Characterizing the contribution of hippo pathway dysregulation to sarcomagenesis

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CHARACTERIZING THE CONTRIBUTION OF HIPPO PATHWAY DYSREGULATION TO SARCOMAGENESIS

by

Sarah Lynne Hall

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology in the Graduate College of The University of Iowa

August 2017

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This is to certify that the Master’s thesis of

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“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.”

-Thomas A. Edison
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ABSTRACT

Sarcomas are cancers of mesenchymal origin. Though they comprise 15-20% of childhood cancers, and have a 5-year survival rate of 16% for metastatic disease, few targeted therapies exist, and the underlying mechanisms of their development are poorly understood.

Transcriptional coactivators TAZ and YAP promote cell growth and proliferation, and are constitutively activated in a number of carcinomas. Accompanying TAZ/YAP activation in these cancers is decreased expression of Hippo pathway kinases MST1/2 and LATS1/2. As the Hippo pathway is the primary negative regulator of TAZ/YAP, this provides a potential mechanistic explanation for constitutive TAZ/YAP activation. TAZ and YAP are also thought to play a prominent role in sarcomagenesis, as TAZ-CAMTA1 and YAP-TFE3 gene fusions are the specific initiating events leading to formation of epithelioid hemangioendothelioma (EHE), a vascular sarcoma. However, the mechanisms causing constitutive activation of wild-type TAZ/YAP in sarcomas have not been well-characterized.

The purpose of this study was to determine if Hippo pathway dysregulation occurs in sarcomas with constitutively active, wild-type TAZ/YAP, as well as the mechanisms by which this regulation is lost. We also investigated whether TAZ/YAP could be therapeutically targeted in sarcomas using verteporfin, a small-molecule inhibitor of the TAZ/YAP-TEAD interaction. To address these questions, sarcoma cell lines and patient clinical samples were utilized.

Using 159 patient tumor sections, we constructed a tissue microarray, stained for activated (nuclear localized) TAZ/YAP, and Hippo kinases MST1/2 and LATS1/2. A majority of sarcomas contained activation of both TAZ and YAP, while significant decreases in MST1/2 and LATS1/2 expression were observed. Results indicated a majority of tumors which stained positively for nuclear-localized TAZ/YAP also contained loss of expression of at least one of the four kinases evaluated.
All cell lines evaluated via immunofluorescence also had constitutively active (nuclear) TAZ/YAP when grown to confluence, which suggested they were no longer being negatively regulated by the Hippo pathway. In ~50% of lines, protein loss of MST1/2 and LATS2 occurred and mRNA expression of MST1 and MST2 was notably decreased in ~50%, although loss of LATS1 and LATS2 was minimal.

Potential mechanisms which could account for Hippo kinase loss were next investigated. It was found that protein degradation diminished MST2 in 25% of cell lines. Regulation by epigenetic modifications was also investigated; hypermethylation accounted for slightly reduced MST1/2, while 67% of lines had histone deacetylation in both kinases. Whether TAZ/YAP can be therapeutically targeted using verteporfin was also tested; treatment significantly inhibited anchorage-independent growth, proliferation, and TAZ/YAP transcriptional activity in sarcoma cell lines.

Our results collectively demonstrate TAZ/YAP activity can be targeted in sarcomas with verteporfin, and their constitutive activation is due to loss of MST1/2 and LATS2 kinase expression through protein degradation, histone deacetylation, and promoter hypermethylation. Such findings enhance our current comprehension of the molecular events which promote sarcomagenesis; this knowledge also opens up the possibility of creating targeted pharmacological interventions.
Sarcomas are cancers occurring in mesenchymal tissue, such as bone and muscle. They comprise a significant portion of childhood cancers (15-20%), and 5-year survival rates for patients with metastatic disease are poor (16%); however, current treatment options are limited, and commonly used therapies are minimally effective in advanced-stage disease. Therefore, identification of new therapeutic targets, pharmacological interventions, and elucidation of the underlying mechanisms which lead to sarcoma development is necessary.

Two transcriptional coactivators thought to play a role in sarcoma development are TAZ and YAP, which are inhibited by the Hippo pathway. Hippo pathway inhibition of TAZ/YAP is shown to be decreased in several cancers, including breast and liver. Consequently, TAZ/YAP remain activated and contribute to uncontrolled tumor growth.

The purpose of this study was to determine whether silencing of the Hippo pathway activates TAZ/YAP in sarcomas, and if so, how this occurs. The extent of TAZ/YAP activation was first evaluated, and a majority of patient tumor samples and cell lines were found to contain activated TAZ/YAP. We next investigated whether TAZ/YAP activation in these sarcomas is due to loss of Hippo regulation. Results showed significantly decreased expression of Hippo pathway components MST1, MST2, and LATS2, indicating the pathway can no longer inhibit TAZ/YAP activity. Protein degradation, promoter hypermethylation, and histone deacetylation were then discovered to be mechanisms which enable decreased MST1/2 expression. Finally, we tested whether TAZ/YAP can be therapeutically targeted using verteporfin, and found treatment successfully stunted cell growth, proliferation, and TAZ/YAP transcriptional activity.
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CHAPTER 1: INTRODUCTION

Sarcoma Incidence and Characterization

Incidence and Affected Populations

Sarcomas are cancers derived from mesenchymal cells. Being of mesenchymal lineage, sarcomas may arise throughout the body from various tissue types, including: fibrous tissue, skeletal muscle, smooth muscle, vascular tissue, nerve-sheath, cartilage, and bone (Table 1). As with most cancers, sarcomas are common in older adults; a study by Ferrari et al. (2011) found the majority of patients affected are >20 years of age (94.4%)\(^1,2\). However, sarcomas are unique in that they also make up a significant portion of childhood cancers. Around 20% of all pediatric cancer patients are diagnosed with sarcomas, while 5.6% of all sarcoma patients are ≤20 years old\(^1\). The most common histological type affecting the pediatric population is alveolar rhabdomyosarcoma (ARMS), while undifferentiated pleomorphic sarcoma (UPS) is the most common subtype in adults\(^1,4\). Significant differences in tumor stage do not occur between age groups; however, survival is markedly improved for soft-tissue sarcoma patients <50 years old, with 88.8% ± 0.2% survival, vs. 40.3% ± 0.3% in patients ≥50 years \(^1\).

Although comprising 1% of all cancers, the 5-year survival rate is poor, particularly if the sarcoma is late-stage or metastatic. Overall, sarcoma patient survival rates are 83% if localized, 54% for regional, and 16% for those with metastases \(^5\). With few exceptions, targeted therapies have not been developed to treat sarcomas, regardless of stage. The first line of treatment is generally surgery, with the goal being removal of the tumor and surrounding affected areas, while still preserving function. Though amputation was once the gold standard of treatment, today salvage surgery is common for most sarcomas with the exception of osteosarcoma, embryonal rhabdomyosarcoma, and alveolar rhabdomyosarcoma. Osteosarcomas are treated...
using en bloc resections\textsuperscript{6}, which entail removing large sections of the tumor and affected area of bone. These resections offer significant advantages as opposed to surgeries removing the entire bone through amputation, namely, that unaffected areas surrounding the tumor remain intact, thereby maintaining structural integrity and limb function\textsuperscript{6}. Similarly, embryonal and alveolar rhabdomyosarcomas are commonly treated using a three-pronged approach: en-bloc surgical excision and chemotherapy in addition to external-beam radiation\textsuperscript{7}.

**Characterization, Common Genetic Alterations, and Disease Progression**

Over 50 histological types of sarcoma have been identified, many of which have distinct initiating events. These initiating events can manifest as either simple or complex karyotypes. For example, sarcomas such as embryonal rhabdomyosarcoma and UPS contain primary mutations or translocations which drive tumorigenesis, and many of the remaining chromosomes also contain abnormalities. On the other hand, sarcomas such as ARMS contain a singular \textit{PAX3-FOXO1} chromosomal translocation which drives sarcomagenesis\textsuperscript{3}, yet the other chromosomes remain functional and intact (Table 2).

Though tumor karyotyping gives insight into specific genetic and chromosomal alterations, it is not always a precise indicator of the natural history of disease progression in the clinical setting\textsuperscript{8}. Therefore, sarcomas are further classified according to grade and stage. Grade assesses the degree of mitotic activity and necrosis present in a tumor; the most widely used grading system is the FNCLCC (Fédération National des Centres de Lutte Contre le Cancer). Stage indicates both the size and level to which a tumor has spread within the body. The following tables indicate levels of sarcoma grading and staging (Table 3, Table 4).

Considering the variability of tumor origin, cause, and patient age in sarcomas, a common target would be helpful in treatment, as would identification of a unifying biomarker to detect the early stages of sarcomagenesis.
TAZ, YAP, and the role of the Hippo Pathway in their Regulation

**TAZ and YAP: Structure, Function, and Downstream Targets**

First characterized by Sudol *et al.* (1995), YAP (Yes-Associated Protein) is a 493 amino acid, 65kD protein which binds to the SH3 (Src homology 3) domain of the Yes oncogene product through a proline-rich WW domain. The YAP protein is encoded by the *YAP1* gene, and has 8 transcript variants. Human YAP mRNA expression is abundant in highly regenerative tissues, though not commonly found in the brain, spleen, or leukocytes.

Later, Kanai *et al.* (2000) identified a YAP paralog transcriptional coactivator, TAZ (Transcriptional co-activator with PDZ-binding motif). TAZ is a 50kD protein containing 395 amino acids, and contains 45% homology to YAP. The TAZ protein, encoded by the *WWTR1* gene, is expressed in humans as a 6.0kb mRNA transcript in all tissues excluding peripheral blood leukocytes, and the thymus; with the highest expression in the kidney, heart, placenta, and lung.

Both YAP and TAZ do not contain DNA binding domains of their own, therefore, they must complex with other transcriptional coactivators. TAZ/YAP contain a TEAD (TEA Domain Transcription Factor/Transcriptional Enhancer Factor) binding domain in the N-terminus which complexes with TEAD transcription factors, most notably TEAD4, to activate transcription of genes controlling cell growth (Figure 1). Both proteins also contain WW domains (named after two conserved tryptophans), although they differ in that YAP contains two, while TAZ has one. Within the WW domain is a PPXY proline-rich motif, which complexes with other PPXY-containing proteins such as transcription factor Runx. A 14-3-3 protein binding domain is also present in the N-terminus centered on serine-89 in TAZ and serine-127 in YAP. In their C termini, both proteins contain a transactivation domain as well as a PDZ binding motif. The PDZ binding domain is thought to play a role in the nuclear localization of TAZ/YAP. Studies have
shown that the PDZ domain in these proteins also interacts with the PDZ domains of other proteins, such as NHERF and NHERF-2\textsuperscript{11,12}. In TAZ specifically, the last 4 C-terminal amino acids are critical to this function\textsuperscript{11,12}. These proteins are important intermediaries in the Hippo pathway, and target upstream proteins such as Merlin\textsuperscript{13}.

TAZ and YAP function in promoting cell growth and proliferation through their two primary downstream transcriptional targets Connective Tissue Growth Factor (\textit{CTGF}) and Cysteine-Rich Angiogenic Inducer 61 (\textit{CYR61}). CTGF is a secreted cysteine-rich matricellular protein, and contains 4 domains which can bind and regulate the activity of cytokines and growth factors such as: TGF-\textit{β}, VEGF, BMP4, LRP6, and FGF\textsuperscript{14}. Through these interactions, CTGF has been shown to influence cell migration, apoptosis, metastasis, angiogenesis, vascular remodeling, and extracellular matrix formation\textsuperscript{15,16}. CYR61, like CTGF, is part of the CCN family of proteins and is also composed of 4 conserved domains: IGF-1-like, von Willebrand factor type C repeats, thrombospondin type 1 repeats, and cysteine knots in the C-terminal domain\textsuperscript{17}. CYR61 can be located intracellularly, or interact with the extracellular matrix, and also functions in regulating cell proliferation, adhesion, apoptosis, migration, and extracellular matrix formation\textsuperscript{17}.

Apart from their homology, YAP and TAZ differ in their overall function and location in several ways as well. First, YAP interacts with the SH3 domain of YES protein and contains a proline-rich region; both features which TAZ lacks\textsuperscript{11}. In their interaction with the plasma membrane, YAP interacts with N-terminal and C-terminal PDZ domains in NHERF and NHERF-2, while TAZ binds only to NHERF-2 through its N-terminal PDZ domain\textsuperscript{11,12}. Despite these differences, both TAZ and YAP are shown to be negatively regulated through key serines by upstream kinases in the Hippo pathway.
The Hippo Pathway: Components and Function in Drosophila

Discovered originally in *Drosophila*, kinases Hippo and Warts play a role in regulation of organ size and are key components within the Hippo pathway. Hippo is a member of the Ste20 kinase family, and Warts is part of the NDR family of kinases. Salvadore (Sav) is a scaffolding protein, which contains two WW domains in addition to a coiled-coil domain. Together, Sav and Warts have been shown to interact to control cell growth and apoptosis. Mutations to Warts have been shown to cause tissue overgrowth, and these mutations phenocopy those found in Salvadore, suggesting these proteins work together within a common pathway. Hippo and Warts also interact via Salvadore, through their respective WW domains/PPXY motifs. Together, Hpo, Wts, and Sav interact within the Hippo pathway as a kinase cascade, whose ultimate target is Yorkie. Hippo has been shown to interact with Sav, and thus phosphorylate Warts downstream. The Hippo-Sav complex is critical to the function of Hippo phosphorylating Warts. In turn, Warts complexes with its adaptor protein Mats, which then allows phosphorylation of downstream transcription factor Yorkie at S138. This phosphorylation site is a critical regulator of Yki function; when phosphorylated, Yki is localized to the cytoplasm, and interacts with 14-3-3 protein through phospho-S138. Yorkie is subsequently targeted by ubiquitin-ligases for degradation.

