation by MST2, it is found that LATS1 can phosphorylate YAP, and potentially TAZ as well, at their LATS1-specific phosphorylation motifs (HxRxxS) located at Ser61, Ser109, Ser127, Ser164 and Ser397 of YAP and Ser89, Ser66, Ser117 and Ser311 of TAZ (93, 113). Phosphorylation at these sites promotes cytoplasmic retention of YAP and TAZ, thereby inhibiting their function as transcription coactivators (93, 113). Two particularly well-studied transcriptional targets of TAZ and YAP are *Cyr61* and *CTGF* (117, 118). It has been shown that YAP and TAZ modulate cell growth differentiation and apoptosis by transcriptional activation of *CTGF* and/or *Cyr61* (117, 118). Both *Cyr61* and *CTGF* are potent oncogenes and have been implicated in a number of human malignancies (119-121). Therefore, through repressing the function of YAP and TAZ, it is thought that LATS1 plays a critical role in suppressing the expression of their downstream oncogenic targets.

**1.3.4 Regulation of LATS1**

Previous work from our lab and others established that LATS1 is a master regulator of the Hippo-LATS pathway and its activity is controlled mostly in a manner similar to that described in *Drosophila* (97). In the mammalian system, MST1/2, which are orthologs of *Drosophila* Hippo, can activate and stimulate LATS1 kinase activity through phosphorylation of LATS1 (86, 122-124). WW45 is the mammalian ortholog of *Drosophila* Savaldor (70). Like its *Drosophila* counterpart, WW45 can act as a scaffold.
protein that recruits LATS1 and brings it in close proximity with MST1/2 for phosphorylation (124). As described by Chan and her colleagues, MST2 phosphorylates LATS1 specifically at two critical residues, Ser909 and Thr1079 and phosphorylations of these sites are indicative of increased LATS1 kinase activity (124). In addition to MST1/2, LATS1 is also activated by MOB1B, an ortholog of Mats. Expression of MOB1B coactivator protein together with LATS1 can enhance LATS1-mediated inhibition of cell proliferation and induction of apoptosis, suggesting that MOB1B is also a critical activator of LATS1 (94).

Although much is known regarding to how LATS1 kinase activity could be stimulated and measured at the molecular level, no direct negative regulator of LATS1 has yet been identified. With our knowledge of LATS1, we speculate that LATS1 may be negatively regulated through three potential mechanisms, including 1) transcriptional repression of the LATS1 gene by repressor proteins, 2) dephosphorylation and inactivation of LATS1 by phosphatases, or 3) downregulation of LATS1 proteins by ubiquitin-mediated degradation. However, the genes responsible for these mechanisms have not yet been identified. Exploration of how LATS1 may be negatively regulated will reveal novel therapeutic strategies for restoring LATS1 activity in tumours with decreased function of LATS1.

1.4 E3 ubiquitin ligases and their roles in cancer

1.4.1 Concept of E3 ubiquitin ligases

Ubiquitination is a highly specific, energy dependent process that the cell commonly uses to target proteins for degradation. It is a fundamental biological process
for fine-tuning the turnover of cellular proteins, and is generally carried out by a set of three enzymes, namely the E1, E2 and E3 enzymes (125-127). The E1 enzyme, also known as ubiquitin activating enzyme, is responsible for catalyzing the first step of ubiquitination, where a molecule of ubiquitin becomes activated to its ubiquitin-adenylate form via the use of ATP energy (128-130). Upon its activation by E1, the ubiquitin gets transferred to an E2 ubiquitin-conjugating enzyme and from E2, it is delivered to an E3 ubiquitin ligase that specifically interacts with its substrate protein to catalyze the formation of the ubiquitin tag (125, 131) (Figure 3).

Depending on the length of the ubiquitin tag, ubiquitinated proteins may encounter different fates (132). For instance, most polyubiquitinated proteins, or proteins tagged with an ubiquitin chain of four or more residues, are recognized and processed for degradation by the 26S proteasome, a complex with strong proteolytic activity (132, 133). On the other hand, proteins that are monoubiquitinated, or tagged with just one ubiquitin residue, are generally targeted for destruction by the lysosome, an organelle containing both proteases and lipases activity (132, 134).

The molecular mechanisms underlying protein ubiquitination has attracted a lot of research attention since its discovery. In particular, E3 ubiquitin ligases, which serve as specific-substrate recognition elements of the ubiquitin-proteasome system (UPS), have been extensively studied (135, 136). To date, about 1000 E3 ubiquitin ligases have been identified in humans and they can be categorized into two major classes: 1) the RING (Really Interesting New Gene) class and 2) the HECT (Homologous to E6-AP C-terminus) class E3 ubiquitin ligases (136-139). These two classes of E3 ubiquitin ligases differ both in their modular design and their mechanism of action. For instance, while the
Figure 3. Mechanism of ubiquitination. During ubiquitination, ubiquitin (Ub) is activated by E1 using ATP energy and is subsequently transferred to the E2 conjugating enzyme. E2 either transfers activated ubiquitin directly to the substrate protein or to E3 ubiquitin ligase, which then catalyzes ubiquitination of the substrate protein.
RING class E3 ubiquitin ligases are characterized by the presence of a cysteine-rich RING domain, the HECT class E3 ubiquitin ligases are defined by the presence of a C-terminal HECT ligase domain that has intrinsic ligase activity (138, 139). Unlike the RING E3 ubiquitin ligases that catalyze ubiquitination of their substrate proteins by acting as a scaffold protein between E2 and the substrate protein, HECT E3 ubiquitin ligases directly use their HECT ubiquitin ligase domain to catalyze ubiquitination of their substrates (138, 139) (Figure 4). Interestingly, it was found that deregulation of RING and HECT E3 ubiquitin ligases occurs in various human cancers, suggesting their potential value as therapeutic targets for cancer treatment (136, 140, 141).

1.4.2 Role of E3 ubiquitin ligases in cancer

Accumulating evidence in the recent years has suggested that E3 ubiquitin ligases play a critical role in cancer development and progression. Firstly, as it was previously described, protein degradation mediated by E3 ubiquitin ligases is one of the most critical events driving the proliferation of a cell (142). For a cell to divide and proliferate, it requires degradation of specific cyclins and cell cycle regulators at specific times (142). During the mitotic phase, for instance, degradation of Cyclin B is required before a cell can exit from telophase and divide into its daughter cells (143). At the G1/S checkpoint, it is required that Cyclin D is degraded prior to progression to the S phase (144, 145). Without the proper functions of E3 ubiquitin ligases, specific cyclin and cell cycle regulators may aberrantly accumulate in the dividing cells, leading to loss of cell cycle controls, which may ultimately contribute to tumour formation (144, 145). Secondly, various E3 ubiquitin ligases are frequently overexpressed in human cancers. Examples of
Figure 4. Mechanism of RING and HECT E3 ubiquitin ligases. (A) RING E3 ubiquitin ligases have no intrinsic ubiquitin ligase activity. They act as scaffold proteins by bringing substrate proteins into close association with E2 conjugating enzymes. (B) HECT E3 ubiquitin ligases possess intrinsic ligase activities for the ubiquitination of their substrate proteins. HECT E3 ubiquitin ligases act as acceptors of activated ubiquitin from E2 conjugating enzymes and transfer ubiquitin directly to their substrate proteins.
these E3 ubiquitin ligases include, but are not limited to, Mdm2, WWP1, BCA2, Cul4A, RNF11 and XIAP (146). Interestingly, many of these E3 ubiquitin ligases have been shown to promote degradation of tumour suppressors or cell cycle regulators and their amplification frequently promotes cell growth, cell proliferation and cell survival in human cancers (146). Thirdly, although the mechanism remains unclear, it has been shown that the commercially available proteasome inhibitor Bortezmib is effective against various cancers, including myelomas, mantle cell lymphoma and brain cancers (147). This suggests that specific targeting of the protein proteolytic system may be a useful strategy for the treatment of cancers. Finally, the development of small molecule inhibitors for specific E3 ubiquitin ligase such as Mdm2 has also demonstrated promise in the treatment of various cancers (148, 149). Collectively, the evidence suggests that E3 ubiquitin ligases are specific cellular regulators and identification of small molecular inhibitors of E3 ubiquitin ligase may be a novel method for developing effective, non-toxic drugs for cancer therapy.

1.4.3 Nedd4 family of E3 ubiquitin ligases

The Nedd4 family of E3 ubiquitin ligases is a specific class of HECT E3 ubiquitin ligases consisting of nine members (150), which are Itch, Nedd4-1, Nedd4-2, WWP1, WWP2, Smurf 1, Smurf 2, Bul1 and NEDL2. Members of this family are conserved from Drosophila to mammals and they function in a wide spectrum of cellular processes, including signal transduction, transcription, cell cycle regulation, apoptosis and differentiation (150).
In general, members of this family are characterized by a unique modular design that includes an N-terminal C2 domain, multiple WW domains and a C-terminal HECT ubiquitin ligase domain (150). While their C2 domains contain a membrane targeting function, their WW domains are responsible for recognition of ubiquitin substrates (150). Since WW domains predominantly recognize proline rich regions, many Nedd4 E3s specifically recognize substrates harboring a proline rich PY or PPxY motif (112, 150), suggesting that these E3 ubiquitin ligases play a unique role in the negative regulation of substrates containing proline-rich motifs.