Functionally, this trimer protein complex and their regulation of Yorkie as part of the Hippo pathway has important implications in controlling apoptosis and cell growth. For instance, Hippo, Salvadore and Warts function together to downregulate DIAP1, a RING-finger protein which inhibits apoptosis by binding to and inhibiting caspases. Pantalacci *et al.* (2003) reported Hippo kinases specifically promote the downregulation and ubiquitin-mediated degradation of DIAP1. Additionally, knockdown of Hippo (Hpo) in *Drosophila* leads to profound overgrowth of the eye imaginal discs and head, while overexpression of Yorkie also leads to uncontrolled
growth of wing discs\textsuperscript{27}. Moreover, Hippo has been shown to downregulate the presence of cyclin E, a modulator of cells’ transition from G1 to S phase\textsuperscript{19, 20, 28, 29}.

**Hippo Pathway Components in Mammals and their Regulation of TAZ and YAP**

Overall, the Hippo pathway is highly conserved between mammals and *Drosophila*; both adaptor proteins and kinases have ortholog counterparts. For instance, adaptor protein WW45/SAV1 in mammals is orthologous to Sav in *Drosophila*. It also contains two WW domains, which facilitate interaction between upstream MST1/2, the mammalian counterparts to Hippo, and their phosphorylation targets Large Tumor Suppressor kinase (LATS)1 and 2. MOB1, the human ortholog to Mats, is phosphorylated by upstream kinase MST1/2 at threonine 35, and allows interaction between downstream Warts ortholog LATS1/2 and its phosphorylation target TAZ/Yorkie ortholog YAP\textsuperscript{30}. Similarly, phosphorylation of TAZ and YAP occurs at regulatory serines analogous to S138 in Yorkie (Figure 2). In terms of their structures, MST1/2 contain an N-terminal kinase domain (aa26-281 in MST1, aa1-297 in MST2), along with a C-terminal SARAH domain, so named for its interaction with Sav/Rassf/Hpo, and which also allows dimerization (aa432-480 in MST1, aa436-491 in MST2). MST2 also contains an inhibitory domain from aa327-392. LATS1/2 contain C-terminal kinase domains from aa705-1130 in LATS1, and aa670-1088 in LATS2. Both contain PPXY motifs; LATS1 features 2 at aa373-376 and aa556-559, while LATS2 has this motif at aa515-518. The central feature of PPXY is its ability to interact with the WW-domains of TAZ/YAP; as such, the second PPXY motif in LATS1 is within the YAP interaction domain (aa526-655) (Figure 3).

Although it was known that *Drosophila* components of the Hippo pathway have orthologous counterparts in mammals with a variety of functions, it was not until 2007, that Dong *et al.* demonstrated the physiological importance of these proteins in mammals, and how they formed a complex signaling cascade\textsuperscript{18}. Taken together, their findings illustrate the
interaction of mammalian Hippo pathway components, and their regulation of TAZ/YAP as follows: the Hippo pathway is composed of a series of serine/threonine kinases including MST1/2 and LATS1/2 which negatively regulate the human Yorkie orthologs TAZ (Transcriptional Activator with PDZ motif) and its paralogue YAP (Yes Associated Protein) through phosphorylation of multiple serines\(^ \text{18} \). The serines playing the most significant role in their regulation are Ser89 in TAZ and Ser127 in YAP, which are also part of 14-3-3 protein binding sites. When these serines are phosphorylated, 14-3-3 protein binds to TAZ and YAP, promoting a shift in their localization from the nucleus to the cytoplasm. Once in the cytoplasm, these proteins are targeted for ubiquitin-mediated degradation by U3 ubiquitin ligases (Figure 2).

**Significance of TAZ/YAP Regulation by Hippo: An Important Interaction in Cancer**

Two conditions critical for phosphorylation, and thus activation, of Hippo kinases are cell confluence and detachment. In normal mesenchymal cells, the Hippo pathway phosphorylates and inactivates YAP/TAZ when cells become confluent or detached \(^ \text{31} \). In addition to these being important cues developmentally, they are also important obstacles which must be overcome by neoplastic cells. Given the primary function of TAZ/YAP in facilitating cell growth and proliferation, and how uncontrolled growth/proliferation as a result of constitutive TAZ/YAP activation are key features of cancer, the Hippo pathway can serve as an important tumor suppressor pathway.

**Alternative Regulation of TAZ and YAP**

Although the Hippo pathway is recognized as the primary tumor suppressor pathway governing TAZ/YAP activity, Hippo-independent mechanisms can regulate TAZ/YAP in cancer as well. Components in several major pathways including PI3K, Wnt, GPCRs, and MAPK can serve
as upstream interacting factors and downstream effectors which cross-talk with TAZ, YAP, and the Hippo pathway.

**Inhibition of TAZ/YAP by Phosphorylation of N-Terminal and C-Terminal**

**Phosphodegrons**

Both proteins contain a C-terminal phosphodegron which is serially phosphorylated at S311 by LATS and S314 by CK1, and subsequently targeted for degradation by SCF U3 ubiquitin ligase\(^3^0\). TAZ contains an additional N-terminal phosphodegron, which is phosphorylated by LATS1/2. However, Huang *et al*. (2012) revealed that TAZ can also be phosphorylated in its N-terminal phosphodegron at Ser58 and Ser64 by glycogen synthase kinase (GSK3)\(^3^0,3^2\). As GSK3 activity is inhibited through its phosphorylation by PI3K and AKT, this suggests a role for the PI3K pathway in the activation of TAZ/YAP as well\(^3^2\).

**MTOR and PI3K Pathway Cross-talk with TAZ/YAP**

High levels of TAZ expression have been observed in PI3K-driven cancers\(^3^0\), and the interaction of TAZ/YAP, LATS, and PI3K pathway component mTOR has a demonstrated effect on tumor growth\(^3^3\). Cross-talk between the Hippo pathway and mTOR was originally discovered in *Drosophila*\(^3^4-3^6\), while later work has shown this occurs in mammals as well\(^3^3\).

In non-neoplastic cells, YAP’s activity and promotion of organ growth is negatively regulated by LATS1/2 phosphorylation. At the same time, LATS1/2 negatively regulate mTOR complexes 1 and 2, and as a secondary outcome, decrease S6K phosphorylation by mTOR at Thr389 and AKT phosphorylation at Ser473\(^3^1,3^6\). Elimination of Hippo pathway regulation by LATS1/2 knockdown led to decreased YAP phosphorylation as well as activation of mTOR complexes 1 and 2 (mTORC1/2), along with upregulation of mTORC substrates S6K Thr389, and AKT Ser473\(^3^3,3^6\). Furthermore, overexpression of YAP in the absence of LATS1/2 coincided with
increased AKT phosphorylation at Ser473, and S6K phosphorylation at Thr389. This discovery implied YAP itself could upregulate mTOR activity.  

Further investigation into the underlying mechanisms governing YAP activation of mTOR revealed YAP overexpression and knockdown did not affect expression of mTOR interacting proteins TSC2, AKT, Raptor, and Rictor. Instead, YAP overexpression lead to downregulated pTEN, a potent tumor suppressor and PI3K inhibitor. Decreased PTEN expression was not found to be an outcome of disrupted protein stability or transcription, but through micro-RNA-mediated interference. Specifically, YAP upregulates expression of miR-29c, which subsequently downregulates pTEN transcription by binding to its 3'-UTR.  

This discovery was also shown to have functional relevance, as miR-29c expression through YAP transcription promotes cyclin D1 expression, with outcomes of increased cell proliferation and increased organ size. However, treatment with either rapamycin or LY294002 (a PI3K, and thus mTOR inhibitor) mitigated these effects, establishing a way in which YAP activation of the PI3K pathway can be targeted therapeutically. When tested in-vivo, administration of LY294002 in YAP transgenic mice also decreased mTOR activation, as well as cell size, growth, and hyperplasia.  

**Cross-talk Between the Wnt Pathway, β-Catenin, and TAZ/YAP**  

Two studies have proposed a role for the Wnt pathway in regulation of TAZ/YAP, through β-catenin cross-talk. The first, by Azzolin et al. (2012) formulates that TAZ acts as a second messenger of β-catenin. The Wnt pathway, which prevents β-catenin phosphorylation and accumulation, was also shown to cause TAZ accumulation, and TEAD upregulation. Much like the regulation of β-catenin in the absence of Wnt signaling, TAZ degradation is also mediated by the destruction complex; composed of Axin, GSK3, and APC. In particular, TAZ was shown to bind to GSK-3-phosphorylated β-catenin in the absence of Wnt, and that this was
necessary for B-TrCP-ubiquitin-mediated degradation of both proteins\textsuperscript{38}. These findings vary significantly from what was previously believed about the importance of the Hippo pathway in regulation of TAZ/YAP, and suggests that the Wnt-pathway and β-catenin can play a dominant role in governing T/Y activity\textsuperscript{38}. It also illustrates a previously undiscovered role of TAZ in tumors with active β-catenin signaling\textsuperscript{37, 38}.

A second study by Rosenbluh \textit{et al.} (2012) proposes a mechanism distinct from that of Azzolin \textit{et al.} (2012), which states YAP complexes with β-catenin interacting transcription factors TBX5 and YES1 to induce expression of \textit{BCL2L1} and \textit{BIRC5} in cells with Wnt/β-catenin activity\textsuperscript{39}. This large-scale study utilized siRNA screens in 85 cancer cell lines, and identified upregulated YAP, YES1, TBX5, and pro-survival Hippo pathway transcriptional targets \textit{BIRC5} and \textit{BCL2L1}\textsuperscript{37}. Furthermore, association of YAP with β-catenin and TBX5 was reported to regulate pro-survival gene expression, and contribute to cancer transformation. The study also identified this pathway as a potential therapeutic target through use of YES1-selective inhibitor Dasatinib, which prevents formation of the pro-survival YES1-TBX5-YAP complex and subsequent growth in colon cancer cells, though not in healthy colon cells\textsuperscript{39}.

In contrast to the findings of Azzolin (2012), the Rosenbluh study found that TAZ knockdown did not reveal a connection to β-catenin, unlike YAP\textsuperscript{39}. Importantly, it defined a major area of uncertainty within the field since the discovery of YAP/YES1 by Sudol (1995): it delineated the interaction and relationship between YAP (Yes-Associated interacting Protein) and YES1 itself\textsuperscript{9, 39}. Nevertheless, neither paper defined the exact relationship by which the Wnt pathway and Hippo kinases interact\textsuperscript{37}.

However, this unknown was partially addressed in a previous study by Zhou, D \textit{et al.} (2009), which discovered the presence of MST1/2 Hippo signaling lead to inhibition of the Wnt pathway and both cytoplasmic/phosphorylated β-catenin and YAP\textsuperscript{31}. Interestingly, this study
also noted that in the absence of MST1/2, LATS1/2 phosphorylation did not significantly change, suggesting that kinases other than MST1/2 act to phosphorylate LATS as well\textsuperscript{31}.

**MAP4K-Mediated Inhibition of TAZ and YAP**

Meng et al. (2015) also found that in HEK293A cells, LATS1/2 phosphorylation still occurs even in the absence of MST1/2. Their work determined MST-independent activation of LATS is mediated through upstream signaling by members of the MAP4K family\textsuperscript{25}. Like their Ste20 family counterparts MST1/2, MAP4Ks phosphorylate the LATS1/2 hydrophobic motif under several conditions, such as 2-DG treatment or serum starvation\textsuperscript{25, 40}. Moreover, MAP4K4 phosphorylation of LATS1/2 also leads to inhibition of YAP and transcription of downstream YAP-TEAD targets \textit{CTGF} and \textit{CYR61}. This study is important, as it shows YAP can still be negatively regulated in the absence of MST1/2 regulation; however, it did not investigate whether this is the case in the context of cancer\textsuperscript{25}.

**GPCR-Mediated Phosphorylation and Activation of TAZ/YAP**

Upstream G protein coupled receptors (GPCRs) have also been identified as an alternative means to Hippo pathway regulation of TAZ/YAP activity. For instance, signaling through Gs-coupled receptors can increase YAP phosphorylation and decrease its activity. Interaction between factors such as glucagon or epinephrine and their respective Gs receptors contributes to phosphorylation of LATS1/2, with inhibition of YAP activity as the outcome\textsuperscript{41, 42}.

Conversely, thrombin binding to PAR1 induces YAP activation\textsuperscript{33, 41}, as does LPA and S1P\textsuperscript{41}. Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) have been shown to disrupt Hippo regulation of TAZ/YAP by activating upstream G\(\alpha\) subunits G12/13 and Gq/11 coupled receptors, which inhibit LATS1/2 phosphorylation of YAP at S127. As a result, TAZ/YAP become constitutively activated and expression of their transcriptional targets \textit{CTGF}, \textit{CYR61}, and
**ANKRD1** is upregulated\(^41\). Unlike LATS1/2, LPA and S1P did not affect MST1/2 phosphorylation status. These results were also observed *in-vivo* using an LPA transgenic mouse model\(^41\). Though GPCR signaling through TAZ/YAP is a transient event, these particular receptors have been shown to contribute to cell proliferation through dephosphorylation of TAZ/YAP, and through activation of Rho GTPases\(^41\).

Yu *et al.* (2012) showed Rho and actin cytoskeleton remodeling control LATS1/2 phosphorylation\(^41\); however, it was not until work by Feng *et al.* (2014) that the mechanistic links between upstream G\(\alpha\) subunits and TAZ/YAP activation were revealed\(^43\). This study showed Trio and its dependent GTPases RhoA and Rac1 act as intermediaries between upstream G\(\alpha\) subunits and downstream YAP by remodeling cellular G-actin to F-actin, which binds YAP-inhibiting AMOT, allowing for YAP activation\(^43\). In addition, it was discovered that gain of function mutations of GNAZ and GNA11 lead to G\(\alpha\)q activation of YAP through Trio interaction with Rho/Rac, which polymerize actin and cause release of YAP from Angiomotin (AMOT), while simultaneously inhibiting LATS1/2 phosphorylation of YAP within the context of uveal melanoma\(^41, 43\). Notably, this discovery highlights the involvement of several TAZ/YAP regulatory mechanisms in mammals, in addition to those present in *Drosophila*\(^43\).

**Mechanotransduction in TAZ/YAP Activation**

Dupont *et al.* (2011) also found that mechanical forces from the extracellular matrix lead to actin remodeling, accompanied by TAZ/YAP activation in a Hippo-independent manner\(^44\). Mechanical strain at the ECM can also activate JNK kinases, which inhibit LATS1/2 and promote TAZ/YAP nuclear localization\(^44\). Conversely, LKB1, a regulator of cell polarity, activates Hippo kinases and causes TAZ/YAP phosphorylation through upstream MARK kinase and Scribble. In addition, LKB1 activates AMPK, which can also directly inhibit YAP by phosphorylation at Ser94\(^28, 42\).
The Crumbs polarity complex also forms an important link between cell density and the negative regulation of TAZ/YAP\(^{45}\). Crumbs is assembled at the apical surface of polarized epithelial cells and connects cell density information to downstream activity of both TAZ and YAP\(^{46,47}\). Like the Hippo pathway, Crumbs directly interacts with the WW domains and PPXY motifs in TAZ/YAP through its CRB3 and PALS subunits and promotes their phosphorylation in response to cell-cell contact inhibition\(^{45,47,48}\). As a result, it also controls SMAD2/3 localization and suppresses TGF-\(\beta\) signaling and associated EMT\(^{45}\).

**Other TAZ/YAP Transcriptional Binding Partners**

Downstream of these Hippo-independent pathways, TAZ/YAP also partner with other transcription factors to influence cellular outcomes. A novel finding by Eisinger-Mathason et al. (2015) demonstrated FOXM1 to be a YAP/TEAD binding partner, and also a transcriptional target of YAP, which contributes to proliferation and tumorigenesis of a small subset of soft-tissue sarcomas in the absence of Hippo regulation\(^2\).

Nevertheless, there is still much to be investigated in regards to how TAZ/YAP are activated in cancer; whether in a Hippo-independent manner, or by dysregulation of kinases within the Hippo pathway itself. Evidence indicates that much of the cross-talk between TAZ/YAP, Hippo kinases, and other pathways is largely dependent on the tissue of study and context. Many of these studies have focused on cross-talk within liver, colon, and breast cancers, but the occurrence of pathway cross-talk and dysregulation in sarcomas remains largely unknown, and requires further study.

**The Significance of TAZ and YAP in Cancer**

Several studies have provided insight into the oncogenic roles of TAZ and YAP in carcinomas, and how their constitutive activation promotes several hallmarks of cancer,
including: proliferation, invasion, metastasis, resistance to anoikis, and stemness. The first link between TAZ/YAP, and cancer was established by Chan, et al. (2009) when they demonstrated TAZ expression is prevalent within breast cancer lines and in clinical samples. Moreover, increased TAZ expression correlated with increased invasiveness of the cancer itself. In the context of promoting tumorigenesis, TAZ plays a critical role in promoting the invasion and migration hallmarks of cancer as well. Overexpression of TAZ increased the ability of cells to proliferate, and migrate; it also led epithelial cells to adopt a more mesenchymal, fibroblastic-like appearance. Conversely, shRNA knockdown of TAZ mitigated these effects; thus establishing an important oncogenic role of TAZ within breast cancer, and its potential as a therapeutic target. These findings were also demonstrated in other cancers, including: colon, liver, lung, and thyroid.

In colon cancer, Wang et al. (2013) found increased expression of TAZ/YAP to be correlated clinically with decreased patient survival, and that patients exhibiting both TAZ and YAP activation had the worst prognosis. In-vitro, colorectal cancer cells with TAZ/YAP knockdown showed a significant decrease in their ability to proliferate, invade and metastasize, thus highlighting the importance of TAZ/YAP in several hallmarks of cancer.

The most common genetic aberration in pancreatic ductal adenocarcinomas (PDAC) is the KRASG12D mutation and its associated MAPK signaling; however, a small subset of PDAC and their associated proliferation are driven by YAP overexpression/amplification and interaction with TEAD2 and E2F transcription factors.

Within the context of non-small cell lung cancer (NSCLC), TAZ acts as an oncogene as well. TAZ overexpression in immortalized HBE135 cells increases proliferation, transformation, and anchorage-independent growth, while TAZ knockdown decreases these effects both in-vitro and in-vivo within mice.
p53

Myc-expressing progenitors with increased YAP expression also had a propensity for promoting tumorigenesis, cell growth, and survival in both human hepatocellular carcinoma (HCC) tumors and mouse models of liver cancer. Moreover, shRNA-mediated silencing of YAP expression in these tumor cells significantly decreased cyclin E expression and cell growth both 

in-vitro and when grafted into mice.

TAZ also plays an important oncogenic role in papillary thyroid carcinomas. In thyroid tumors, TAZ expression is increased in comparison to normal thyroid tissue, while its overexpression in-culture leads to EMT and confers a growth advantage.

The Role of TAZ and YAP, and the Hippo Pathway in Sarcomas

Aberrant TAZ and YAP Activity as an Initiating Event in Sarcomagenesis

TAZ and YAP are known to direct important processes in mesenchymal cells. One example is that TAZ has been shown to direct differentiation of pluripotent mesenchymal stem cells (MSC) into either an osteogenic or adipocytic fate based on its transcriptional activation or repression of transcription factors Runx2 and PPAR. Through its WW domain, TAZ interacts with the PPXY motifs of both Runx2 and PPAR, though with two very distinct outcomes. In its interaction with Runx2, TAZ plays a stimulatory role, whereby the expression of osteocalcin is upregulated, and MSCs will ultimately differentiate into osteoblasts. Contrary to Runx2, the interaction of TAZ with PPAR has an inhibitory effect, which leads to adipocytic differentiation.

In relation to the role of TAZ in sarcomas, constitutive activation of TAZ by deletion of regulatory Ser89 in MSCs revealed an even more pronounced decrease adipocytic differentiation. Considering the mesenchymal origin of sarcomas, this suggests constitutive activation of TAZ may lead to the development of sarcomas with an osteoid or musculoskeletal origin, such as osteosarcomas and rhabdomyosarcomas.
These findings were further supported by subsequent studies such as that by Crose, et al. (2014) that characterized the mechanisms by which a PAX3-FOXO1A gene fusion in alveolar rhabdomyosarcoma (ARMS) drives tumor development. The study demonstrated the PAX3-FOXO1A gene fusion upregulated expression of RASSF4, which in turn inhibited MST1 phosphorylation, and caused subsequent Hippo pathway inactivation and TAZ/YAP activation. In addition, they proposed that YAP upregulation in alveolar rhabdomyosarcomas may not only be a result of Hippo pathway dysregulation, but could also be the direct result of RASSF4 activation of YAP in order to promote cell proliferation, evasion of cellular senescence, and tumorigenesis.

Similar findings were presented by Tremblay, A.M. et al. (2014) using an in-vivo mouse model of embryonic rhabdomyosarcoma (ERMS). YAP was found to be highly expressed in ERMS, and overexpression of YAP-S127A mutants exhibited increased cell proliferation and tumorigenesis, while knockdown of YAP1 was shown to mitigate these effects.

**Genetic Alterations of TAZ/YAP in Sarcomas**

The precise mechanisms of TAZ and YAP activation in carcinomas and sarcomas alike have yet to be elucidated, especially since TAZ and YAP are wild-type in the vast majority of cancers. The only consistent genetic alterations of TAZ (WWTR1) and YAP (YAP1) known to exist in sarcomas are the WWTR1-CAMTA1 fusion and YAP1-TFE3 fusion. These fusions specifically lead to development of epithelioid hemangioendothelioma (EHE), a rare vascular sarcoma.

EHE harbors a t(1;3) (p36;q25) chromosomal translocation encoding the WWTR1-CAMTA1 gene fusion. In the TAZ-CAMTA1 fusion, the N-terminus of TAZ (WWTR1) remains intact and still contains the WW and TEAD binding domains, allowing TEAD4 binding and activation of their transcriptional programs. Importantly, the WWTR1-CAMTA1 gene fusion abolishes the normal regulatory function of the Hippo pathway. WWTR1 itself remains intact.
up to exon 2 (type 1 transcript) or exon 3 (type 2 transcript), and retains its Ser89/14-3-3 binding site; this is fused in-frame to exons 9-23 of CAMTA1.

Thus, the C-terminus of the CAMTA1 portion of the protein contains a strong nuclear localization signal (NLS), and results in constitutive localization of the TAZ-CAMTA1 fusion to the nucleus. This occurs despite phosphorylation of Ser89 in the 14-3-3 binding site of the N-terminal TAZ portion\(^5^6\). Without negative regulation by interaction with 14-3-3 protein, TAZ remains constitutively active within EHE. This constitutive activation has been shown to largely direct a TAZ-transcriptional program, rather than a CAMTA1 transcriptional program; though CAMTA1 makes up 90% of the gene fusion\(^5^6, 5^7\).

The YAP1-TFE3 (YT) gene fusion was discovered later, although it has not been as well-characterized as the WWTR1-CAMTA1 fusion. What is known, however, is that exon 1 of YAP1 is fused to either exons 4 or 6 of TFE3 - a transcription factor belonging to the MiT family\(^5^7, 5^8, 5^9\). Within this particular gene fusion, most of the TEAD binding site remains intact, but the Ser127 Hippo phosphorylation site and 14-3-3 binding site of YAP1 is lost, along with the WW domains, transactivation domain, and PDZ binding motif\(^5^7, 5^8\). Consequently, it is thought that YAP1 provides a stronger promoter for TFE3 activity in tumors\(^5^8\).

**Oncogenic Contributions of TAZ-CAMTA1 to Sarcomagenesis**

Recently, the TAZ-CAMTA1 (TC) fusion has been shown to transform cells via constitutive activation of the TAZ portion of the fusion\(^5^6, 6^0\). When expressed in non-transformed HEK-293 or NIH-3T3 cell lines, TAZ-CAMTA1 has been shown to contribute to several hallmarks of cancer, including detached growth in soft agar, proliferation, and resistance to anoikis\(^5^6\). However, deletion of the CAMTA1 NLS significantly decreased the ability of cells to exhibit these hallmarks. Although no longer responsive to Hippo pathway regulation, the TAZ portion still contains a functional TEAD binding domain which is necessary for carrying out its transcriptional
program. Hence, this binding can be successfully targeted by therapeutic intervention using verteporfin (VP), a heme derivative shown to disrupt TAZ/YAP’s interaction with TEAD transcription factors. These chromosomal rearrangements highlight the importance of activated TAZ/YAP in cancer, as well as their specific contributions to sarcomagenesis.

**Hippo Pathway Dysregulation as a Means of TAZ/YAP Constitutive Activation and Sarcomagenesis in-vivo**

Nevertheless, TAZ/YAP constitutive activation through gene fusions are not the only means by which sarcomagenesis is shown to occur. It is thought that Hippo becomes suppressed by mechanisms unknown, allowing TAZ and YAP to localize to the nucleus and act as oncoproteins. Alternatively, it has been proposed that mutations or deletions of Hippo kinases may render them unable to regulate TAZ/YAP and thus contribute to tumorigenesis. This hypothesis has been demonstrated using in-vivo mouse models.

For example, Zhou, D. et al. (2009) found that conditional homozygous knockout of both MST1/2 in the liver contributes to hepatocellular carcinoma (HCC) development. Without MST1/2, resistance to Fas-ligand-induced apoptosis and overgrowth of liver cells was increased. In the absence of MST1/2, phosphorylation of YAP at Ser127 did not occur, and HCC developed; this highlights the important role of MST1/2 as tumor suppressors. Re-expression of MST1/2 had the opposite effect, as it both promoted YAP negative regulation and abrogated tumorigenesis. This mouse system provided proof that MST1/2 are highly expressed in epithelial liver cells, and homozygous loss of Hippo kinases is both necessary and sufficient for tumor formation. Yet, it is unclear whether the liver is particularly susceptible to transformation due to Hippo pathway dysregulation, or whether these findings are applicable to mesenchymal neoplasms.
Counter to MST1/2 knockout, loss of LATS1 in mice was shown to specifically lead to sarcomagenesis. Mice contained an inserted neomycin cassette corresponding to aa 756-1130 of human LATS1, which through homologous recombination, led to effectively disrupted mouse Lats1 expression\textsuperscript{63}. Importantly, soft-tissue sarcoma formation occurred rapidly in this model, with tumors apparent at 4-10 months. In addition, Lats1\textsuperscript{-/-} mice were shown to have decreased survival, and those with homozygous Lats1 knock-out were observed in numbers below Mendelian ratios (8% vs an anticipated 25%)\textsuperscript{63}. Although the study proved the importance of LATS1 \textit{in-vivo} as a tumor suppressor, there is the necessity for a more comprehensive genetic model to better recapitulate sarcoma development \textit{in-vivo}; ideally one which links decreased Hippo kinase expression to TAZ/YAP activation as well.

\textbf{Dysregulation of the Hippo Pathway in Sarcomas}

\textit{Potential Mechanisms of Hippo Pathway Dysregulation}

Studies have also established that increased TAZ and YAP activation in cancer is a result of the Hippo pathway being unable to effectively negatively regulate TAZ and YAP, although the mechanisms by which this deficiency occurs are not completely understood at present.

One possible mechanism for decreased negative regulation by Hippo kinases in cancer may be due to mutations in targets of the pathway, rather than the pathway itself; however, no mutations in TAZ or YAP have been identified\textsuperscript{24}. This suggests that changes to regulatory kinases within the Hippo pathway are a more likely explanation for activated TAZ/YAP. Inherited mutations of NF2 upstream of TAZ/YAP have been shown to result in the development of Type 2 Neurofibromatosis, yet this mutation has not been previously implicated in the tumorigenesis of other more common sarcomas\textsuperscript{24,64}. Mutations in Hippo kinases MST1/2 and LATS1/2 are not
common occurrences in human cancer, but epigenetic silencing of their expression has been identified\textsuperscript{24, 65-67}.

\textit{Epigenetic Silencing of the Hippo Pathway}

Dysregulation of Hippo kinases by epigenetic mechanisms, such as promoter hypermethylation, has also been proposed as an explanation for the oncogenic properties of TAZ/YAP. Much of the work examining hypermethylation of Hippo kinases has primarily focused on LATS1/2. In one such study using breast cancer, LATS1/2 human tumors contained significant promoter hypermethylation, with 56.7\% (17/30) of tumors containing LATS1 hypermethylation, and 50\% (15/30) of tumors containing hypermethylated LATS2\textsuperscript{65}. Moreover, LATS1/2 mRNA expression was significantly decreased in hypermethylated tumors, as compared to controls; and decreased mRNA expression correlated with increased tumor size, frequent metastasis to lymph nodes, and an absence of ER/PR receptor expression\textsuperscript{65}.