Increasing research on this family of E3 ubiquitin ligases has uncovered a potential role for its members to be involved in the development of human cancers (151). For instance, while Nedd4-1 has been shown to ubiquitinate and degrade the PTEN tumour suppressor (152), WWP1 and Itch can ubiquitinate and degrade the p63 tumour suppressor (153, 154), suggesting a role for the Nedd4 family members in the negative regulation of tumour suppressors. In addition, many Nedd4 family members are also found amplified in human cancers and their over-expression in various cell types has been shown to promote cell transformation, cancer cell invasion and metastasis. WWP1, for example, has demonstrated oncogenic potential in both breast and prostate cancers (155, 156) and is frequently upregulated in human carcinoma cell lines (157). On the other hand, expression of Smurf 2 also promotes a more aggressive phenotype and poor prognosis in esophageal squamous cell carcinoma (158). Together, this evidence suggests a potential role for the Nedd4 family of E3 ubiquitin ligases in the regulation of cancer development and progression.
1.5 Itch

Among the Nedd4 family of E3 ubiquitin ligases, Itch, also known as atrophin-interacting protein 4 (AIP4), has gained increasing research interest in the recent years (159).

1.5.1 Origin of Itch

The Itch gene was first discovered in 1995 by Copeland and his colleagues during their study of mouse coat color change. It was found that a specific mutation, known as $a^{18H}$, on mouse chromosome 2 could lead to expression of a darker coat color (160). Mice harboring this mutation displayed a distinct skin scratching phenotype and various autoimmune defect symptoms (160). These mice eventually died at 6-8 months of age due to hypoxia, a condition that is associated with pulmonary inflammation (160). Because of the distinct skin scratching phenotype in these mice, the $a^{18H}$ mice are known as the “Itchy mice” and the gene responsible for it was later named as Itch (160).

1.5.1 Structure and function of Itch

Studies of the human Itch gene revealed that it encodes a Nedd4 family E3 ubiquitin ligase that is 854 amino acids (aa) in length and has a relative molecular weight of 113 kDa (159). Like other Nedd4 family members, Itch is a monomeric protein (159). It has a N-terminal C2 domain for membrane targeting, four WW domains at aa.195-246 for recognition of proline rich (PY, PPXY, PPPPP, PPPPY, PPLP) sequences and a C-terminal HECT ubiquitin ligase domain responsible for its catalytic activity (159) (Figure 5).
**Figure 5. Domain structure of Itch E3 ubiquitin ligase.** The Itch E3 ubiquitin ligase is a monomeric protein consisting of an N-terminal C2 domain for membrane targeting, four Group I WW (W, Tryptophan) domains for protein-protein interaction, and a C-terminal HECT E3 ubiquitin ligase domain for catalyzing ubiquitination of its substrate proteins.
Since its discovery, many substrates of Itch have been identified and the list continues to grow (159). Substrates of Itch generally include transcription factors (e.g. Notch, p63 and p73), transmembrane receptors (e.g. CXCR4 and ErbB4) and protein kinases (e.g. PKC-θ) and they are involved in a number of biological processes, which include signal transduction, transcription, cell differentiation, cell growth, cell death and the immune response (159).

Among the many functions of Itch, its role in T cell differentiation and immune response has been most characterized (161). In particular, it has been found that Itch plays a direct role in the degradation of two critical substrates responsible for the differentiation of helper T cells in our immune system (161). Under normal physiological conditions, production of helper T cells, either Th1 or Th2, requires differentiation from a precursor Thp cell (162). While differentiation to Th1 cells requires exposure of Thp to IL-12 cytokines, differentiation to Th2 cells requires exposure to IL-4 cytokines (162). Itch, in particular, is found to be the primary E3 ubiquitin ligase responsible for degrading the two transcription factors, JunB and c-Jun, that activates the expression of IL-4 (163, 164). By keeping JunB and c-Jun at low levels, Itch therefore prevents the expression of IL-4 and production of Th2 cells under normal conditions (162). In addition to JunB and c-Jun, Itch also mediates degradation of other immune substrates, including the Notch receptors (165), phospholipase C-γ1 (PLC-γ1) (166) and protein kinase C (PKC) (166), all of which are well-established molecular players with roles in cell differentiation and morphogenesis. Together, these findings not only explain the autoimmune defects observed in Itchy mice, but also help to highlight the role of Itch in immune cell regulation.
1.5.3 Itch in cancer

Despite its well characterized role in the immune response and T-cell differentiation, the function of Itch in cell growth and cell death control remains an area of research that awaits further clarification. Increasing evidence has demonstrated that Itch is a potential oncogene that can promote the initiation and progression of cancers. Firstly, the human *Itch* gene, located at chromosome 20q11.22, is found highly amplified in anaplastic thyroid carcinomas (ATC) (167). While the copy number amplification of Itch is associated with the increased growth phenotypes in ATC cells, knockdown of the amplified Itch by small interfering RNA (siRNA) reduced the proliferation of these cells, supporting the potential role of Itch in driving cell cycle progression (167). Second, like other Nedd4 family members, Itch is responsible for ubiquitination and degradation of multiple proline-rich tumour suppressor proteins. p63 and p73, for instance, are well established targets of Itch (154, 168). Both p63 and p73 belong to the same protein family as the p53 tumour suppressor and share functional similarities with p53 in inducing apoptosis upon DNA damage and cell stress (169, 170). This suggests a potential regulatory role for Itch in apoptosis. In addition to p63 and p73, Itch is also responsible for degradation of the Smad2 tumour suppressor (171). Loss or downregulation of Smad 2 is observed in a number of human cancers, including colorectal cancers, breast cancers and prostate cancers (172, 173). Together, this evidence suggests a role for Itch in the negative regulation of proline-rich tumour suppressors. Further studies of how Itch might mediate degradation of its targets may provide the basis for development of novel cancer therapeutics.
1.6 Rationale, Hypothesis and Objectives

Despite our increasing knowledge of the tumour suppressive function of LATS1 in humans, little is known regarding how LATS1 is regulated at the molecular level. Although many upstream activators of LATS1, such as MST1/2 and MOB1, have been reported, direct negative regulators of LATS are yet to be identified.

To investigate how LATS1 is regulated at the molecular level, we used an unbiased proteomic approach to screen for in vivo binding partners of LATS1. Through this screen, we revealed that Itch, a Nedd4 family E3 ubiquitin ligase, is a binding partner of LATS1. Since Itch is an E3 ubiquitin ligase that frequently targets ubiquitination and degradation of its binding partners, we hypothesized that Itch may be a novel negative regulator of LATS1 protein stability and its tumour suppressive function.

To test this hypothesis, I pursued the following experimental objectives. First, I validated Itch as a binding partner of LATS both in vitro and in vivo. Second, I determined the specific functional domains mediating LATS1/Itch interaction. Third, I explored the effect of Itch on LATS1 protein stability and determined that LATS1 is a specific ubiquitin substrate of Itch. Fourth, I determined the nature and fate of Itch mediated ubiquitination of LATS1. Finally, to determine the functional significance of Itch/LATS1 interaction, I also studied the effect of Itch on LATS1 tumour suppressor activity and its downstream signaling.
CHAPTER 2
MATERIALS AND METHODS

2.1 Plasmid construction

The LATS1-PPxY-WT (aa. 526-655) and LATS1-PPxY-Mut (aa. 526-655) plasmids were constructed as described (113). For construction of Itch-WPI, Itch C830A-WPI and Itch-WW-Mut-WPI lentiviral plasmids, Itch cDNA was first PCR amplified using Itch-specific primers (see Appendix 1) from Itch-myc, Itch-C830A-myc and Itch-WW-Mut-myc plasmids (all were provided by Dr. Antony Pawson), digested by PmeI, and subsequently cloned into the PmeI site of the WPI lentiviral vector. For construction of Itch-WW-pGEx4T1 plasmid, aa. 288-476 region of Itch cDNA was first PCR amplified using Itch-WW primers (see Appendix 1) from the Itch-myc plasmid, digested by BamH I/NotI, and subsequently cloned into the BamH I/NotI site of the pGEx4T1 vector. For construction of LATS1-Y376A-FLAG, LATS1-Y559A-FLAG and LATS1-Y376A+Y559A-FLAG, overlapping PCR was performed using LATS1-mutagenic primers (see Appendix 1).

2.2 Cell culture and transfection

HeLa (human cervical cancer cell line), MDA-MB-231 (human breast cancer cell line), MCF-7 (human breast cancer cell line), HEK293T (human embryonic kidney cell line) and COS-7 (monkey kidney cell line) cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Invitrogen), whereas MCF10A (human immortalized mammary epithelial cell line) cells were maintained in Dulbecco's Modified Eagle's Medium
Nutrient Mixture F12 Ham (DMEM-F12) supplemented with 5% horse serum, 20 ng/ml hEGF, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 100 ng/ml cholera toxin, 2.5mM L-glutamine, and 1% penicillin-streptomycin. All cells were cultured in 5% CO₂ at 37°C.

For transfection, cells were counted with a hemocytometer and seeded in tissue culture dishes with growth medium without any antibiotics one day prior to transfection. On the day of transfection, cells were 60-80% confluent. Plasmid DNA was mixed with Opti-MEM Reduced Serum Medium and Lipofectamine 2000 (Invitrogen) transfection reagent at a 1:3 ratio at room temperature (RT) for 20-30 min. The complex mixture was then added dropwise to the cells and the plate was swirled gently to ensure even dispersion. Cells were then incubated in a 37°C, 5% CO₂ incubator. At 24 hours post-transfection, the medium and complexes were removed and replaced with normal growth medium with antibiotics. At 48 h post-transfection, cells were harvested and extracted as described in Section 2.6.

2.3 SILAC (Stable isotope labeling with amino acids in cell culture) proteomics screen

HEK293T cells were cultured as followed. Arginine- and Lysine-deficient medium (Thermo scientific) was used for SILAC experiments, where one cell population was supplemented with light lysine and arginine (L-Lysine-2HCl and L-Arginine-HCl) and another was supplemented with heavy lysine and arginine (13C₆-Lysine-2HCl and 13C₆-Arginine-HCl). Cells were grown in the “heavy” or “light” media for at least six cell divisions prior to cell lysis in NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1.0% Nonidet P-40). For GST-pulldown assay, 200 µg of
LATS1-PPxY-WT-GST or LATS1-PPxY-Mut-GST fusion protein cross-linked to glutathione-Sepharose 4B beads by DMP was mixed with 20mg of heavy or light cell lysates, followed by overnight incubation on a rotator at 4˚C. The beads were then washed four times with NP40 lysis buffer. Bound proteins were eluted with 100 μL of 0.1M glycine-HCl (pH 2.5) two times. Elutes from GST-pulldown of both PPxY-WT-GST and PPxY-Mut-GST were pooled and mixed, dialyzed against 10 mM NH4HCO3 overnight, freeze dried in a vacuum concentrator, and in-solution digested by trypsin. Tryptic peptides were analyzed by nano LC-MS/MS.

2.4 Lentivirus production, purification and titering

To produce lentivirus expressing a gene of interest, a 150mm dish of 60-80% confluent HEK293T cells were transfected with 7.5μg of lentiviral transfer plasmid that encodes the target gene, 5.6μg of PAX packaging plasmid, and 1.9μg of MD2G envelope plasmid. At 24 h post-transfection, the growth medium and complexes were removed, washed twice with 1x PBS, and replaced with Opti-MEM Reduced Serum Medium containing 10mM sodium butyrate. 24 hours after sodium-butyrate treatment, conditioned medium containing lentivirus were collected, filtered through a 0.45μm filter, and concentrated to a final volume of around 250μl using a Centricon-20 ultra filtration column (Millipore Corp).

For virus titration, 4×10^5 HeLa cells were seeded into each well of a 24-well plate. One day after plating, cell numbers per well were counted and cells were infected overnight with serial dilutions of the lentiviral stock and cultured in Opti-MEM Reduced Serum Medium supplemented with 8 μg/ml polybrene. At 24 h post-infection, medium
was replaced with normal growth medium and incubated for an additional 24 h under a 37°C, 5%CO₂ atmosphere. For titering, cells were harvested and the proportion of GFP-positive (virus containing) cells for a given virus dilution was counted. The final virus titer was calculated according the following formula: Transduction units/ml (TU/ml) = (cell number at the time of infection) × (percentage of GFP-positive cells) × (dilution factor)

2.5 Establishment of Itch over-expression and Itch knockdown stable lines

To generate Itch over-expressing stable lines, HeLa cells with low endogenous Itch levels were infected with either WPI, Itch, Itch-C830A or Itch-WW-Mut lentivirus at Multiplicity of infection (MOI) of 2. At two days post-infection, cells were analyzed under the Nikon Eclipse TE-2000U Inverted Fluorescent Microscope to ensure 100% infection (GFP-positive). Over-expression of Itch, Itch-C830A or Itch-WW-Mut was observed by comparison to the WPI control using western blotting with the mouse anti-Itch monoclonal antibody (BD Transduction Laboratories). To generate Itch-knockdown stable cell lines, MDA-MB-231 cells with high endogenous Itch levels were infected with either pGIPZ control vector or shItch lentivirus. At two days post-infection, cells were selected in DMEM growth medium containing 1 µg/ml puromycin for over 10 days. Puromycin-selected cells were expanded and collected for western blot analysis. Knockdown of Itch was confirmed by comparison of Itch levels in MDA-MB-231-pGIPZ and MDA-MB-231-shItch cells using the anti-Itch (BD Transduction Laboratories) antibody. After screening with five different shItch lentivirus, shItch-1 and shItch-2, which target different regions of Itch mRNA (shItch-1, GGCAAGAACTTATTTGCAA;
shItch-2, CCGACAAATACAAATACAA), demonstrated the most knockdown in MDA-MB-231 cells and they were selected for use in further studies.

2.6 Protein extraction, quantitation and western blot

For protein extraction, adherent cells in tissue culture dishes were placed on ice, washed twice with ice cold 1× PBS, and resuspended in a NP40 lysis buffer (1% NP40, 150mM NaCl and 1× Complete EDTA-free protease inhibitor (Roche)) unless otherwise stated. After 30 min of incubation on ice, cell suspension was centrifuged at 12,000g for 10 min at 4°C. The supernatant was transferred to a fresh tube on ice and stored at -80°C until needed. To prepare for western blot analysis, protein samples were quantitated using a DC protein assay kit (Bio-Rad), prepared as equal aliquots (10µg) and boiled in 1× protein loading buffer with 4.2% β-mercaptoethanol for 5 min. The boiled samples were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane and blotted in either 5% milk in Tris-Buffered Saline Tween-20 (TBST) or 5% BSA in TBST. After blotting, the membrane was probed with primary antibody and the respective horseradish peroxidase (HRP) conjugated secondary antibody. The proteins were then detected using an enhanced chemiluminescence (ECL) reagent and developed using x-ray film.

2.7 Co-immunoprecipitation (Co-IP)

For interaction of LATS1-FLAG with Itch-myc or Itch-C830A-myc, COS-7 cell lysates expressing the respective proteins were harvested and 1mg of each protein lysate
sample was precleared, immunoprecipitated with 2ug of anti-Myc 9E10 (Roche) antibody and detected with western blot analysis using anti-FLAG M2 (Sigma) antibody.

For interaction of endogenous LATS1 with endogenous Itch, 1mg of MDA-MB-231 cell lysates expressing high level of endogenous LATS1 and Itch were precleared, immunoprecipitated with either the control anti-FLAG M2 (Sigma) antibody or the anti-LATS1 Y03 antibody and detected with western blot analysis using the anti-Itch (BD Transduction Laboratories) antibody. All blots for immunoprecipitation were stripped and re-blotted using the immunoprecipitation antibody to ensure successful immunprecipitation of target proteins.

2.8 Glutathione-S-transferase (GST) fusion protein production

For GST-fusion protein production, transformed E.coli strain BL21 were grown in culture at 37°C with shaking at 300rpm. Upon reaching an OD$_{600}$ of 0.6, the GST fusion protein was induced with addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) at a final concentration of 0.4mM, followed by incubation overnight at RT with shaking at 125rpm. On the day of harvest, overnight bacterial culture expressing the GST-fusion proteins of interest was collected by centrifugation and the resulting bacterial pellet was resuspended in 1× PBS and subjected to 3-4 cycles of protein sonication using a Sonic Dismembrator Model 500 (Fisher). The sonicator setting at each cycle is as followed: 15sec/cycle duration, 0.5sec on time, 0.5 sec off time and 60% amplitude. The resulting mixture is then centrifuged at 6000g for 15min at 4°C to remove cell debris. The soluble fraction containing the GST-fusion proteins of interest was then purified using GSB (Glutathione Sepharose 4B) beads and eluted. Concentrations of GST-fusion proteins in
the eluate were quantitated by comparison to a range of BSA standards (0.1-4 µg) on SDS-PAGE gel.

2.9 GST pull-down assay

For GST pull-down assays, about 100µg of protein lysate expressing LATS1-FLAG, LATS1-Y376F-FLAG, LATS1-Y559F-FLAG or LATS1-Y376F/Y559F-FLAG was mixed with GSB beads and 10µg of GST (control), Itch, Itch-WW Mut, or WW domain GST fusion proteins at 4 °C with rocking for 2 hrs. To remove non-specific binding proteins, the GSB beads were washed four times with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1.0% Nonidet P-40). The beads were then resuspended in 2×SDS sample buffer, boiled, and centrifuged. The resulting supernatants was subjected to western blot analysis using anti-FLAG M2 (Sigma) antibody.

2.10 Indirect immunofluorescent assay

For analysis of LATS1 and Itch subcellular localization, COS-7 cells were grown on polylysine-coated coverslips in a 24-well plate and co-transfected with LATS1-FLAG and Itch-Myc. 16 h post-transfection, cells were fixed in 4% formaldehyde for 15 min and permeabilized in 0.2% Triton X-100 in PBS for 2 min. The coverslips were incubated in blocking buffer (10% BSA and 5% goat serum in 1×PBS) for 1 h at RT, followed by incubation with mouse monoclonal anti-FLAG M2 (Sigma) and rabbit polyclonal anti-Myc A14 (Santa Cruz) primary antibodies at 1:1000 at RT for 1 h. Alex Fluor (AF) 488 (Green) anti-rabbit IgG and AF 555 (red) anti-mouse IgG secondary antibodies were applied at a dilution of 1:100. DAPI (3 µg/ml) was used for nuclear staining.
For analysis of YAP subcellular localization, HeLa cells expressing WPI, Itch, Itch-C830A, and Itch-WW-Mut were processed as described above except that rabbit polyclonal anti-YAP antibody (Santa Cruz) (1:200 dilution) and AF555 anti-rabbit IgG (1:100 dilution) were used as primary and secondary antibodies, respectively. Images were obtained with a Nikon Eclipse TE-2000U Inverted Fluorescent Microscope at 600× magnification.