Similarly, in astrocytomas, Jiang \textit{et al.} (2006) found significant promoter hypermethylation of LATS1 (63.7\%) and LATS2 (71.5\%)\textsuperscript{66}. In addition, increased promoter hypermethylation correlated with the development of malignant lesions, and overall expression of LATS1/2 at a transcriptional level was decreased with increasing pathological grade\textsuperscript{66}. Promoter hypermethylation was also investigated in 2 astrocytoma cell lines, and treatment of cells with 5-Aza-2\textsuperscript{\textprime} -deoxycytidine (Decitabine) rescued LATS1/2 expression to induce apoptosis\textsuperscript{66}. In soft-tissue sarcomas, LATS1 gene expression was also surveyed in 50 patient tumor samples using RT-PCR, and 14\% (7/50) contained decreased expression, 1/7 due to a missense point mutation, and 6/7 due to CpG promoter hypermethylation\textsuperscript{68}.

The overall expression of MST1/2 and its correlation with promoter hypermethylation have been significantly less studied than LATS1/2; however, research by Seidel \textit{et al.} (2007) investigated both LATS1/2 and MST1/2 hypermethylation frequencies within the same subset of
soft-tissue sarcomas$^{67}$. In contrast to the findings of the Hisaoka et al. (2002) study$^{68}$, the Seidel study demonstrated a less than 7% incidence of LATS1 promoter hypermethylation, and LATS2 was not found to be hypermethylated in any of the examined samples (0/41)$^{67}$. Intriguingly, MST1 and MST2 were frequently hypermethylated in primary soft tissue sarcomas, with MST1 hypermethylation in 37% (19/52) and MST2 in 20% (12/60)$^{67}$ (Table 5). Promoter methylation of MST1 in Saos-2, A204, RD, and SK-LMS-1 cells was also observed, and decreased mRNA expression was found to accompany MST1 hypermethylation in primary sarcomas as well$^{67}$. Whether promoter hypermethylation plays a significant role in regulating TAZ/YAP activation in additional sarcoma cell lines as well as other clinical sarcoma subtypes remains to be seen. If so, this mechanism may be an attractive therapeutic target in sarcomas.

**Therapeutic Interventions**

Therapeutic targeting of epigenetic modifications in cancer has primarily focused on promoter hypermethylation and histone deacetylation. Two attractive drug classes which inhibit these processes are DNA methyltransferase (DNMT) inhibitors such as 5-Azacytidine, and histone deacetylase (HDAC) inhibitors like Trichostatin A. 5-Azacytidine (5-Aza) is a highly toxic cytidine analog, which is incorporated into DNA, and inhibits DNMT activity by formation of a covalent bond with the enzyme$^{69}$. Although unstable and quickly degraded in culture, 5-Aza has been shown to decrease DNA methylation and increase expression of previously hypermethylated genes at concentrations of 1-10µM in many models of cancer, including leukemias, melanoma, lung, and breast cancer$^{69}$,$^{70}$. Trichostatin A (TSA), meanwhile, is a cytostatic, antifungal antibiotic which arrests cell cycle progression from G1 to G2; though it was later found to be a specific inhibitor of HDACs both in in-vitro and in-vivo models of breast carcinoma$^{71}$. In breast cancer cell lines, 2µM TSA treatment led to histone H4 hyperacetylation, and inhibited cellular proliferation$^{71}$. TSA administered subcutaneously with doses up to 5mg/kg
was not found to be toxic in rats with NMU-induced mammary carcinoma\textsuperscript{71}. It did, however, decrease malignant tumor growth and formation in a dose-dependent manner, presumably through inducing differentiation\textsuperscript{71}.

TAZ and YAP themselves have also emerged as important potential therapeutic targets in cancer. Liu-Chittenden, \textit{et al.} (2012) first discovered the YAP-TEAD interaction could be targeted using verteporfin (Visudyne)\textsuperscript{61}. Verteporfin (VP) is a heme analog approved for clinical use as a photosensitizer in patients with neurovascular macular degeneration\textsuperscript{61}. In murine models of hepatocellular carcinoma with Yap-overexpression and in Yorkie-overexpressing \textit{Drosophila} S2 cells though, VP was shown to inhibit Yap-Tead2/Yorkie-Sd complex formation through selective binding to Yap/Yorkie\textsuperscript{61}. Furthermore, with VP concentrations of 2.5µM-10µM, liver growth was substantially decreased, and mRNA expression of downstream Yorkie target gene Diap1 was significantly reduced in S2 cells with 200nM VP treatment\textsuperscript{61}.

Work by Jiao \textit{et al.} (2014) and Zhang \textit{et al.} (2014) has also suggested that VGLL4, a transcription co-factor protein, can act as an inhibitor of YAP activity by competing with YAP for TEAD binding. These studies show VGLL4 expression \textit{in-vitro} and \textit{in-vivo} decreased tumor formation, growth, and cell proliferation in both gastric and lung cancers\textsuperscript{72,73}.

**Purpose of Study: Rationale for the Analysis of Hippo Pathway Dysregulation, TAZ/YAP Activation in Sarcomas, and Application of Targeted Therapies**

Despite these findings, however, the prevalence and effects of Hippo pathway dysregulation \textit{in-vitro} and \textit{in-vivo} has not been extensively studied in sarcomas. Previous work by Fullenkamp \textit{et al.} (2016) has demonstrated TAZ/YAP activation in a significant portion of sarcomas\textsuperscript{74}. A tissue micro array (TMA) containing 159 primary sarcomas was constructed, and samples stained for TAZ/YAP. As TAZ/YAP are transcriptional coactivators, their activation is
evident when they are localized to the nucleus. Overall, it was found that TAZ was activated in 66% of sarcoma samples, whereas YAP was activated in 50%.

These results demonstrated TAZ/YAP are activated in a significant portion of sarcomas, and also showed the clinical relevance of TAZ/YAP activation in sarcomas. Interestingly, the TMA results showed TAZ and YAP were differentially activated within certain sarcoma subtypes. This discovery opens up the possibility that TAZ/YAP each carry out separate transcriptional programs/functions in certain subtypes; although further study is required.

Utilizing well-differentiated (WDLPS) and de-differentiated liposarcoma (DDLPS) TMA samples, activation of TAZ/YAP was analyzed, and a correlation between the portion of activated TAZ/YAP in these samples and sarcoma grade was noted; WDLPS are low-grade (grade 1) and exhibited significantly less TAZ/YAP activation than DDLPS samples, which are high grade (grade 2-3).

Data analyzed from The Cancer Genome Atlas (TCGA) also showed that in patients with undifferentiated pleomorphic sarcoma (UPS) and de-differentiated liposarcoma (DDLPS), WWTR1 (TAZ) and YAP1 (YAP) gene expression correlated with decreased survival, further solidifying their clinical relevance.

These findings were also relevant to study within a laboratory setting. Knockdown of both TAZ and YAP in SK-LMS-1 cells decreased proliferation, and TAZ KD reduced colony formation in soft agar. Not only does this demonstrate that TAZ and YAP play a crucial role in cancer cell survival, but they do so by specifically affecting the cancer hallmarks of proliferation, and detached growth. In HT1080 and SK-LMS-1 sarcoma cell lines, TAZ and YAP were targeted therapeutically. Treatment of both cell lines with verteporfin (VP) decreased colony formation in soft agar. This result was confirmed by analyzing TAZ/YAP transcriptional target
CTGF expression via qRT-PCR, which showed a decrease in CTGF expression with increasing VP administration.

Sarcoma cell lines HT1080 and SK-LMS-1, when grown to confluence and observed via immunofluorescence, showed nuclear-localized, and thus activated TAZ/YAP. This supports previous studies showing activation of TAZ/YAP is evident within sarcomas. Thus far, however, the mechanisms by which their activation occurs (presumably by the dysregulation of the Hippo pathway) is not fully understood. Several mechanisms for dysregulation have been proposed, yet they have not been thoroughly investigated for their applicability within clinical samples, nor have they been extensively studied in sarcoma cell lines. Additionally, the effects of verteporfin and other therapeutic reagents have been shown to decrease TAZ/YAP activation and transcription in cancers, yet evidence showing whether they significantly decrease hallmarks of cancer in sarcomas both in-vivo and in-vitro, and whether these treatments effectively compensate for loss of Hippo kinase regulation, is lacking.

Study Aims

1) Conduct a broad survey of TAZ and YAP activation and Hippo pathway dysregulation in multiple sarcomas

   a) Evaluate whether expression of Hippo kinases is decreased in sarcoma subtypes exhibiting TAZ/YAP activation (clinical sample and cell line studies)

   b) Evaluate potential mechanistic reasons why decreased Hippo kinase expression occurs using therapeutic interventions
<table>
<thead>
<tr>
<th>Sarcoma Type</th>
<th>Tissue of Origin</th>
<th>Location</th>
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<tbody>
<tr>
<td>Liposarcoma</td>
<td>Adipose</td>
<td>Retroperitoneum, Extremities</td>
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<td>Fibrous</td>
<td>Trunk, Extremities, Head, Neck</td>
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<td>Smooth Muscle</td>
<td>Retroperitoneum, Large veins, Lower extremities</td>
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<td>Uncertain Differentiation</td>
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Table 1: Sarcoma Subtypes and Tissue/Location of Origin
Listed are the major sarcoma subtypes and the tissues in which they arise, as well as typical location throughout the body. As sarcomas originate from cells of mesenchymal lineage, they are often found in fibrous/connective tissue, adipose, muscle, or bone. Adapted from Fletcher, C.D.M. et al. WHO Classification of Tumours of Soft Tissue and Bone (2013).
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<th>Chromosomal Rearrangements</th>
<th>Gene Fusions</th>
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<td>PAX3-FKHR fusion</td>
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<td></td>
<td>t(1;13)(p36;q14)</td>
<td>PAX7-FKHR fusion</td>
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<td>Alveolar soft part sarcoma</td>
<td>t(X;17)(p11;q25)</td>
<td>ASPL-TFE3 fusion</td>
</tr>
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<td>Atypical lipomatous tumor/well differentiated liposarcoma</td>
<td>12q rings and giant markers</td>
<td>HMGIC, CDK4, and MDM2 amplification</td>
</tr>
<tr>
<td>Clear cell sarcoma</td>
<td>t(12;22)(q13;q12)</td>
<td>ATF-1-EWS fusion</td>
</tr>
<tr>
<td>Dermatofibrosarcoma protuberans/giant cell fibroblastoma</td>
<td>t(17;22)(q22;q13)</td>
<td>PDGFB-COL1A1 fusion</td>
</tr>
<tr>
<td>Desmoplastic small round cell tumor</td>
<td>t(11;22)(p13;q12)</td>
<td>WT1-EWS fusion</td>
</tr>
<tr>
<td>Epithelioid hemangioendothelioma (EHE)</td>
<td>t(1;3)(p36;q25)</td>
<td>TAZ-CAMTA1 fusion</td>
</tr>
<tr>
<td></td>
<td>t(11;17)(q2;q25)</td>
<td>YAP-TFE3 fusion</td>
</tr>
<tr>
<td>Ewing’s sarcoma</td>
<td>t(11;22)(q24;q12)</td>
<td>FLI-1-EWS fusion</td>
</tr>
<tr>
<td></td>
<td>t(21;22)(q22;q12)</td>
<td>ERG-EWS fusion</td>
</tr>
<tr>
<td></td>
<td>t(7;22)(p22;q12)</td>
<td>ETV1-EWS fusion</td>
</tr>
<tr>
<td></td>
<td>t(17;22)(q12;q12)</td>
<td>EIAF-EWS fusion</td>
</tr>
<tr>
<td></td>
<td>t(2;22)(q33;q12)</td>
<td>FEV-EWS fusion</td>
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<tr>
<td>Extraskeletal myxoid chondrosarcoma</td>
<td>t(9;22)(q22;q12)</td>
<td>TEC-EWS fusion</td>
</tr>
<tr>
<td>Infantile fibrosarcoma</td>
<td>t(12;15)(p13;q25)</td>
<td>ETV6-NTRK3 fusion</td>
</tr>
<tr>
<td>Myxoid/round cell liposarcoma</td>
<td>t(12;16)(q13;q11)</td>
<td>CHOP-TLS fusion</td>
</tr>
<tr>
<td></td>
<td>t(12;22)(q13;q111-12)</td>
<td>CHOP-EWS fusion</td>
</tr>
</tbody>
</table>

**Table 2: Chromosomal Translocations in Soft Tissue Sarcomas**

Specific chromosomal rearrangements are responsible for several different types of sarcomas; gene fusions of several chromosome combinations may lead to the development of the same sarcoma subtype (such as EHE) however, they are often rare. Adapted from Goldblum J.R., Folpe A.J., and Weiss S.W. Enzinger and Weiss’s Soft Tissue Tumors (2014)75; and Fletcher C.D.M. Diagnostic Histopathology of Tumors (2000)76.
<table>
<thead>
<tr>
<th>Histological parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor differentiation</td>
<td>• Score 1-3: Sarcomas resembling normal adult mesenchymal tissue to poorly differentiated/undifferentiated sarcomas</td>
</tr>
<tr>
<td>Mitotic count</td>
<td>• Score 1-3: 0 to &gt;19 mitoses per 10 HPF</td>
</tr>
<tr>
<td>Tumor necrosis</td>
<td>• Score 0-2: no necrosis to &gt; 50% tumor necrosis</td>
</tr>
<tr>
<td>Histological grade</td>
<td>• Grade 1-3: based on total score of 2-8 points</td>
</tr>
</tbody>
</table>

Table 3: FNCLCC Sarcoma Grading
Tumors are clinically scored using the FNCLCC scale, out of 2-8 total points based on histological parameters such as: differentiation, number of mitoses present, percentage of necrosis present, and histological grade. Poor clinical prognosis correlates with increasing tumor grade/score. Adapted from Fletcher, C.D.M. et al. WHO Classification of Tumours of Soft Tissue and Bone (2013)\(^8\).
### Tumor stage | Phenotypic parameters
--- | ---
**Early** | Small tumors- ≤ 5 cm in diameter  
Superficial- located above the fascia  
Non-metastatic
**Advanced** | Larger tumors- > 5 cm in diameter  
Deep seated- located below the fascia  
Metastatic

Table 4: AJCC Sarcoma Staging System

Sarcoma tumors are grossly staged as early or advanced. This classification is dependent upon their size, localized spread, as well as whether or not metastasis is present. Adapted from Goldblum J.R., Folpe A.J., and Weiss S.W. Enzinger and Weiss’s Soft Tissue Tumors (2014)\textsuperscript{75} and Fletcher, C.D.M. et al. WHO Classification of Tumours of Soft Tissue and Bone (2013)\textsuperscript{8}. 
Figure 1: Domains of WWTR1 and YAP1
TAZ and YAP are transcriptional coactivators. Both contain a TEAD binding domain, a critical serine (S89 in TAZ, S127 in YAP) whose phosphorylation is necessary for 14-3-3 binding. Both contain two conserved tryptophan (WW) domains, although YAP contains two, while TAZ only contains one; these interact with PPXY motifs in several transcription factors. A transactivation domain (TAD) is also present, with a central coiled-coiled domain. Finally, both proteins have a PDZ binding motif, thought to aid in nuclear localization and interact with other PDZ-containing proteins.
The Hippo pathway is a series of serine/threonine kinases activated in normal, growing cells when cells become confluent or detached. Upstream Merlin/NF2 phosphorylates MST1/2, which subsequently complexes with scaffolding SAV1 and MOB1 to phosphorylate LATS1/2. LATS then phosphorylates YAP/TAZ, which localize to the cytoplasm and are targeted for degradation by ubiquitin ligases. Adapted from Eisinger-Mathason T.S., et al. Deregulation of the Hippo pathway in soft-tissue sarcoma promotes FOXM1 expression and tumorigenesis (2015)².