2.11 Cycloheximide time-course analysis

To measure the effect of Itch over-expression on LATS1 turnover, COS-7 cells cultured in 100mm tissue culture plates were transiently transfected with either LATS1-FLAG alone or LATS1-FLAG together with Itch-Myc. At 5 h post-transfection, cells were passed into five 60mm plates and maintained in DMEM / 10%FBS growth medium. At 16 h post-transfection, culture medium was replaced by DMEM / 10%FBS supplemented cycloheximide (20 µg/ml) and cells were lysed at 0, 4, 8, 12 and 24 hours following cycloheximide treatment. The resulting lysates were then analyzed by western blotting using anti-FLAG M2 (Sigma) and anti-Myc 9E10 (Roche) antibodies.

To analyze the effects of Itch knockdown on LATS1 turnover, MDA-MB-231 cells expressing lentiviral vector (pGIPZ) or shItch-1 were seeded into five 60mm tissue culture plates one-day post-treatment. On the day of cycloheximide treatment, cells were at 30% confluence and culture medium was changed to DMEM / 10% FBS supplemented with cycloheximide (20 µg/ml). Cells were lysed at 0, 4, 8, and 12 h following cycloheximide treatment and the resulting lysates were analyzed by western blotting using anti-LATS1 Y03 and anti-Itch (BD Transduction Laboratories) antibodies.
2.12 Proteasome inhibitor or lysosome inhibitor treatment

To test the effect of proteasome inhibitor (MG132) or lysosome inhibitor (Baf A1) on Itch mediated LAT51 degradation, COS-7 cells cultured in 100mm tissue culture plates were transfected with either LAT51-FLAG alone or LAT51-FLAG together with Itch-myc. At 24 h post-transfection, cells were passed to 60mm plates with 30% confluency in either normal growth medium, medium with 20µM proteasome inhibitor MG132, or medium with 1.0uM lysosome inhibitor Baf A1. At 24 h post-treatment, cells were extracted and analyzed by western blotting using anti-FLAG M2 (Sigma) and anti-Myc 9E10 (Roche) antibodies.

2.13 In vitro and in vivo ubiquitination assay

For in vitro ubiquitination assay, 1mg of cell lysate expressing LAT51-myc was immunoprecipitated with protein G beads conjugated to anti-Myc 9E10 antibody. After four washes with modified RIPA buffer (2mM Tris-HCl, pH7.5; 5 mM EDTA, 150 mM NaCl, 1%NP40, 1% Sodium Deoxycholate, 0.025% SDS), the beads containing the immunoprecipitates were incubated in 30µl of ubiquitination conjugation reaction buffer [2mM ATP, 0.5 µg Ubiquitin-FLAG, 0.1 µM ubiquitin activating enzyme (E1), and 0.5 µM ubiquitin conjugating enzyme E2 UbcH7 (Boston Biochem) either in the absence or presence of Itch-GST (E3) or Itch-C830A-GST at 30°C for 90 min. As a negative control, Itch-GST alone was also incubated with ubiquitin conjugation reaction buffer. The beads containing ubiquitinlated LAT51 were spun down, washed 4 times with modified RIPA (to eliminate all other proteins in the reaction mix), and resuspended in 2×SDS sample
buffer (200mM Tris-HCl, pH 6.8, 200 mM DTT, 20% glycerol, 4% SDS, and 0.02% bromophenol blue), and boiled at 100°C for 5 min, followed by western blot analysis using anti-FLAG M2 (Sigma) antibody.

For in vivo ubiquitination assay, eight 60mm plates of 60-80% confluent HEK293T cells were transiently transfected with the indicated plasmids. At 24 h post-transfection, cells were passed to 100mm plates in DMEM / 10% FBS growth medium supplemented with 5µM MG132. At 24 h post-treatment, cells were lysed with modified RIPA buffer and 1mg of each lysate sample were precleared with Protein G beads, immunoprecipitated by anti-FLAG M2 (Sigma) antibody, followed by western blot analysis with anti-HA Y11 (Santa Cruz) antibody.

2.14 Cell proliferation assay

To examine the effect of Itch over-expression on cell proliferation, triplicates of 4×10^4 HeLa cells stably expressing either WPI, Itch, Itch-C830A or Itch-WW-Mut were seeded into each well of a 24-well plate and cells were counted everyday for 3 consecutive days following plating. The effect of Itch, Itch-C830A or Itch-WW-Mut on cell viability is determined by comparison to the WPI vector control. For statistical analysis, the mean and SD of three triplicate samples were shown. Significant difference between each group was calculated by performing a Student’s t test.

2.15 Colony formation assay

To examine the effect of Itch over-expression or Itch knockdown on colony forming ability, two colony formation assays were performed. In the first assay,
triplicates of $1 \times 10^3$ HeLa cells stably over-expressing WPI, Itch, Itch-C830A, or Itch-WW-Mut were seeded to 100mm plates and cultured for 10 days. The formed colonies were stained with 0.005% crystal violet in 20% methanol for 1 hr at RT. The colonies numbers in each plate were then counted using a Bio-Rad Gel Doc System. Standard deviations were representative of data from three repeated experiments. In the second assay, MDA-MB-231 cells were transiently infected with lentivirus expressing pGIPZ (vector), shItch-1, shItch-1/shLATS1, shItch-2, or shItch-2/shLATS1 at MOI of 2. One day after infection, triplicates of $1 \times 10^3$ cells of each cell line were seeded into 100 mm plates and cultured for 10 days. Staining and counting of formed colonies were performed as described above. For statistical analysis, the experiments were repeated three times. The mean and SD of three independent experiments are shown. Significant difference between each group was calculated by performing a Student’s t test.

2.16 Trypan blue exclusion assay

To study the effect of Itch over-expression on LATS1 pro-apoptotic function, a trypan blue exclusion assay was performed. In this assay, triplicates of $4 \times 10^4$ MCF7 breast cancer cells were seeded into each well of a 24 well plate 1 day prior to lentiviral infection. On the day of infection, cells were counted and subsequently infected with WPI, LATS1, LATS1 together with increasing MOI (4 or 6) of Itch lentivirus. At four days post-infection, both detached and adherent cells were collected, centrifuged and resuspended in 100ul growth medium. To determine the % cell death in each sample, 50μl of cell resuspension were mixed with 50μl of 0.4% trypan blue dye (Sigma) that
selectively stains dead cells. The numbers of blue (dead) and white (live) cells were counted and % cell death was determined.

To study the effect of Itch-knockdown on LATS1 apoptotic function, triplicates of 4x10^4 MDA-MB-231 cells stably expressing either pGIPZ, shItch-1 or shItch-2 were seeded to each well of a 24 well plate 1 day prior to lentiviral infection. On the day of infection, cells were counted and subsequently infected with WPI or LATS1 were infected at an MOI of 2. At four days post-infection, detached and adherent cells were collected, and percentage of cell death was measured as described above. For statistical analysis, the mean and SD of the triplicate samples were shown. Significant difference between each group was calculated by performing a Student’s t test.
CHAPTER 3

3.1 Identification of Itch as a binding partner of LATS1 by SILAC

LATS1 is a tumour suppressor with critical roles in the development and progression of human cancers (97). Despite its importance, little is known regarding to how LATS1 is regulated at the molecular level. To identify direct cellular regulators of LATS1, we first sought to identify novel interacting proteins of LATS1 using a proteomic approach.

Through previous work in our lab, it is known that LATS1 interacts with its binding partners mainly through its two PPxY motifs (PPxY\textsuperscript{376} and PPxY\textsuperscript{559}) (113). Both PPxY motifs are known for their abilities to recognize and interact with WW domains-containing proteins (113). It is known that a single point mutation of the conserved tyrosine (Y) residue on the PPxY motif can completely disrupt its affinity towards WW domains (113). To screen for novel binding partners of LATS1 in vivo, we therefore generated two LATS1 deletion (amino acids 526-655) GST fusion proteins containing either wild-type (PPxY\textsuperscript{WT}) or mutant (PPxF\textsuperscript{Mut}, negative control) motif and used them as bait in a recently developed SILAC proteomic screen. Endogenous proteins that are capable of binding to wild-type (PPxY-WT), but not mutant (PPxY-Mut) GST fusion proteins of LATS1 in the screen may be novel binding partners of LATS1.

In this SILAC analysis, proteins in HEK293T human embryonic kidney cells were first metabolically labeled, respectively, by either heavy (H) [\textsuperscript{13}C\textsubscript{6}-Arg and \textsuperscript{13}C\textsubscript{6}-Lys] or light (L)[\textsuperscript{12}C-Arg and \textsuperscript{12}C-Lys] amino acids. The resulting “heavy” and “light” SILAC lysates were then incubated, respectively, with PPxY-WT-GST and PPxY-Mut-GST fusion proteins, followed by GST-pulldown and LC MS/MS
mass-spectrometry analysis. Our LC MS/MS analysis identified 118 proteins, of which only two proteins, YAP (gi5174751) and Itch (gi27477109), are specific LATS1-binding proteins with H/L ratios of >1.5. Interestingly, peptides from both Itch and YAP are labeled only by the heavy isotope (Figure 6A and 6B), suggesting that they specifically bind to wild-type rather than mutant PPxY-GST fusion proteins. On the other hand, peptides from cytoplasmic Actin (gi4501885), a non-specific protein, was labeled equally by both heavy and light isotopes and has a H/L ratio of 1.05 (Figure 6C).