Figure 2: TAZ, YAP, the Hippo Pathway, and their Regulation in Mammals
The Hippo pathway is a series of serine/threonine kinases activated in normal, growing cells when cells become confluent or detached. Upstream Merlin/NF2 phosphorylates MST1/2, which subsequently complexes with scaffolding SAV1 and MOB1 to phosphorylate LATS1/2. LATS then phosphorylates YAP/TAZ, which localize to the cytoplasm and are targeted for degradation by ubiquitin ligases. Adapted from Eisinger-Mathason T.S., et al. Deregulation of the Hippo pathway in soft-tissue sarcoma promotes FOXM1 expression and tumorigenesis (2015)².
Figure 3: The Functional Domains of MST1/2 and LATS1/2
The above schematics of MST1/2 and LATS1/2 illustrate their functional domains. MST1/2 contain an N-terminal kinase domain which targets LATS1/2, along with a C-terminal SARAH domain which allows dimerization and interaction with Sav/Rassf/Hpo. Adapted from Liu G, et al. Structure of SARAH domain provides insights into its interaction with RAPL (2014)77.

LATS1/2 contain C-terminal kinase domains which phosphorylate TAZ/YAP at S89 and S127 respectively. Both contain PPXY motifs; LATS1 contains two, and LATS2 contains one. The presence of a PPXY motif is critical for LATS interaction with the WW-domains of TAZ/YAP. Adapted from Visser S and Yang X. LATS tumor suppressor: a new governor of cellular homeostasis (2010)78.
### Table 5: Proportion of Soft-Tissue Sarcomas Containing Promoter Hypermethylation of MST/LATS

**A.** Methylation frequency of Hippo kinases; adapted from Seidel C, et al. Frequent hypermethylation of MST1 and MST2 in soft tissue sarcoma (2007)\(^\text{67}\). Fractions and percentages of sarcoma tumor samples which contain promoter hypermethylation of MST1/2 show hypermethylation frequency of MST1 (37%) and MST2 (20%). LATS 1 has minimal promoter hypermethylation (7%), while LATS2 was not hypermethylated in any of the tumor samples\(^\text{67}\).

**B.** Adapted from Hisaoka M, et al. Molecular alterations of h-warts/LATS1 tumor suppressor in human soft tissue sarcoma (2002)\(^\text{68}\). Of 50 soft tissue sarcomas categorized into 18 tumor subtypes, only 3 contained decreased LATS1 expression: MFH (11%), Leiomyosarcoma (43%), and Myxoid Liposarcoma (60%). Of sarcoma samples with decreased LATS1 expression, a majority had promoter hypermethylation\(^\text{68}\).

<table>
<thead>
<tr>
<th>Sarcoma Type</th>
<th>MST1</th>
<th>MST2</th>
<th>LATS1</th>
<th>LATS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposarcoma</td>
<td>2/8</td>
<td>2/13</td>
<td>1/13</td>
<td>0/9</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>5/10</td>
<td>0/13</td>
<td>1/11</td>
<td>0/11</td>
</tr>
<tr>
<td>MFH and MFS</td>
<td>2/11</td>
<td>4/13</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>4/5</td>
<td>0/3</td>
<td>0/3</td>
<td>0/4</td>
</tr>
<tr>
<td>Neurogenic sarcoma</td>
<td>1/2</td>
<td>0/3</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>1/6</td>
<td>3/6</td>
<td>1/4</td>
<td>0/5</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>1/3</td>
<td>0/3</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Other (Unclassified)</td>
<td>2/6</td>
<td>2/3</td>
<td>0/3</td>
<td>0/4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>19/52</td>
<td>12/60</td>
<td>3/43</td>
<td>0/41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sarcoma Type</th>
<th>Decreased LATS1 Expression</th>
<th>Hypermethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFH</td>
<td>1/9 (11%)</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>3/7 (43%)</td>
<td>2/3 (60%)</td>
</tr>
<tr>
<td>Liposarcoma (Myxoid)</td>
<td>3/5 (60%)</td>
<td>3/3 (100%)</td>
</tr>
</tbody>
</table>

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CHAPTER 2: THE PRESENCE OF HIPPO DYSREGULATION IN SARCOMAS AND
MECHANISMS BY WHICH IT OCCURS

Rationale

The connection between Hippo pathway dysregulation and cancer has recently become a growing field of study. The constitutive activation of TAZ/YAP in cancer has been established\(^4^9\),\(^5^3\),\(^5^5\), and loss of Hippo kinase expression in a small subset of studies has also been noted\(^2\),\(^2^5\). Nevertheless, decreased expression of these kinases has not been broadly surveyed across a multitude of sarcoma cell lines, or in patient sarcoma samples. In cancer cell lines, TAZ/YAP have shown constitutive nuclear localization, suggesting their activity plays a role in cancer\(^4^9\); however, prior to Fullenkamp et al. (2016), large-scale tissue microarrays had not been constructed to determine the extent of TAZ/YAP activation in patient samples. Due to the small number of sarcoma patient samples included in these early studies, the results provided limited insight into the role that TAZ/YAP individually play in a clinical setting. In addition, this technique has also only been used minimally to evaluate Hippo kinase expression, and not concurrently in the same sample subset with TAZ/YAP activation. Hippo kinase expression has been characterized primarily at a protein level by western blot in a small number of primarily rhabdomyosarcoma and leiomyosarcoma cell lines, but for other common sarcoma subtypes, this is a major unknown requiring analysis.

Moreover, the mechanism by which this observed loss occurs is still not fully understood. Whether the primary mechanism driving Hippo pathway dysregulation both in sarcoma cell lines and patient samples occurs because of alterations in TAZ/YAP, or in the kinases themselves, is unknown. Whether dysregulation of Hippo kinases occurs exclusively at a protein level, through epigenetic regulation, at a transcriptional level, or by a
combination/previous unidentified mechanism is also unknown; though studies have shown both promotor hypermethylation, and protein degradation to be plausible explanations within a few cell lines. Such knowledge could have a major impact on the methods currently used to treat sarcoma patients. The extent of therapies has largely been focused on the location of the tumor, rather than the molecular mechanisms governing its growth/survival. Combinatorial approaches, apart from radio/chemotherapy partnered with surgery, have been largely unsuccessful, and understudied. Identification of a therapeutic biomarker opens the door to targeted, personalized treatment with increased efficacy.

In this chapter, the above concerns will be addressed. TAZ/YAP activation as well as MST1/2 expression will be dually studied in 159 patient samples using a tissue microarray, and their correlation to patient outcomes will be analyzed. In sarcoma cell lines, serines in TAZ/YAP targeted by the Hippo pathway will be sequenced for mutations which render them resistant to regulation by Hippo pathway signaling. Fluorescence in-situ hybridization (FISH) studies will be performed to evaluate the presence of genomic amplifications/deletions of the Hippo kinases. Identification of the mechanisms of Hippo dysregulation will also be studied at the protein level in 13 sarcoma cell lines using western blotting, and MG132 experiments. At a transcriptional level, Hippo kinase expression will be surveyed using RT-PCR. Levels of TAZ/YAP targets, such as CTGF, will also be evaluated. At an epigenetic level, promotor hypermethylation will be evaluated using bisulfite-based amplicon sequencing.

Cell lines exhibiting decreased kinase mRNA will subsequently be treated with histone methyltransferase inhibitor 5-Azacytidine and Trichostatin A to see if these therapeutic interventions can restore expression to normal levels.
**Materials and Methods**

**Cell Culture**

13 sarcoma cell lines from the American Type Culture Collection (ATCC) were cultured at 37°C, 5% CO₂ in either DMEM, McCoy’s 5A, or RPMI 1640 (Gibco by Life Technologies, Carlsbad, CA) media with 10% FBS and 1% Penicillin and Streptomycin (Gibco by Life Technologies, Carlsbad, CA) as specified by ATCC guidelines.

**Tissue Micro-Array/Immunohistochemistry**

159 primary human sarcoma tissue samples were obtained from the University of Iowa Department of Pathology. 1.0mm sample cores were formalin-fixed and paraffin embedded, then stained by for TAZ/YAP activation and Hippo kinase loss with the following antibodies: anti- TAZ mouse monoclonal 1H9 at a dilution of 1:50 (Cat no. LSC173295; Lifespan Biosciences, Seattle, WA; USA). Anti-YAP rabbit polyclonal at 1:100 dilution (Cat no. sc-15407; Santa Cruz Biotechnology, Santa Cruz, CA; USA). Anti-MST1 rabbit polyclonal at 1:200 (Abcam, cat no. ab52641), anti-MST2 rabbit polyclonal at 1:2000 (Thermo Scientific, cat no. PAS-17691). Anti-LATS1 (G16) goat polyclonal (Santa Cruz, cat no. sc-12494). Anti-LATS2 rabbit polyclonal 1:100 (Abcam, cat no 70565). Tissue cores were assembled using an MTA-1 tissue arrayer (Beecher Instruments, Sun Prairie, WI; USA), and analyzed by several pathologists using the World Health Organization (WHO) classification criteria. Endothelial cells in blood vessels were used as internal controls, which have been shown previously to express high levels of TAZ/YAP. Staining for Hippo kinases was compared to normal mesenchymal tissue.

**Immunofluorescence**

Cells were either plated sparsely or to confluence, fixed in 4% paraformaldehyde in PBS for 15 minutes, washed in PBS, then permeabilized/blocke'd in 0.3% Triton-X with 3% Fetal Bovine
Serum (FBS) for 30 minutes. TAZ (at 1:200) and YAP (at 1:200) primary antibodies (Cat no. sc-15407; Santa Cruz Biotechnology; Santa Cruz, CA, USA) were used to stain samples overnight, while Alexa Fluor-488 secondary antibodies (Cat no. A11034 and A11029; Life Technologies, Carlsbad, CA) were used at a 1:200 dilution for 1 hour for fluorescence visualization. Vectashield fluorescent mounting media with DAPI was also applied for nuclear staining (Vector Laboratories, Inc.; Burlingame, CA; USA). TAZ/YAP localization was visualized using the Leica DM IL LRD fluorescence scope, DFC3000G camera, and AF600 Modular System Software system (Leica Microsystems, GmbH, Wetzlar, Germany).

**TAZ/YAP Exon Sequencing**

The following primers were designed to sequence exons 2 and 6 of *WWTR1* (transcript variant #2, 400aa), which contain series #66, 89, 117, and S311 targeted by the Hippo pathway, and synthesized by IDT (Integrated DNA Technologies, Iowa City, IA) (*Table 6*). In addition, the following primers (IDT, Iowa City, IA) were designed to sequence exons 1, 2, and 7 in *YAP1* (transcript variant #3, 488aa), which contain Hippo-targeted serines #61, 109, 127, 164, 381 (*Table 6*):
<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Exon 2 Primers (S66, 89, 117):</th>
<th>Exon 6 Primers (S311):</th>
<th>YAP1 Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward: 5’-GCCTAGCTCGTGGCGGAAGAGATCCTGC-3’</td>
<td>Forward: 5’-GCAGCATGGCAACAATGCACCTAG-3’</td>
<td>Exon 1 Primers (S61):</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GCAGTGCCAGCTCGTGGTCAG-3’</td>
<td>Reverse: 5’-CTACCTGTATCCATCTCATCCACATTGCTG-3’</td>
<td>Forward: 5’-CGCCGGGCATCAGATCGTGC -3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse: 5’-GGACGACTCCAGTTCCACCTCGC -3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exon 2 Primers (S109, 127, 164):</td>
</tr>
<tr>
<td></td>
<td>Forward: 5’-GACCCTTGAACCTGCACCCACTGC -3’</td>
<td>Forward: 5’-GTCCTGGTGCCTCCACCTCCCTGC -3’</td>
<td>Forward: 5’-GTCTCTGGTGGGCTACCTGGAG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GTCTTTGCCATCTCCACCTGC -3’</td>
<td>Reverse: 5’-GTCTGATCCATCTCCACACTGTTCCAG-3’</td>
<td>Reverse: 5’-CTGTATCCACTCCACACTGTTCCAG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exon 7 Primers (S381):</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Forward: 5’-GTCTCTTGTGGCACCACCCACCTGGAG-3’</td>
</tr>
</tbody>
</table>

**Table 6: WWTR1/YAP1 Sequencing Primers.**
Sequencing primer design and the exons containing Hippo-targeted serines within each gene.
Table 7: PCR Programs Utilized to Amplify WWTR1 exons 2, 6 and YAP1 exons 1, 2, 7

Gradient PCRs were performed to amplify genomic DNA sequences for exons containing Hippo-targeted serines in WWTR1(TAZ) and YAP1(YAP).