YAP is a WW domain-containing protein and a known binding partner and kinase substrate of LATS1 (113). Itch, on the other hand, is a WW-domain-containing protein and an E3 ubiquitin ligase known to promote protein ubiquitination and degradation (159). It has been previously suggested that LATS1 may be regulated through protein ubiquitination by E3 ubiquitin ligases (115), but it is unclear which E3 ubiquitin ligase specifically targets its ubiquitination and degradation. This screening result suggests the possibility that LATS1 may be a specific ubiquitin substrate of the Itch E3 ubiquitin ligase. To further confirm endogenous Itch is pulled down by LATS1-PPxY-WT rather than PPxY-Mut GST fusion proteins of LATS1, we performed a GST pulldown assay using the same conditions as those used for SILAC screening. Interestingly, our result showed that endogenous Itch from HEK293T cell lysate specifically binds to PPxY-WT-GST, rather than PPxY-Mut-GST (Figure 6D), suggesting that Itch may be a strong binding partner of LATS1.
Figure 6. Identification of LATS1-interacting proteins by SILAC. (A–C)
Quantification of heavy and light peptides. A section of the mass spectrum of peptides from (A) Itch (NYEQEQLQR), (B) YAP (TANVPQTVPMR), and (C) Actin (HQGVMVGGMQK) are shown. The pair of light (L) and heavy (H) labeled peptides were identified by MS/MS measurements, and their H/L ratios were determined by the intensities of monoisotopic ions. The heavy $^{13}$C isotope labeled peptides are indicated with solid dots, whereas the corresponding light peptides, which are 6 kDa smaller, are labeled with unfilled dots. Note that because of the inability of binding to PPxY-Mut-GST, the light peptides for both Itch and YAP were missing, whereas both heavy and light peptides were detected for nonspecific binding to the Actin protein with an H/L ratio of 1.05. (D) Confirmation of PPxY motif–Itch interaction by GST pull-down assay. Lysate from HEK293T cell was pulled down by either PPXY-WT or PPXY-Mut-GST fusion proteins, followed by western blotting using anti-Itch antibody. Ponceau S was used to stain fusion proteins on the membrane. Note that equal amounts of PPxY-WT and PPxY-Mut GST fusion proteins were used for the GST pull-down assays. The SILAC experiment is performed by Dr. Zhonghua Zhou and the mass spectrometry analysis is performed by Dr. Yin-Min She and Dr. Terry D. Cyr from Health Canada.
3.2 Validation of Itch as a novel binding partner of LATS1

3.2.1 Interaction of LATS1 and Itch in vivo

Our finding that the Itch E3 ubiquitin ligase can bind to LATS1 suggests the possibility that Itch may be a novel regulator of LATS1 and may be responsible for promoting its ubiquitination and protein turnover. To further verify Itch as a bona fide binding partner of LATS1 in vivo, we performed two separate co-immunoprecipitation experiments. As expected, in our first experiment, LATS1 co-immunoprecipitates with Itch in cell lysates overexpressing both LATS1 and Itch (Figure 7), demonstrating a strong binding affinity between LATS1 and Itch. To further test if the interaction between LATS1 and Itch is physiologically significant, we also performed a separate co-immunoprecipitation experiment, in which human breast cancer cells MDA-MB-231 were used to analyze the binding between endogenous LATS1 and endogenous Itch analyzed. We found that endogenous LATS1 robustly co-immunoprecipitates with endogenous Itch (Figure 8), suggesting that the LATS-Itch interaction is physiologically relevant.

3.2.2 Co-localization of LATS1 and Itch in vivo

Since LATS1 and Itch demonstrated strong physical interaction in vivo, we next investigated if LATS1 and Itch may colocalize with each other by indirect immunostaining. As expected, upon staining of COS7 cells transfected with both LATS1 (green) and Itch (red), a strong overlapping signal (yellow) was observed in the cytoplasm (Figure 9). This suggests that LATS1 co-localizes with Itch in the same subcellular compartment and that their interaction is physiologically significant.
Figure 7. Interaction of ectopically expressed LATS1 and Itch in vivo. COS7 lysates expressing either LATS1-FLAG or LATS1-FLAG together with Itch-Myc or Itch-C830A-Myc were immunoprecipitated with anti-Myc antibody, followed by western blotting with anti-FLAG antibody. Ponceau S staining of antibody heavy chain indicates that equal amounts of anti-Myc antibody were used.
Figure 8. Interaction of endogenous LATS1 and Itch in vivo. Protein lysates from MDA-MB-231 cells were immunoprecipitated with either control anti-FLAG antibody or anti-LATS1 antibody, followed by western blotting with anti-Itch antibody. Ponceau S staining of antibody heavy chain indicates that equal amounts of control anti-FLAG and anti-LATS1 antibodies were used.
Figure 9. Subcellular colocalization of LATS1 and Itch. LATS1-FLAG and Itch-Myc were cotransfected into COS7 cells, followed by immunostaining with anti-FLAG and anti-Myc primary antibodies and their corresponding AF488 anti-mouse IgG and AF555 anti-rabbit IgG secondary antibodies.
3.2.3 Interaction of LATS1 and Itch in vitro

A) Functional domains of Itch interacting with LATS1

After our validation that Itch is an intrinsic binding partner of LATS1, we sought to determine the functional domains on Itch that bind to LATS1. As mentioned previously, LATS1 mediates its protein–protein interaction mainly through its PPxY motifs (PPxY^{376} and PPxY^{559}), which have high affinities toward Group I WW domains (113). Given that Itch contains four Group I WW domains (112, 159), we therefore tested if its WW domains are responsible for LATS1–Itch interaction in a GST pull-down assay.

For this assay, a series of Itch GST-fusion proteins were produced, including (1) GST control, (2) Itch-GST, (3) Itch-WW-Mut-GST, which has all four of Itch’s WW domains disrupted and (4) WW-GST, which contains WW domains of Itch (Figure 10). As shown in Figure 11, wild-type LATS1 binds strongly to the Itch-GST fusion protein, but not to the GST control, suggesting that LATS1 and Itch form a stable complex in vitro. No interaction was, however, detected between LATS1 and Itch-WW-Mut-GST, implying that the LATS1-Itch interaction requires intact WW domains of Itch. Consistent with this notion, the four WW domains of Itch (WW-GST) can bind to LATS1 with as strong affinity as the full-length Itch (Figure 11), suggesting that WW domains are the primary interaction domains on Itch for binding to LATS1.

B) Functional motifs of LATS1 interacting with Itch

Finally, to determine if and which PPxY motifs on LATS1 may be responsible for mediating its binding to Itch, additional GST pull-down assays were performed using
Figure 10 Domain structures of GST fusion proteins. Protein constructs of GST, Itch-GST, Itch-WW-Mut-GST and WW-GST are shown. WW, WW domain; W, Trytophan; A, Alanine.
Figure 11. Interaction of LATS1 and Itch in vitro. COS7 lysates expressing either wild-type (LATS1-WT-FLAG), single-PPxY mutants (LATS1-Y376A-FLAG or LATS1-Y559A-FLAG), or the double PPxY mutant (LATS1-Y376A-Y559A-FLAG) of LATS1 was pulled down with either GST, Itch-GST, Itch-WW-Mut-GST, or WW-GST, followed by western blotting for LATS1-FLAG using anti-FLAG antibody. 1/10 input (10μg) represents 1/10 of protein lysate (100 μg) used for GST pull-down. Ponceau S staining indicates that equal amounts of GST fusion proteins were used.
lysates that expresses different PPxY motif mutants of LATS1, namely (1) LATS1-Y376A, which has a the first PPxY motif disrupted, (2) LATS1-Y559A, which has the second PPxY motif mutated, or (3) LATS1-Y376A+Y559A, which has both of its PPxY motifs disrupted. Our result showed that although mutation of a single PPxY motif in LATS1 (LATS1-Y376A or LATS1-Y559A) is sufficient to reduce its binding to Itch-GST, mutation of both PPxY motifs (LATS1-Y376A-Y559A) is necessary to complete abolish its binding to Itch-GST (Figure 11), demonstrating that both PPxY motifs of LATS1 mediate the interaction of full-length LATS1 with Itch WW domains.

3.3 Degradation of LATS1 by Itch

3.3.1 Dose-dependent degradation of LATS1 by Itch

Itch is an E3 ubiquitin ligase known to target tumour-suppressor substrates for ubiquitination and degradation (144, 154, 159). Since LATS1 is a tumour suppressor and a binding partner of Itch, we asked if LATS1 might also be an ubiquitin substrate of Itch. To answer this question, we first tested if over-expression of increasing amounts of Itch can affect LATS1 protein abundance. Our results show that over-expression of Itch induces down-regulation of LATS1 in a dose-dependent manner (Figure 12A), suggesting LATS1 may be a substrate of Itch. On the other hand, cotransfection of LATS1 with increasing amounts of a catalytically inactive mutant of Itch (Itch-C830A-Myc; C, cysteine; A, alanine) abolished Itch’s ability to downregulate LATS1 proteins (Figure 12B), suggesting that the ubiquitin ligase activity of Itch is essential for degradation of LATS1.
Figure 12. Dose dependent down-regulation of LATS1 by Itch. (A) Dose-dependent degradation of LATS1 by Itch. Western blot analysis of LATS1 upon expression of increasing amount of Itch in COS7 cells. (B) Loss of Itch ligase activity abolishes its effect on LATS1. Western blot analysis of LATS1-FLAG upon expression of increasing amount of a catalytically inactive Itch (Itch-C830A-Myc) in COS7 cells. (C) Disruption of Itch WW domains abolishes Itch-induced degradation of LATS1. Western blot analysis of LATS1 upon expression of increasing amounts of Itch-WW-Mut in COS7 cells. (D) Disruption of LATS1 PPxY motifs abolishes LATS1 degradation by Itch. Western blot analysis of LATS1-Y376A-Y559A upon expression of increasing amounts of Itch in COS7 cells. (E) Inability of Itch to degrade NDR1, a homolog of LATS1. Western blot analysis of NDR1-FLAG upon expression of increasing amounts of Itch in COS7 cells. (F) Inability of TrCP, an E3 ubiquitin ligase lacking WW domains, to degrade LATS1. Western blot analysis of LATS1-FLAG upon expression of increasing amounts of TrCP in COS7 cells.
To exclude the possibility that reduced degradation of LATS1 by Itch-C830A ligase-dead mutant is because of reduced binding affinity to LATS1, we performed a Co-IP experiment with LATS1 and Itch-C830A. As shown in Figure 7, similar to Itch-LATS1 interaction, LATS1 is strongly coimmunoprecipitated by Itch-C830A, suggesting that the single-point mutation of Itch did not disrupt normal LATS1–Itch interaction, but affected Itch’s function.

As mentioned previously, we found that LATS1-Itch interaction requires the WW domains of Itch and PPxY motifs of LATS1. To see if disruption of LATS1-Itch binding is sufficient to abolish Itch-mediated downregulation of LATS1, we contransfected LATS1 together with increasing amounts of an Itch-WW mutant, which has all four of its WW domains mutated. Interestingly, mutation of WW domains on Itch prevented dose-dependent degradation of LATS1, suggesting that physical interaction between Itch and LATS1 is essential for downregulation of LATS1 (Figure 12C). Next, we tested if disruption of PPxY motifs of LATS1 affects Itch’s ability to downregulate LATS1. As expected, mutation of PPxY motifs on LATS1 prevented its degradation by Itch (Figure 12D), further supporting the notion that a physical interaction between Itch and LATS1 is required for LATS1 downregulation by Itch.

To confirm that Itch-mediated degradation of LATS1 is specific, we conducted two further experiments. First, we tested whether or not Itch can degrade Ndr1, a LATS1 homolog that lacks a PPxY motif (86). As expected, Itch was unable to degrade Ndr1 (Figure 12E). Next, we tested whether another E3 ubiquitin ligase β-TrCP, which lacks WW domains and targets oncogene products rather than tumour suppressors for degradation (174), can also degrade LATS1. As expected, β-TrCP ubiquitin ligase was
unable to affect the stability of LATS1 (Figure 12F). Together, these experiments strongly suggest that the destabilizing effect of Itch on LATS1 is specific.

### 3.3.2 Itch negatively controls protein stability of endogenous LATS1

Moreover, to further confirm our results from the transient transfection experiments, we used a lentiviral system to stably overexpress Itch in three different human immortalized/cancer cell lines (MCF10A, HeLa, and MCF7) and subsequently analyzed the effect on endogenous LATS1 protein stability. Interestingly, in all three cell lines tested, a marked decrease in endogenous LATS1 was detected upon over-expression of Itch but not the lentiviral vector WPI (Figure 13). This finding suggests that the effect of Itch on LATS1 is physiologically significant.

Finally, to examine whether or not endogenous Itch can directly modulate the stability of endogenous LATS1, we stably knocked down Itch with two shItch constructs in MDA-MB-231 cells, which express high levels of endogenous Itch. As expected, endogenous LATS1 is up-regulated upon knockdown of Itch with either one of the two shItch constructs (Figure 14), indicating Itch is one of the major ubiquitin ligases negatively regulating LATS1 stability in physiological conditions.

### 3.3.3 Itch promotes degradation of LATS1 at the protein level

Additionally, to determine if Itch directly promotes down-regulation of LATS1 at the protein rather than mRNA levels, we performed a cycloheximide time-course experiment in which protein synthesis is blocked and the steady-state levels of LATS1 is measured in the presence or absence of Itch. As shown in Figure 15A and 15B, although
Figure 13. **Downregulation of endogenous LATS1 by Itch.** Western blot analysis of LATS1 in MCF10A, HeLa and MCF7 cells upon expression of Itch and WPI vector control using a lentiviral system.
Figure 14. Knockdown of Itch promotes upregulation of endogenous LATS1 and its downstream signaling. Western blot analysis of endogenous LATS1 and its downstream signaling proteins in MDA-MB-231 cells upon shRNA knockdown of Itch. Two shRNAs targeting different regions of Itch (shItch-1 and shItch-2) were used to knockdown Itch in MDA-MB-231 cells.
Figure 15. Induction of LATS1 protein turnover by Itch upon inhibition of protein synthesis. (A) Cycloheximide time-course analysis of LATS1 degradation after Itch over-expression. COS7 cells expressing either LATS1-FLAG alone or LATS1-FLAG together with Itch-Myc were treated with cycloheximide (CHX) to inhibit protein synthesis. At the indicated time, cells are harvested and analyzed for LATS1 level using an anti-FLAG antibody. (B) Cycloheximide time-course analysis of LATS1 degradation after Itch knockdown. Itch is stably knocked down in MDA-MB-231 by shRNA targeting Itch. Cells infected with pGIPZ vector were used as an shRNA control. At the indicated time after cycloheximide treatment, cells were harvested and analyzed for LATS1 levels using an anti-LATS1 antibody.
over-expression of Itch efficiently facilitates LATS1 turnover, knockdown of Itch inhibits LATS1 degradation upon inhibition of protein synthesis. This finding suggests that Itch induces downregulation of LATS1 directly at the protein level.

3.3.4 Itch promotes ubiquitination of LATS1

Next, we sought to elucidate the molecular mechanism by which Itch degrades LATS1. Given that a key biochemical function of Itch is to catalyze ubiquitination of its interacting proteins, we performed an in vitro ubiquitination assay using purified E1, E2, E3 (Itch-GST) enzymes, FLAG-tagged ubiquitin, immunoprecipitated LATS1-myc and various Itch GST fusion proteins to test if Itch can directly promote ubiquitination of LATS1 in vitro. Our results showed that wild-type Itch (Itch-GST) rather than ligase dead Itch (Itch-C830A-GST) causes ubiquitination of LATS1 in vitro (Figure 16). In addition, we also carried out an in vivo ubiquitination assay examining if Itch can catalyze ubiquitination of LATS1 in cells. As shown in Figure 17 and 18, high molecular weight ubiquitinated forms of LATS1 were detected only upon addition of wild-type Itch, but not the ligase-dead (Itch-C830A) or WW domain mutant (Itch-WW-mut) of Itch. This suggests that Itch, with its ligase activity, directly catalyzes in vivo ubiquitination of LATS1.

3.3.5 Itch degrades LATS1 through the 26S proteasome pathway

In general, ubiquitinated protein can be processed for degradation by either the 26S proteasome or the lysosome (125, 131, 175). To determine which proteolysis
Figure 16. *In vitro ubiquitination of LATS1 by Itch.* Immunoprecipitated LATS1-myc on beads was used as a substrate in an ubiquitination assay with a ligase buffer containing E1, E2, Ubiquitin-FLAG, ATP, and Itch-GST or Itch-C830A-GST. Proceeding the reaction, the beads containing LATS1-myc were washed extensively with modified RIPA buffer, followed by Western blot analysis using anti-FLAG antibody.
Figure 17. *In vivo* ubiquitination of LATS1 by Itch. Ubiquitin-HA and the indicated combinations of Itch-Myc, Itch ligase-dead mutant (Itch-C830A-Myc), and LATS1-FLAG were transfected into COS7 cells. At five hours post-transfection, cells were treated with 5µM MG132 proteasome inhibitor. At 24 hours post-treatment, cells were harvested for immunoprecipitation. Ubiquitinated LATS1 was detected by immunoprecipitation of LATS1 with anti-FLAG antibody, followed by detection of ubiquitin using an anti-HA antibody.
Figure 18. Disruption of Itch WW domains abolishes *in vivo* ubiquitination of LATS1. Ubiquitin-HA and the indicated combinations of Itch-WW-Myc with LATS1-FLAG were transfected into COS7 cells. Potential ubiquitinated LATS1 was detected by immunoprecipitation of LATS1 with anti-FLAG antibody, followed by detection of ubiquitin using an anti-HA antibody.
system is responsible for LATS1 degradation by Itch, we tested the stability of LATS1 upon treatment with either a proteasome inhibitor (MG132) or lysosome inhibitor (Baf A1). Interestingly, although Itch promotes down-regulation of LATS1 under control conditions, this effect is abolished upon addition of the proteasome inhibitor MG132 (Figure 19A). On the other hand, addition of the lysosome inhibitor Baf A1 did not affect Itch-mediated degradation of LATS1 (Figure 19B), suggesting that Itch degrades LATS1 via the proteolytic activity of the proteasome, but not the lysosome. Taken together, our results provide convincing evidence that Itch promotes degradation of LATS1 by catalyzing its ubiquitination and its subsequent proteolysis by the 26S proteasome.