Genomic DNA was isolated from whole-cell lysates using the Quiagen DNeasy Blood and Tissue Kit (Cat no.69504; Quiagen, Germantown, MD). Exons were amplified using PCR, using the following PCR protocol (Table 7) on an Eppendorf Mastercycler Nexus thermocycler (Eppendorf, Hamburg, Germany). Bands at expected molecular weight were extracted and gel-purified using Zymoclean Gel DNA Recovery Kit (cat no. D4008; Zymo Research, Irvine, CA; USA). Yields were quantified using the Nanodrop 2000 system (Thermo Scientific, Waltham, MA), and subsequently sequenced at the Iowa Institute of Human Genetics. Results were analyzed using DNAStar Lasergene 12 Core Suite (DNASTAR, Madison, WI; USA).

**Western Blot**

Cells were grown to 60-80% confluence, harvested using 0.05% Trypsin (Life Technologies, Carlsbad, CA), and lysed using Radioimmunoprecipitation Assay (RIPA) buffer supplemented with Complete Mini EDTA (Roche) and PhosSTOP (Sigma, Cat no. 4906845001), which were prepared according to manufacturer’s guidelines. Following lysis, protein quantification was carried out using Pierce BCA Protein Assay Kit (cat no.23227), and 50ug of each sample was loaded onto the gel (BioRad Mini-PROTEAN TGX Gels 4-15%, cat no. 456-1084). Proteins were transferred to a (PVDF) membrane, blocked in 5% milk, and stained using the following primary antibodies: anti-TAZ(WWTR1) at 1:500 (cat no. HPA007415 ; Sigma-Aldrich, St. Louis, MO), anti-YAP at 1:1000 (D8H1X XP; cat no. 4912; Cell Signaling, Danvers, MA) anti-MST1 at 1:500 (Abcam, cat no. ab52641), anti-MST2 at 1:1000 (Abcam, cat no. 124787), Anti-LATS1 (Santa Cruz, cat no.
Anti-LATS2 (Novus, cat no. NB200-199), and anti-β-Actin at 1:5000 (cat no. a1978; Sigma). Donkey-anti-goat (cat no. 2033), goat-anti-rabbit (cat no. 2054), or goat anti-mouse (cat no. 2055) secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at concentrations of 1:5000 for visualization. Blots were visualized using the BioRad ChemiDoc Touch system (Biorad, Hercules, CA).

**Quantitative Real-Time PCR**

Cells were plated so that they remained 60-80% confluent at time of harvest. Total cellular RNA was collected using TRIzol (Cat no. 15596018; Ambion, Carlsbad, CA), and processed using Purelink DNase Kit (cat no. 12185-010; Invitrogen, Carlsbad, CA) with the PureLink RNA Mini Kit (cat no. 12183018A Invitrogen, Carlsbad, CA). Samples were quantified by Nanodrop, and diluted to 20 ng/µL in H₂O, then loaded as shown below (Table 8) along with Taqman Universal PCR Mastermix (Cat no. 4304437; Applied Biosystems, Foster City, CA) and the appropriate primer/probe pair. The following primer/probe pairs were purchased from Integrated DNA Technologies (Coralville, IA): MST1 (Cat no. Hs.PT.58.20611087); MST2 (Cat no. Hs.PT.58.15648886); LATS2 (Custom order: Probe=5’-56FAM/ACAGGACAG/ZEN/CATGGAGCCCAG, Primer 1=5’-AACTCACAGATTTCGGCCTC-3’, Primer 2= 5’-ACACCGACAGTTAGACACATC-3’); CTGF (Custom order: Probe=5’/56FAM/TGCGAAGCT/ZEN/GACCTGGAAGAGAAC, Primer 1=5’-ACTTCACAGATTTCGGCCTC-3’, Primer 2= 5’-ACACCGACAGTTAGACACATC-3’); CTGF (Custom order: Probe=5’/56FAM/TGCGAAGCT/ZEN/GACCTGGAAGAGAAC, Primer 1=5’-ACTTCACAGATTTCGGCCTC-3’, Primer 2= 5’-ACACCGACAGTTAGACACATC-3’); CYR61 (Cat no. Hs.PT.58.27029250). The following reference gene primers/probes were used as controls, and were also purchased from IDT:. B-actin (Cat no.N001101.1) RPOL-2 (Cat no. Hs.PT.58.25515089). Samples were analyzed using the ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative quantitation was calculated using the standard Delta-delta Cₜ equation and the geometric mean of β-Actin and RPOL-2 Cₜ results as reference control values (Table 9).
### Table 8: Loading Protocol for qRT-PCR

Reagents were added to 20µL per well as described.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted cDNA</td>
<td>5µL</td>
</tr>
<tr>
<td>Primer/Probe pair</td>
<td>1µL</td>
</tr>
<tr>
<td>PCR Mastermix</td>
<td>10µL</td>
</tr>
<tr>
<td>H₂O</td>
<td>4µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20µL</strong></td>
</tr>
</tbody>
</table>

### Calculation

#### Formula used

ΔΔCT = \( \frac{2^{(C_{GOT}-X_{GOT})}}{2^{(C_{RB}-X_{RB})}} \)

\[ C_{RB} = \sqrt{(C_{Bct})(C_{Rct})} \]

\[ X_{RB} = \sqrt{(X_{Bct})(X_{Rct})} \]

### Table 9: Calculation of ΔΔCT

**A.** The ΔΔCT was calculated for each gene in each cell line, using the above standard equation. The geometric mean was utilized to average CT values of β-Actin and RPOL-2 reference genes. C=Untreated control sample, X= Treated sample, GOI=Gene of Interest CT value, RB= Geometric mean of RPOL2 and B-Actin CT values, BCt=Beta-Actin CT value, RCt= RPOL-2 CT value. **B.** Example calculation of MST2 ΔΔCT in RD and HT1080 cells as compared to GCT.
**Soft Agar**

A 0.5% (final concentration) of low melt agarose was used as the bottom layer. The top layer was composed of $5 \times 10^3$ HT1080 or SK-LMS-1 cells suspended in 2mL of 0.35% (final concentration) low-melt agarose in complete growth medium.

**Proliferation Assay**

500-1000 SK-LMS-1 and HT1080 TAZ/YAP knockdown cells and corresponding parental control cells were plated in 96-well plate (100µL per well, 9 replicates per cell line). Proliferation was determined using Dojindo Assay Cell Counting Kit 8 (Dojindo Molecular Technologies, Rockville, MD) and absorbance measured with the BioTek: Synergy h1 Hybrid Reader (Winooski, VT).

**TAZ/YAP Knockdown**

pLKO.1- puromycin constructs were obtained from Sigma-Aldrich (St. Louis, MO, US). Empty vector construct (SHC001), scrambled negative control (SHC002) and TAZ knock-down constructs (Mission shRNA bacterial glycerol stocks; prod no: SHCLNG) TRCN0000319149 (shTAZ#1), TRCN0000319150 (shTAZ#2), TRCN0000319224 (shTAZ#3), TRCN0000370006 (shTAZ#4), TRCN0000370007 (shTAZ#5) were also obtained from Sigma-Aldrich. YAP knock-down constructs were obtained from Addgene: #42540 (shYAP#1), #42541 (shYAP1#2), #27368 (pLKO.1 shYAP#3). To produce lentivirus, pLKO.1 vectors (see below) were transfected (Lipofectamine with PLUS reagent, Invitrogen-Life Technologies) into HEK 293T cells along with pCMV8.12 and pVSVG packaging plasmids. Supernatant was collected at 48 and 72 hrs after transfection, filtered with a 0.45-µm filter (Millipore, US) and supplemented with 8 µg/mL polybrene (Millipore, US). Pooled stable lines were selected for with 1 µg/mL puromycin for two weeks.
**Verteporfin Treatment**

Cells were treated with 10µM Verteporfin (VP) for 12 hours and cultured in soft agar, or analyzed via Dojindo proliferation assay or western blot as described.

**MG132 Treatment**

Cells were treated with MG132 (Sigma, Cat no. C2211) resuspended in DMSO (Fischer, cat no. BP231-100) and diluted in media to a final concentration of 10µM. After 12 hours, cells were harvested and analyzed via western blot.

**5-Azacytidine Treatment**

5-Azacytidine (Sigma, cat no. A1287) was resuspended in 500µL cell culture media, and used at a working concentration of 1-5µM. HT1080, SK-LMS-1, and SW982 cells all received 1µM 5-Aza, while all other cells received 5µM 5-Aza. Cells were treated for 4 days, with media changes and 5-Aza re-application every 24 hours. RNA was collected, processed, and analyzed via RT-PCR as previously described.

**Trichostatin-A Treatment**

Cells were treated with Trichostatin A (Sigma, Cat no. T8552) at a concentration of 0.25µM (0.125µM for SJCRH30 cells due to higher concentrations being toxic). TSA was applied to cells for 12 hours prior to collection. TSA treatment was either in the presence or absence of 1-5µM 5-Azacytidine. Results were compared to untreated controls. Expression of MST1 and MST2 mRNA was analyzed using qRT-PCR as previously described.
Results

Survey of TAZ/YAP Activation in Patient Tumor Samples and Human Sarcoma Cell Lines

To establish the clinical relevance of TAZ/YAP activation, a TMA of 159 patient sarcoma tumors was assembled, and assessed via immunohistochemistry (IHC) for nuclear-localized and thus activated TAZ/YAP. As previously described\(^7\), the majority of patient sample cores contained activated TAZ/YAP. TAZ is noted to be activated in 66% (two-thirds), while YAP is activated in 50% of samples. Interestingly, certain sarcoma subtypes contained a mix of activated TAZ/YAP, while others contained preferential activation of either TAZ or YAP, or neither at all (Table 10). This would suggest that some sarcoma subtypes are more TAZ-dependent, while others are more YAP-dependent. Patient outcomes were correlated with their TAZ/YAP activation, the results of which determined increased TAZ/YAP protein expression and constitutive activation in sarcoma samples negatively correlated with overall patient survival as well as metastasis-free survival (Figure 4A). These findings could also be extrapolated to the relationship between TAZ/YAP expression at a transcriptional level and patient survival. An assembly of TCGA data from DDLPS and UPS adapted from Fullenkamp et al. (2016) showed high WWTR1 (TAZ) and high YAP1 (YAP) gene expression coincided with decreased overall patient survival\(^7\) (Figure 4B).

In order to determine whether the findings in human sarcoma samples were also relevant in a laboratory/in-vitro setting, TAZ/YAP localization in human sarcoma cell lines was determined using immunofluorescence experiments. Results showed TAZ/YAP activation within the SK-LMS-1 leiomyosarcoma line (Figure 5A), and this was also the case for the other sarcoma cell lines analyzed. Activated TAZ/YAP have been demonstrated to play a tumorigenic role in
these cells, and contribute to hallmarks of cancer such as increased proliferation, resistance to anoikis, and anchorage-independent growth\textsuperscript{74, 60}. Utilizing shRNA methods, both TAZ and YAP were separately knocked down in SK-LMS-1 cells, and their contributions to tumorigenesis assessed (Figure 5B). Knockdown of both TAZ/YAP significantly decreased hallmarks of cancer such as proliferation, as determined by MTT assays (Figure 5C). However, only knockdown of TAZ decreased anchorage-independent growth, while knockdown of YAP minimally affected the cells’ ability to grow, as shown by decreased colony formation in soft agar compared to controls (Figure 5D). This suggests that TAZ plays a role in resistance to anoikis in SK-LMS-1, yet YAP does not. This experimental finding again supports the hypothesis that TAZ/YAP initiate similar, but not identical transcriptional programs.

**Effects of Therapeutically Targeting the TAZ/YAP-TEAD Interaction in Sarcomas**

Not only were TAZ/YAP shown to play a role in sarcomagenesis, but therapeutically targeting TAZ/YAP in sarcoma cells was shown to successfully reverse their effects on growth and proliferation as well (Figure 6A-B). Treatment of SK-LMS-1 and HT1080 cells containing activated TAZ/YAP with verteporfin (VP) significantly decreased colony formation in soft-agar in a dose-dependent manner. Treatment with 10µM VP was also shown to specifically disrupt the interaction of TAZ/YAP and TEAD, due to its ability to cause a decrease in the transcription of CTGF, a primary TAZ/YAP target (Figure 6C). Again, these observed effects were more prominent in HT1080 rather than SK-LMS-1, suggesting that SK-LMS-1 is TAZ-dependent, and perhaps CTGF is the primary transcriptional target of YAP, but not TAZ. Therefore, we repeated the experiment, this time looking for changes in CYR61 expression- a possible downstream target of TAZ activity (Figure 6D). Results indicated a modest dose-dependent decrease in CYR61 expression with VP addition, indicating that it is likely a target of TAZ in SK-LMS-1 and that the TAZ-TEAD interaction is indeed disrupted by VP.
The effectiveness of VP in reducing outcomes of growth and transcription of TAZ/YAP targets CTGF and CYR61 in-vitro introduces the possibility for use of this drug in sarcoma patients in the future. However, extensive experimentation is still necessary, as there are several practical considerations which must be taken into account when using VP to treat sarcomas in-vivo. Foremost, VP has yet to be tested and approved for clinical use in sarcomas. VP was originally FDA-approved for use in photodynamic therapy to treat macular degeneration\textsuperscript{79, 80}, but its use in-vivo, particularly in treating sarcomas, has been limited. Nevertheless, results have been positive, in that VP has been show to successfully hinder tumor growth in prostate cancer xenografts\textsuperscript{81} and uveal melanoma xenografts\textsuperscript{82}, as well as hepatomegaly in mice with YAP overexpression or NF2 knockout\textsuperscript{61, 83}. Of concern are also issues of toxicity and off-target effects. VP was discovered to inhibit growth of breast, liver, bone and cervical cancer cell lines, even the absence of YAP, TAZ, or both, which suggests a function independent of YAP-TEAD inhibition\textsuperscript{84}. When further investigated in colorectal cancer (CRC) cells and an in-vivo CRC model, oligomerization of signaling proteins by non-enzymatic cross-linking was determined to be the mechanistic explanation\textsuperscript{84}. Still, treatment was somewhat specific, as non-cancerous cells were able to clear these oligomers, yet neoplastic cells could not\textsuperscript{84}.

**Mechanistic Studies- Investigating Serine Mutation as Cause for TAZ/YAP Activation**

In an in-vitro setting, it has been shown that mutation of serines targeted by the Hippo pathway to alanine leads to deregulation of TAZ/YAP, as well as their constitutive activation in cancer\textsuperscript{85}. Therefore, we initially hypothesized that TAZ/YAP activation may be due to their inability to respond to Hippo pathway signaling. To determine the mechanisms causing constitutively activated TAZ and YAP, mutational analysis was conducted on serines in TAZ/YAP which are targeted by Hippo kinases in sarcoma cell lines. Sequencing of Hippo-targeted serines
in both TAZ (WWTR1) and YAP (YAP1) via exon sequencing in sarcoma cell lines and breast cancer lines revealed that neither TAZ nor YAP contained mutations (Table 11). This suggests TAZ/YAP still retain the ability to be negatively regulated by upstream Hippo signaling in sarcomas. As such, we next investigated whether constitutive activation of TAZ/YAP could be due to deregulation of the Hippo kinases themselves.

**Hippo Kinase Expression in Patient Tumor Samples and Human Sarcoma Cell Lines**

We first focused on assessing the extent of decreased Hippo kinase expression within patient samples using our previous TMA, and staining for MST1/2 and LATS 1/2 (Figure 7). In this way, we were able to correlate patient outcomes and TAZ/YAP activation with diminished Hippo kinase expression. The results of these experiments demonstrate that decreased Hippo kinase expression occurs in a significant portion of sarcomas (Table 12). Overall, MST1 loss occurred in 47% (71/150) samples, while MST2 loss was observed in 31% of samples. The overlap of both decreased MST1 and MST2 occurred in 20% of samples. Analysis of LATS1 expression showed 22% (34/153) of samples contained decreased expression, and 17% (26/157) of samples had decreased LATS2.

Going one step further, the percentage loss of Hippo kinase expression was next identified in samples without TAZ/YAP activation (negative), and those which had activated TAZ, YAP, or both (positive) (Table 13). Of 177 tumor cores, 143/177 (81%) samples stained positively for activated (nuclear localized) TAZ/YAP, and 34/177 (19%) were negative for staining of either protein. MST1/2 were significantly decreased in TAZ/YAP positive tumors, with MST1 loss occurring in 52% (75/143), and MST2 loss in 25% (36/143). LATS1/2 were also lost in a significant portion of TAZ/YAP positive tumors, 27% (38/143) for LATS1, and 20% (28/143) for LATS2. Notwithstanding, the relationship between activation and kinase loss was not always clear-cut. For example, there was also decreased kinase expression in the 34 tumors negative
for TAZ/YAP activation, with loss of MST1 occurring in 52%, MST2 in 50%, LATS1 24%, and LATS2 in 15%. With these results confirming TAZ/YAP activation occurs simultaneously with loss of Hippo kinase expression in patient tumors, we next analyzed the prevalence of Hippo dysregulation within human sarcoma cell lines \textit{in-vitro}.

For cell-based \textit{in-vitro} experiments, GCT was utilized as the control cell line due to previous results (Figure 8A) showing that it had similar protein expression of MST1/2 and TAZ/YAP as compared to immortalized, non-transformed cell line MCF10a. MCF10a is widely considered the gold standard of control cell lines in human cancer cell studies, and had also been used in numerous prior studies as a control, though its applicability as an epithelial line to cancers of mesenchymal origin (such as sarcomas) was a major shortcoming.

When Hippo kinase expression was analyzed \textit{in-vitro} at a protein level via western blot, a majority of sarcoma cell lines exhibited decreased Hippo kinase expression, with exception of LATS1. For instance, 9/12 cell lines showed a decrease in MST1 expression, while 7/12 cell lines exhibited decreased MST2 expression, 11/12 had decreased LATS2, and LATS1 was decreased in 3/12 (Figure 8B). When quantified by comparing specific kinase expression:Ponceau-S stained total protein, and using a threshold of 2-fold decrease, actual numbers of cell lines containing decreased kinases were as follows: MST1=7/12, MST2=5/12, LATS2=7/12, LATS1=1/12 (Figure 8C-D).

Thirteen sarcoma cell lines were analyzed for their kinase expression using qRT-PCR to observe whether the results of decreased MST1/2 were also present at an mRNA level. The reference genes used were β-Actin and RPol2, and were chosen because their expression had the least variability across a majority of sarcoma cell lines (Figure 9A). The $\Delta\Delta C_T$ was calculated using a standard equation, with the geometric mean representing the average expression of both reference genes (Table 9A-B). Using a 2-fold threshold as a cutoff point, 50% of cell lines
exhibited significantly decreased MST1, while 58% of cells had reduced MST2 expression (Figure 9B). LATS1 expression was not substantially decreased in a majority of cells (17%) (Figure 9C). Interestingly, LATS2 was not decreased at an RNA level, suggesting protein degradation is the primary regulatory mechanism which accounts for its observed loss at a protein level by western blot (Figure 8D). Considering the results for MST1 and MST2; however, we next wondered if epigenetic mechanisms such as promoter hypermethylation or histone deacetylation play a role in mRNA expression, or if proteasomal degradation could account for the observed decreases in protein expression.

**Mechanistic Studies- Investigating Dysregulation of MST1 and MST2 Hippo Kinases**

In order to ascertain the mechanisms causing decreased MST/LATS protein expression in both patient tumors and human cell lines, we first investigated whether degradation could account for the observed depletion in MST1/2 and LATS1/2 within a majority of sarcoma cell lines. Therefore, our first experiment utilized MG132 to inhibit proteasomal degradation, and western blotting to analyze effects on MST1/2, and *LATS1/2 expression (Figure 10A-B. *Data for LATS2 not shown- confirmation of antibody specificity is necessary). If degradation were a major mechanism of decreased Hippo kinase expression, we would expect a significant increase in protein accumulation with 10μM MG132 treatment; however, this was not observed in any lines for MST1, or LATS1 kinases in sarcoma lines. LATS2 expression was also tested as with MST1/2 and LATS1, although confirmation of the specificity of anti-LATS2 antibody used is necessary to further analyze the results. MST2, on the other hand, exhibited several cell lines which had protein accumulation with MG132 treatment, including: SK-LMS-1, RD, SW982, A204, Saos-2, SNF02.2, SNF96.2, SJCRH30, and SW872 (Figure 10A). However, quantitation showed only SK-LMS-1, Saos-2, and SJCRH30 lines had a definitive increase (2-fold) in protein with
treatment as compared to DMSO controls (Figure 10B); suggesting that proteasomal degradation is the primary mechanism of decreased protein expression in these cells.

With the loss of Hippo kinase expression at a transcriptional level, we next investigated if epigenetic mechanisms such as promoter hypermethylation and histone deacetylation could account for these results. In terms of the individual contributions of promoter hypermethylation to Hippo kinase expression, TCGA data showed a small, but notable negative correlation ($r=-0.31$) between promoter hypermethylation and expression of MST2 kinase (Figure 11A). As we postulated promoter hypermethylation could account for the decreased transcriptional expression of MST1/2 in sarcoma cell lines, we treated cells with concentrations of 1-10μM DNA-methyl transferase inhibitor 5-Azacytidine, and analyzed kinase expression in treated vs. untreated cells via qRT-PCR. 1μM 5-Aza was used for HT1080, SKLMS, and SW982, as 5-Azacytidine was shown to be toxic to these lines at higher concentrations; all other cells were treated with 10μM 5-Aza. Interestingly, 5-Azacytidine treatment increased MST1 and MST2 kinase expression in most lines. However, using a 2-fold increase as the cutoff for significance, results determined only RD (1/12) showed restored MST1 (Figure 12A), and only RD and SW872 cells (2/12) had restored MST2 expression (Figure 13A). Comparing protein and total mRNA results with these findings, DNA hypermethylation was discovered to predominantly contribute to decreased MST1 and MST2 in RD cells (Table 14). To establish whether 5-Azacytidine treatment is also able to rescue decreased MST1/2 protein expression, and at levels comparable to mRNA results, A204 and RD cells were treated with 5μM and 10μM concentrations of 5-Aza, then analyzed by western blot (Figure 14). Experimental results indicated 5μM concentrations of 5-Azacytidine did not increase either MST1 or MST2 protein expression in either cell line. Unexpectedly, however, the 10μM concentration was sufficient to increase MST1 expression in both cell lines (Figure 14).
Despite these findings, the majority of reduced MST1/2 in sarcoma cell lines could not be fully explained by either promoter hypermethylation or protein degradation alone; although these kinases are significantly diminished in a majority of the analyzed cells at both a transcriptional and translational level. This indicates there are likely other epigenetic mechanisms which regulate Hippo kinase expression, especially in the case of cells with decreased protein/mRNA kinase expression that was less than 2-fold, or that could not be increased 2-fold with the administration of 5-Azacytidine (5-Aza) alone. One alternative mechanism of regulation we chose to probe was histone deacetylation. Cells were treated individually with histone deacetylase inhibitor (HDAC inhibitor) Trichostatin A (TSA) at 0.125µM for SJCRH30, and 0.25µM for all other lines to confirm the presence and potential contribution of histone deacetylation. Analysis using qRT-PCR revealed HDAC activity could account for decreased MST1/2 levels, and that deacetylase activity was inhibited in 8/12 lines; as TSA treatment amplified MST1/2 expression at least 2-fold in treated cells versus untreated (Figure 12B, Figure 13B).

The previous results present a strong argument for the individual role of histone deacetylation in decreasing MST1/2 expression. However, promoter hypermethylation was also present for MST1/2 in lines which contained histone deacetylation; therefore, we next investigated the possibility that both hypermethylation and deacetylation are mechanisms which coexist and decrease Hippo kinase expression. Findings by Jones et al. (1998) indicated that histone deacetylation can also interfere with DNA methyltransferase inhibitor activity (such as 5-Aza), thus masking the overall effects of these drugs. The recruitment of histone deacetylase by MeCP2-bound DNMT1, and their formation of a transcriptional repressor complex at methylated residues provides a mechanistic explanation for the reduced efficacy of DNMT inhibitors in the presence of histone deacetylation (Figure 11B). Work by others further...
validated these results, and showed that combined HDAC inhibitor + DNMT inhibitor treatment could increase previously downregulated tumor suppressor expression more than each drug individually\textsuperscript{87,88}.

To exclude the possibility of underestimating the contributions of methylation, and to determine if the reason why increased MST1/2 expression with 5-Aza was not always at or above the 2-fold threshold is due to interference by histone deacetylation, we treated cells individually with either 0.25µM histone deacetylase Trichostatin-A (TSA), or 1-5µM DNA methyltransferase 5-Aza, or with both therapies combined (Figure 12C, Figure 13C). 1-5µM 5-Aza concentrations were used here, rather than the previously used 1-10µM concentrations, as combination of 10µM with 0.25µM TSA proved to be cytotoxic (data not shown). Results indicated that both TSA+5-Aza synergistically increased MST1 expression in RD cells, as well as MST2 in A204 cells more than each drug individually. This result implies histone deacetylation and methylation interference was present and inhibiting the full therapeutic effect of each drug in these particular cell lines. RD and A204 cells were previously shown to have significantly decreased Hippo kinase expression at an RNA level, which was increased at least 2-fold with individual 5-Azacytidine or Trichostatin A treatment; yet the findings of TSA + 5-Aza experiments provide further evidence supporting studies which show histone acetylation can mask the effects of 5-Aza treatment\textsuperscript{88}. Taken together, our results demonstrate several mechanisms which regulate Hippo kinase expression in sarcoma cell lines; which specific mechanism is responsible for decreased MST1/2 at a protein or mRNA level is largely cell-line/context dependent.
Discussion

Though TAZ, YAP, and the Hippo pathway have previously been shown to play a role in several types of cancer, the mechanisms by which they contribute to tumorigenesis, particularly in sarcomas, was not well-delineated. Furthermore, parallels between TAZ/YAP activation and Hippo kinase loss were lacking, particularly within the context of patient outcomes.

Patient Tumor Evaluation: TAZ/YAP Localization, Kinase Expression, and Study

Innovation

Our studies have demonstrated the constitutive activation of TAZ/YAP in a majority of sarcomas, with YAP being activated in 50% of sarcoma samples, and TAZ activated in 66%. Interestingly, TAZ and YAP have somewhat independent activation status within the same sarcoma subtypes, that is, their activation in certain tumor subtypes varies from one-another. For instance, the myxoid/round cell liposarcoma subtype contains only activated YAP (78%), and no activation of TAZ (0%), which suggests a potential reliance of this subtype on the transcriptional activity of YAP instead of TAZ. This also suggests specific roles for each transcriptional coactivator, and that each carry out separate transcriptional programs.

In addition to TAZ/YAP activation, a novel component of our research is the inclusion of all four core Hippo kinases in our TMA expression analysis, using the same subset of tumors. The extent of decreased MST1/2 expression in sarcoma tumors has not, to our knowledge, been previously identified. Moreover, antibodies and primers used within our TMA and cell-based assays were specifically selected because they recognize catalytically active MST1/2 (NM_006282.2/NM_006281) and LATS1/2 (NM_004690.3/NM_014572). This is a consideration which has not been addressed in previous studies evaluating Hippo kinase expression levels; a potential outcome of which could be skewed results which do not accurately represent the
expression of the kinase isoforms that regulate TAZ/YAP in cancer. In addition to activated TAZ/YAP, patient tumor samples contain significantly decreased MST1 (47%), MST2 (30%), LATS1 (22%) and LATS2 (17%) protein expression.

**Hippo Pathway Dysregulation in the Presence and Absence of TAZ and YAP Activation**

Following analysis of overall Hippo kinase expression in each subtype, loss of expression was then evaluated in samples either with or without TAZ/YAP activation. Results denoted either TAZ, YAP, or both were activated in a majority of tumors, and were accompanied by loss of expression of at least one Hippo kinase. This provides a justification for further study of Hippo pathway dysregulation in sarcomas, because it suggests decreased Hippo kinase expression exclusively leads to nuclear localization of TAZ/YAP. Such was the case in undifferentiated pleomorphic sarcoma, which also did not have loss of Hippo expression in TAZ/YAP negative samples.