3.4 Itch suppresses LATS1-mediated downstream signaling

3.4.1 Itch over-expression inhibits LATS1 signaling

Since LATS1 is the central player of the Hippo-LATS signaling pathway, we want to see whether down-regulation of LATS1 by Itch could affect components of Hippo-LATS signaling. To date, the YAP and TAZ transcriptional coactivators are the only well established kinase substrates of LATS1 (93, 113). Phosphorylation of YAP or TAZ oncoproteins by LATS1 is known to inhibit their activities, thereby suppressing their transcriptional activation of various downstream oncogenes, such as Cyr61 and CTGF (93, 113, 118) (Figure 1). To test if Itch degradation of LATS1 can affect LATS1’s ability to phosphorylate YAP and TAZ and inhibit transcription of Cyr61 and CTGF oncoproteins, we used the lentiviral system to overexpress Itch, Itch ligase-dead mutant (Itch-C830A) and Itch binding mutant (Itch WW-mut) in HeLa cells, which
Figure 19. Downregulation of LATS1 by Itch requires an intact 26S proteasome, but not lysosome. (A) Proteasome inhibitor blocks Itch-induced LATS1 degradation. COS7 cells transfected with either LATS1-FLAG alone or LATS1-FLAG together with Itch-Myc were treated with either DMSO (control) or MG132 proteasome inhibitor. (B) Lysosome inhibitor fails to block Itch-induced LATS1 degradation. COS7 cells transfected with either LATS1-FLAG alone or LATS1-FLAG together with Itch-Myc were treated with either DMSO (control) or Baf A1 lysosome inhibitor.
express low levels of Itch and high levels of LATS1. Interestingly, upon over-expression of Itch, not only is endogenous LATS1 efficiently down-regulated, but phosphorylated levels of YAP and TAZ (pYAP and pTAZ) are also reduced (Figure 20). This finding suggests that over-expression of Itch strongly suppresses LATS1’s kinase activity. Furthermore, we also found that Itch over-expression promoted up-regulation of endogenous Cyr61 and CTGF (Figure 20), suggesting that Itch promotes transcriptional activity of YAP and TAZ by downregulating LATS1. Moreover, it is also found that the effect of Itch on LATS1 and its downstream signaling is dependent on its ligase activity and its interaction with LATS1. This is because stable over-expression of Itch-C830A or Itch-WW-mut mutants in HeLa cells has no effect on levels of LATS1, pYAP, pTAZ, Cyr61, and CTGF (Figure 20).

3.4.2 Itch knockdown promotes LATS1 signaling

In contrast to Itch over-expression, knockdown of Itch by two Itch shRNAs (shItch-1 and shItch-2) in MDA-MB231 cells, which express high levels of endogenous Itch, has an opposite effect on LATS1 and its downstream signaling (Figure 14). Upon knockdown of Itch by either of the two shItch constructs, endogenous LATS1 level is upregulated and its kinase activity is enhanced as demonstrated by increased phosphorylation of both YAP and TAZ. Protein levels of Cyr61 and CTGF also decreased accordingly upon knockdown of Itch, suggesting that Itch directly regulates LATS1 protein stability and its effect on downstream signaling.
Figure 20. Itch over-expression inhibits LATS1-mediated signaling. Western blot analysis of LATS1 and its downstream signaling proteins upon Itch over-expression. HeLa cells were stably infected with lentivirus expressing the vector control (WPI), Itch, Itch ligase-dead mutant (Itch-C830A), or Itch with dysfunctional WW domains (Itch-WW-Mut).
3.4.3 Itch over-expression inhibits cytoplasmic retention of YAP

Finally, because we previously showed that LATS1 inhibits YAP by phosphorylating and sequestering YAP in the cytoplasm, and loss of LATS1 causes enhanced nuclear translocation of YAP (113), we further examined whether over-expression of Itch affects subcellular localization of YAP. Interestingly, over-expression of wild-type Itch, which leads to reduced LATS1, rather than its mutants (Itch-C830A or Itch-WW-mut), induced enhanced nuclear localization of endogenous YAP in HeLa cells (Figure 21).

3.5 Itch inhibits LATS1 tumour suppressive function

3.5.1 Itch over-expression inhibits LATS1 function

The key tumour suppressor functions of LATS1 include its ability to inhibit cell proliferation (such as in HeLa cells) or induce apoptosis (such as in MCF7 and MDA-MB-231 cells) (94, 113-115). Using established Itch over-expressing HeLa cells (Figure 20), we found that over-expression of wild-type rather than ligase-dead (Itch-C830A) or WW domain mutant (Itch-WW-mut) Itch caused enhanced cell proliferation and colony formation in culture (Figure 22 and 23), suggesting that interaction and ubiquitination/degradation of LATS1 is essential for the Itch-induced phenotype. In addition, by transient over-expression of LATS1 alone or together with Itch in MCF7 breast cancer, we also found that over-expression of wild-type Itch rather than its mutants dramatically inhibits LATS1-induced cell death in a dose-dependent manner (Figure 24).
Figure 21. *Itch affects subcellular localization of the LATS1 kinase substrate, YAP.*
HeLa cells were stably infected with lentivirus expressing the vector control (WPI), Itch, Itch ligase-dead mutant (Itch-C830A), or Itch with dysfunctional WW domains (Itch-WW-Mut). Established HeLa cells were subjected to immunostaining using rabbit anti-YAP primary antibody and AF555 anti-rabbit IgG secondary antibody (red). DAPI is used for nuclear staining (blue).
Figure 22. Itch enhances proliferation of cervical cancer HeLa cells. Cell proliferation was analyzed using HeLa cells stably expressing the vector control WPI, Itch, Itch ligase-dead mutant (Itch-C830A), or Itch mutant with defective WW domains (Itch-WW-Mut). This experiment is performed by Dr. ZhongHua Zhou.
Figure 23. Itch negatively regulates LATS1 mediated inhibition of colony formation. Colony formation was analyzed using HeLa cells stably expressing the vector control WPI, Itch, Itch ligase-dead mutant (Itch-C830A), or Itch mutant with defective WW domains (Itch-WW-Mut). This experiment is performed by Dr. Zhonghua Zhou.

* indicates statistically significant difference (P < 0.05).
Figure 24. Itch negatively regulates LATS1 mediated induction of apoptosis. MCF7 cells were infected with lentivirus expressing LATS1 alone or together with increasing amount of Itch, Itch ligase-dead mutant (Itch-C830A), or Itch mutant with defective WW domains (Itch-WW-Mut). Percent cell death was measured 4 days post-infection using the trypan blue exclusion assay. This experiment is performed by Dr. Zhonghua Zhou.

* indicates statistically significant difference (P < 0.05).
3.5.2 Itch knockdown enhances LATS1 function

To test whether down-regulation of endogenous Itch has any effect on LATS1 function, we knocked down endogenous Itch using two different Itch shRNA constructs (Figure 25). Our results showed that knockdown of Itch by two different shRNAs targeting Itch (shItch-1 or shItch-2) in MDA-MB231 cells caused enhanced endogenous LATS1 and pYAP and reduced number of colony formation (representing survival tumour cells) in culture (Figure 26). Interestingly, this phenotype can be reversed by knocking down LATS1 back to its original levels using shLATS1 (Figure 26), suggesting that activation of LATS1 is responsible for Itch knockdown-induced apoptosis. Together, these results provide convincing evidence that Itch is indeed a potent negative regulator of LATS1 in cancers.
Figure 25. Enhancement of LATS1-mediated apoptosis by Itch knockdown. MDA-MB-231 cells expressing the pGIPZ vector control or shItch-1 or shItch-2 were infected with LATS1 lentivirus. Percent of cell death (Upper) was measured at 4 days post-infection using the trypan blue exclusion assay. Expression of LATS1, Itch, and YAP/pYAP (Lower) was examined by Western blot. * indicates statistically significant difference (P < 0.05).
Figure 26. Knockdown of endogenous Itch inhibits colony formation through up-regulation of LATS1. MDAMB-231 cells were transiently infected with lentivirus expressing pGIPZ vector (control), shItch-1, or shItch-2 alone or together with shLATS1, followed by Western blot analysis of protein expression (Lower) and colony formation assay (Upper). All numbers are mean and SD of three independent experiments.
CHAPTER 4

DISCUSSION

4.1 Identification of Itch as the first negative regulator of LATS1

The Large Tumour Suppressor 1 (LATS1) is a potent tumour suppressor found downregulated in many human cancers (97). Studies of LATS1 revealed that LATS1 is a serine/threonine kinase that functions as a central player of the Hippo-LATS tumour suppressive signaling pathway (97). In this pathway, LATS1 transmits upstream tumour suppressive signals from MST1/2, WW45 and MOB1 to downstream oncoproteins YAP and TAZ, leading to their inhibition and subsequent regression of tumourgenic phenotypes (97).

Although many studies previously elucidated how LATS1 might signal and function in a cell, little is known regarding its mechanisms of regulation. Through an unbiased proteomics screen, we identified Itch, a HECT class E3 ubiquitin ligase, as a unique binding partner and negative regulator of LATS1. We found that Itch promotes ubiquitination and downregulation of LATS1 by the 26S proteasome (Figure 27). Most interestingly, by destabilizing LATS1, Itch demonstrated strong inhibitory effects on LATS1 downstream signaling, cell proliferation and apoptotic function in various cancer cell lines. This finding is also supported by a recent study, where they find that Itch promotes overall tumourigenicity of the cells by enhancing cell proliferation, cell survival and promoting the epithelial-mesenchymal transition (EMT) (176). Together, these findings suggest that Itch is a unique negative regulator of LATS1.

4.2 Nedd4 family E3 ligase as potential negative regulators of LATS1
Figure 27. Regulation of LATS1 at the molecular level. At the molecular level, FAT, Expanded and Merlin acts to activates MST2. MST2, upon activation, phosphorylates and activates both LATS1 and its coactivator MOB, leading to full activation of LATS1. LATS1 can also be inhibited by Itch E3 ubiquitin ligase, which promotes its ubiquitination and degradation through the 26S proteasome pathway.
Our finding that Itch is a negative regulator of LATS1 has several implications. First, since Itch is a member of the Nedd4 family of HECT E3 ubiquitin ligases (150), it is possible that other Nedd4 E3s may also participate in the negative regulation of LATS1.

As mentioned previously, Itch interacts with LATS1’s PPxY motifs through its WW domains. Interestingly, WW domains is a characteristic present in all members of the Nedd4-family of E3 ubiquitin ligases and many members demonstrate high binding affinities towards proline-rich PY or PPxY motifs containing proteins (150). For instance, the PPxY motif containing tumour suppressor p63 is found ubiquitinated and degraded not only by Itch (154), but also by WWP1, a distinct member of the Nedd4 family of E3 ubiquitin ligases (153). An additional example is the ErbB4 tumour suppressor, which is found negatively regulated by Itch, WWP1 and Nedd4, all of which are members of the Nedd4 family of E3 ubiquitin ligases (177-179). Together, these evidences suggest that many Nedd4 family of E3 ubiquitin ligases share similar substrate specificity and functional redundancy. Moreover, like Itch, many members of the Nedd4 family E3 ubiquitin ligases also demonstrate an oncogenic capacity and have been implicated in the development of various cancers (151). For instance, it is well established that the Nedd4 plays a role in the ubiquitination of the PTEN tumour suppressor, whose loss of function is linked to development of brain, breast, prostate and lung cancers (152, 180). Additionally, while Itch is found over-expressed in anaplastic thyroid carcinoma (167), WWP1 is also found over-expressed in prostate and breast cancers (155, 156). Together, these evidences suggest that members of the Nedd4 family E3 ubiquitin ligases may play a role in cancer development and may be interesting targets for cancer therapeutics.
Further examination on how these ubiquitin ligases may coordinate with Itch in regulating LATS1 stability during tumourigenesis may help reveal a novel set of molecular antagonists for the Hippo-LATS signaling pathway.

4.3 Role of Itch in Hippo signaling and biological functions

Since LATS1 is a central player of the Hippo-LATS1 pathway, which simultaneously regulate various biological processes, such as tumourigenesis, metastasis, drug resistance, organ size control, stem cell differentiation, and neural dendrite growth (97), our study is unique in presenting a potential role for Itch in the regulation of all these fundamental biological processes. Studies of Itch recently revealed that Itch plays a role in the negative regulation of the Notch signaling pathway, which, like the Hippo-LATS pathway, is a critical developmental pathway conserved from Drosophila to humans (181). While the Hippo-LATS pathway controls the growth and proliferation of cells, the Notch signaling pathway is responsible for committing undifferentiated cells to a quiescent state (182). The finding that Itch can uniquely regulate both the Notch and Hippo-LATS pathways suggests a potential crosstalk event between these pathways. In fact, although the mechanism remain elusive, one publication recently revealed that the Hippo-LATS pathway plays a role in enhancing Notch signaling (183), supporting the notions that signals for cell proliferation and differentiation has to be tightly coupled during development (184). Studies on how Itch may play a role in the coupling of Notch and Hippo-LATS signaling during development may help further our understanding of the molecular mechanisms involved in the development, differentiation, and morphogenesis of vertebrates.
4.4 Downregulation of LATS1 by Itch as a mechanism for tumour development

Since both LATS1 and Itch are found co-expressed in a broad spectrum of human cell types, it is possible that Itch over-expression or enhanced Itch-mediated downregulation of LATS1 and other tumour suppressors may be an active mechanism leading to cancer development. To date, genetic studies on the LATS1 gene has suggested that LATS1 is frequently downregulated in human cancers through loss of heterozygosity on chromosome 6q24-25 (100-104) or through promoter hypermethylation, which limits transcription of LATS1 (107-110). It is however, not known whether or not LATS1 may also be downregulated directly at the protein level in human cancers. Future studies comparing the protein expression profiles of LATS1 and Itch in human cancers may help reveal a novel mechanism for downregulation of LATS1 in cancers.

4.5 Competitive binding to Itch by LATS1 as a mechanism for regulation

Through this study, we verified that the LATS1-Itch interaction is mediated through the PPxY motifs of LATS1 and WW domains of Itch. Interestingly, PPxY motifs of LATS1 are well-established binding modules for the LATS1 kinase substrates (93, 113). YAP, for instance, is a kinase substrate of LATS1, and it possesses WW domains that strongly recognize LATS1’s PPxY motifs (113). It is therefore possible that Itch, with four WW domains, may compete with YAP for binding to LATS1, as been recently described between YAP and Itch for binding to p73 (185), another PPxY-motif containing tumour suppressor degraded by Itch (168). We speculate that competition between Itch and WW domain-containing substrates of LATS1, such as YAP and TAZ,
may be a mechanism by which LATS1 regulates its function. While increased binding to YAP promotes phosphorylation of YAP and inhibition of tumourigenic events, increased binding to Itch promotes degradation of LATS1, leading to increased YAP activity and tumourigenesis. Taken together, our findings suggest that Itch may play a role in defining the fine balance between cell death and cell survival in human cancers. Development of strategies that specifically disrupt the LATS1/Itch interaction may therefore be useful for driving tumour suppression in cancers.

4.6 Potential for regulation of Itch by LATS1

Finally, although our study demonstrated a role for Itch in negative regulation of LATS1, it is possible that LATS1 may also negatively regulate Itch’s activity by phosphorylating Itch. As mentioned previously, it is well established that kinase substrates of LATS1, such as YAP and TAZ, are WW domain containing proteins with an oncogenic function (93, 113). Interestingly, not only is Itch a WW-domain-containing oncoprotein, it also possesses a LATS1-specific phosphorylation site (113), HFRVWS\textsuperscript{85} (H, Histidine; F, Phenylalanine; R, Arginine; V, Valine; W, Tryptophan; S, Serine). Since it is known that LATS1 generally promotes functional inhibition of its kinase substrate and that Itch plays a role in degrading a variety of tumour suppressors, we speculate that LATS1 may also be involved in the activation of multiple tumour suppressors, such as p63 and p73, by phosphorylating and inactivating Itch (154, 168). Indeed, several recent studies also described a role for serine/threonine or tyrosine kinases in modulating Itch E3 ubiquitin ligase activity through phosphorylation (186, 187). Therefore, it will be very
interesting to further explore if LATS1 may also play a role in the positive regulation of other tumour suppressor pathways.

4.7 Future directions

The present study explores the role of the Itch E3 ubiquitin ligase in the negative regulation of LATS1 in human cancers. Further studies in this field should provide insight into how Itch and LATS1 may be used as molecular markers in cancer detection and treatment. Analysis of the protein expression profiles of Itch and LATS1 across different cancer types, for instance, will be useful for determining the role of these proteins in cancer development and progression.

In addition, in the view of the large number of Nedd4 family of E3 ubiquitin ligases that share similar functions as Itch (188), our study may be a beginning for understanding of how these E3 ubiquitin ligases might together coordinate and negatively regulate tumour suppression. Beyond Itch, it will be interesting to elucidate the physiological function of each E3 member of the Nedd4-family and determine whether and how the distinct E3 ubiquitin ligases may inhibit LATS1 and the Hippo-LATS signaling pathway.

Future work directed towards the identification of small molecular inhibitors that may disrupt Itch/LATS interaction using high-throughput screening methods may also reveal new exciting opportunities for cancer treatment. As described by various recent studies, specific therapeutics disrupting E3 ubiquitin ligase / tumour suppressor complexes is one of the few modern cancer treatments that has demonstrated substantial anti-tumour effects (189, 190). Two small molecule inhibitors of the Mdm2-p53 complex, for instance, were found effective in various types of p53 negative tumours.
Together, our study reveals a new opportunity for development of specific small molecule inhibitors that may be effective against LATS1-negative tumours.

4.8 In summary

In conclusion, our study has identified the Itch E3 ubiquitin ligase as a unique negative regulator of the Large Tumour Suppressor 1 (LATS1) and the Hippo-LATS signaling pathway. Through ubiquitination and subsequent downregulation of LATS1 proteins, Itch directly controls LATS1 protein stability and its tumour suppressive functions. Studies of how Itch and LATS1 interaction may play a role in tumourigenesis using mice or clinical samples may help identify novel therapeutics effective against LATS1 deficient cancers in humans.
REFERENCES


118. Lai D, Ho KC, Hao Y & Yang X (2011) Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF. *Cancer Res*


APPENDIX 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequence</th>
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<tr>
<td>Itch, sense primer</td>
<td>5'-GCAGATCCGGTGTATGTCTGACAGTGGA TCACAAC-3'</td>
</tr>
<tr>
<td>Itch, antisense primer</td>
<td>5'-GTAACAATGCGGCCGCTTTACTACTCTTGT CCAATCTCTTTCTTC-3'</td>
</tr>
<tr>
<td>Itch-WW, sense primer</td>
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<tr>
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