Remarkably, loss of MST1/2 and LATS1/2 also occurred in a number of tumors lacking TAZ/YAP activation, including: chondrosarcoma (grade 2), high-grade osteosarcoma, clear cell sarcoma, and myxoid/round cell liposarcoma. This outcome indicates TAZ/YAP activation may not be a requirement for sarcomagenesis in a small subset of sarcomas, but loss of kinase expression may. Alternative pathways regulating TAZ/YAP phosphorylation can offer a rationalization for these observations.

In sarcomas lacking TAZ/YAP activation and with loss of Hippo kinase expression, negative regulation of TAZ/YAP may be due to MAP4K, which functions in the same way as MST1/2 to phosphorylate LATS1/2, and inhibit TAZ/YAP. GSK3β may also partially account for the noted results through phosphorylation of the N-terminal phosphodegron in TAZ, causing its cytoplasmic sequestration and destruction. A third possibility could be Gs–receptor mediated inhibition of TAZ/YAP by phosphorylation of LATS, though this would not be a likely explanation.
in subtypes with decreased LATS1/2 expression\textsuperscript{41}. Yet another contributing factor could be mechanotransduction and CRUMBS complex activity, which phosphorylates TAZ/YAP in response to cell density, and independently of Hippo signaling\textsuperscript{45}.

One finding of interest was that total loss of MST2 occurred in a greater proportion of TAZ/YAP negative samples (50%), versus TAZ/YAP positive samples (25%). Otherwise, the proportions of decreased expression remained relatively constant between groups: MST1 (~52%), LATS1 (~26%), and LATS2 (~17%). Although there may not be a single method of alternative regulation which is responsible for the observed results, the previously listed signaling pathways could potentially play a role.

An outstanding question in the field of Hippo pathway research is whether loss of only one pathway component is necessary for dysregulation and resulting TAZ/YAP activation, or if loss of multiple kinases is required instead. Based on the observed outcomes of the TMA immunohistochemistry, it appears that the answer is largely dependent on the sarcoma subtype, grade, and patient/tumor variation. For instance, in pleomorphic liposarcoma, the sole TAZ/YAP negative sample had loss of only 1 kinase- MST1. Taking this into consideration, it could be postulated that loss of one kinase is not sufficient to activate TAZ/YAP; however, sole loss of MST1 also occurred in 17% of TAZ/YAP positive tumors of this subtype. Moreover, some subtypes also contained loss of more than one kinase, which did not contain activated TAZ/YAP; two examples are adult-type rhabdomyosarcoma and well-differentiated liposarcoma. In a majority of samples, loss of more than one kinase often occurred in sarcomas with activated TAZ/YAP, which suggests loss of a single kinase is not sufficient to cause TAZ/YAP activation; however further study and data analysis is required. In sum, delineating the precise mechanisms of sarcomagenesis, and the relationship between Hippo dysregulation and TAZ/YAP activation, is crucial for understanding the complex regulatory mechanisms involved in sarcoma development.
activation is complicated; in most cases, the implication stands that there are other interacting factors which influence the degree of TAZ/YAP activation and loss of kinase expression.

**Sarcoma Cell Lines: Post-Translational Modifications in Hippo Pathway Deregulation**

In support of the TMA findings, there was significant loss of MST1/2 and LATS2 protein expression in sarcoma lines, though loss of LATS1 was negligible. Although decreased kinase expression is not as pronounced in TMA samples as with sarcoma cell lines, these observations could be explained in-part by the fact that the TMA is a heterogenous mix of samples. Because of this, some tumor subtypes contain a majority of TAZ/YAP activation accompanied by significantly decreased kinase expression, while others have minimal, if any TAZ/YAP activation or loss of Hippo expression. This leads to a lower average loss of kinase expression and TAZ/YAP activation than in cell lines, which, as confirmed by our prior IF results, all show activated TAZ/YAP as a result of decreased kinase expression in a majority of samples (Table 10, Figure 5A). It is also important to remember that the number of patient tumor samples far exceeds the number of available sarcoma cell lines; this could contribute to the observed disparities.

Proteasomal degradation was also investigated as a potential means by which protein expression of Hippo kinases was decreased. As expected, results indicated MG132 treatment did not affect LATS1 or MST1 expression; however, MST2 accumulation with treatment occurred in 9 lines. This was surprising, as Ho et al. (2011) demonstrated ITCH-ligase activity in HEK cells which accounted for LATS1 protein degradation\(^8^9\). One possible explanation for this disparity could be that their study utilized only one cell line, whereas we assessed 13 lines. Another explanation could be the difference between the cell lines analyzed- Ho et al. evaluated degradation in an epithelial line, and our cells are mesenchymal/sarcomas\(^8^9\). Nevertheless, not all decreases in protein expression could be explained solely by degradation; therefore, we further investigated translational expression as well. The mRNA results obtained mirrored the
protein findings for MST1/2, though not LATS2, which did not have decreased mRNA expression. This suggests decreased LATS2 protein expression is due to post-translational mechanisms of regulation, such as degradation by the proteasome. Another explanation for the increased LATS2 mRNA results could be the observations of Dai et al. (2015) and Moroishi et al. (2015), which show LATS2 is part of a negative feedback loop, where increased YAP expression and activation significantly upregulates LATS2 transcription.85,90.

The Contributions of Epigenetic Regulation to Hippo Expression in-vitro

TCGA data reported by Eisinger-Mathason et al. (2015) showed that of 261 soft-tissue sarcomas within the dataset, 24% contained copy number variations (CNV) of Hippo pathway components (of which 68% were LATS2, 34% SAV1, and 24% NF2). However, the majority (76%) of sarcomas did not have CNV, suggesting other mechanisms which contribute to dysregulation of Hippo kinases.2 Though this was not investigated in the previous study, two such mechanisms identified here are epigenetic repression by promoter hypermethylation and histone acetylation.

Our findings demonstrated decreased MST1/2 protein and RNA expression in a number of cell lines could be explained in part through promoter hypermethylation. These conclusions were congruent with work by Seidel et al. (2007) showing promoter hypermethylation was present in MST1 (37%) and MST2 (20%) in soft tissue sarcomas, and at least a 50% (3/6) decrease in MST1 mRNA expression in primary sarcomas. Our experimental results did not indicate that hypermethylation of LATS1/2 is a contributing factor to decreased protein expression in cell lines, nor did a majority of sarcoma tumor samples contain decreased LATS expression. Similar to our findings, the Seidel study (2007) determined LATS1 had minimal hypermethylation (7%; 3/43 samples) and LATS2 (0/41) was not hypermethylated at all.67 This was in contrast to other studies which indicated LATS1 was hypermethylated in 6/7 patient
tumors\textsuperscript{68}, and that promoter hypermethylation of LATS1/2 occurred in 56.7% and 50.0% of breast cancer tumor samples\textsuperscript{65}.

TCGA data analyzing 261 soft-tissue sarcomas indicated a modest negative correlation between MST2 promoter hypermethylation and MST2 mRNA expression (r= -0.31). The results indicated two distinct populations within the data set: one with high CpG island methylation and low MST2 expression, and another with low CpG methylation and low MST2 expression. One possible interpretation of this is that promoter hypermethylation plays a significant role in regulating MST2 expression in the CpG\textsuperscript{Hi} MST2\textsuperscript{Lo} group, though not in the CpG\textsuperscript{Lo} MST2\textsuperscript{Lo} group. However, when tested for significance using a Chi-square analysis, the differences between the two groups were not significant ($\chi^2=12.939$, data not shown). Results of this analysis indicate that although promoter hypermethylation of MST2 overall can contribute to decreased MST2, there are also other contributing factors which affect Hippo kinase expression.

One such factor tested was histone deacetylation, as deacetylation alone and the interaction of histone deacetylase with DNA methyltransferase is shown to decrease gene transcription\textsuperscript{86}. Indeed, treatment of cells with histone deacetylase inhibitor Trichostatin A (TSA) alone greatly increased MST1 and MST2 mRNA levels in a majority of cell lines by at least 2-fold, suggesting a prominent role in decreased Hippo kinase expression. Interestingly, however, we found that only two cell lines displayed significantly increased expression with combined 5-Aza + TSA treatment: RD for MST1, and A204 for MST2. This would imply HDACs do not interfere with methylation/DNMT inhibitor activity in the majority of sarcoma cells analyzed. It should be considered; however, that the results only give a snapshot of mRNA expression when the global methylation/acetylation status within cells is changed- whether HDACs inhibit certain methylated residues present in sarcomas is not reflected in these results. Therefore, further experimentation is required- not only genome sequencing to determine copy number changes,
but also ChIP/RNA-seq to determine which specific residues are altered and contribute to MST2 suppression, and whether other epigenetic alterations are present which would affect gene expression.
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Table 10: TAZ and YAP are Activated in a Majority of Patient Tumor Samples

TAZ and YAP activation (nuclear localization) in 159 sarcoma samples representing the 17 major sarcoma subtypes. YAP is activated in half (50%) of samples, while TAZ is activated in two-thirds (66%). Adapted from Fullenkamp C.A., et al. TAZ and YAP are frequently activated oncoproteins in sarcomas (2016)\textsuperscript{74}. 
Figure 4: TAZ/YAP Activation Leads to Decreased Survival

A. In all sarcomas, overall survival and metastasis-free survival were significantly decreased in patients with both activated TAZ and YAP (TAZ+YAP+) versus neither and TAZ or YAP alone. B. Kaplan-Meyer curves compare overall patient survival with both high and low WWTR1 (TAZ) and YAP1 (YAP) gene expression in UPS and DDLPS sarcomas. High expression of both TAZ (p=0.04) and YAP (p=0.03) significantly correlates with decreased outcomes. Adapted from Fullenkamp C.A., et al. TAZ and YAP are frequently activated oncoproteins in sarcomas (2016).
Figure 5: YAP is Activated in SK-LMS-1 Cells and T/Y Knockdown Reduces Cell Growth and Proliferation. 

A. Immunofluorescence of nuclei (DAPI) and YAP1 (YAP) staining show an overlap when cells are grown to confluence. 

B. Knockdown of TAZ and YAP in SK-LMS-1 visualized by western blot. 

C. MTT assay comparing proliferation rates of shRNA TAZ/YAP knockdowns to empty vector (EV) and non-targeting (NT) controls. TAZ and YAP KD significantly decreased proliferation. P-value(<0.0001) represents difference in fold-change proliferation at day 4 between non-targeting shRNA (shNT) and shTAZ/YAP#1-2. 

D. Anchorage-independent growth of SK-LMS-1 TAZ/YAP KD cells in soft agar demonstrates KD of TAZ significantly inhibits SK-LMS-1 colony formation, while YAP KD does not. Adapted from Fullenkamp C.A., et al. TAZ and YAP are frequently activated oncoproteins in sarcomas (2016).
Figure 6: Verteporfin Inhibits TAZ/YAP Growth in Soft Agar and Transcriptional Output Through Disruption of TAZ/YAP-TEAD Interaction. A. Soft Agar for HT1080 (shown) and SK-LMS-1 cells (not shown) demonstrates VP inhibition of colony formation in a dose-dependent manner. B. Soft agar colony counts at increasing VP concentrations in HT1080 and SK-LMS-1 cells. C. qRT-PCR assessment of CTGF expression with VP treatment shows decreased expression of TAZ/YAP transcriptional target CTGF; however, this decrease is minimal in SK-LMS-1 cells. D. CYR61 mRNA expression decreases with increasing VP concentration in SK-LMS-1 and HT1080 cells. Adapted from Fullenkamp C.A., et al. TAZ and YAP are frequently activated oncoproteins in sarcomas (2016) 74.
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<tr>
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<td>6</td>
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<tr>
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<table>
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Table 11: Sequencing of MST/LATS-targeted Serines did not Identify Mutations
Exon sequencing of Hippo pathway-targeted serines in TAZ and YAP revealed no mutations which would leave them unresponsive to Hippo regulation in either RD or SK-LMS-1 sarcoma cell lines.
Figure 7: Staining for Hippo Kinase Expression in Sarcoma Patient Tumors

Immunohistochemistry (IHC) of sarcoma patient tumor samples was utilized to determine the percentage of Hippo kinase loss at a protein level. Staining of tumor cores was performed in duplicate and compared to normal mesenchymal tissue. Adapted from Fullenkamp C.A., et al. TAZ and YAP are frequently activated oncoproteins in sarcomas (2016)\textsuperscript{74}.
Table 12: Quantifying Decreased Hippo Kinase Expression in Sarcoma Patient Tumors

Results of TMA staining for nuclear-localized TAZ and YAP again shows significant activation of both (~64%) and (~48%) respectively, with co-activation of TAZ and YAP in ~34% of sarcomas. Staining with anti-MST or anti-LATS antibodies indicates loss of MST1 (47%) and MST2 (31%) occurs in a significant portion of sarcomas, with co-loss occurring in 20% of samples. Loss of LATS1 (22%) and LATS2 (17%) also occurs, though to a lesser extent.

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<th>YAP activated</th>
<th>TAZ/YAP co-activated</th>
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<th>MST2 lost</th>
<th>MST1/MST2 lost</th>
<th>LATS1 lost</th>
<th>LATS2 lost</th>
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<td>0/3 (0%)</td>
<td>2/3 (66%)</td>
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<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
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<td>3/10 (30%)</td>
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<td><strong>30/150</strong></td>
<td><strong>34/153</strong></td>
<td><strong>26/157</strong></td>
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</table>

(64%) and (48%) respectively, with co-activation of TAZ and YAP in 34% of sarcomas. Staining with anti-MST or anti-LATS antibodies indicates loss of MST1 (47%) and MST2 (31%) occurs in a significant portion of sarcomas, with co-loss occurring in 20% of samples. Loss of LATS1 (22%) and LATS2 (17%) also occurs, though to a lesser extent.
Table 13: Hippo Kinase Expression as a Function of TAZ/YAP Activation Status

Table summary of TMA results evaluating Hippo kinase expression in samples with and without activated TAZ/YAP. The majority of sarcomas stained positive for nuclear YAP, TAZ, or both. Most of the TAZ/YAP positive sarcomas also had significant loss of at least one Hippo kinase. Nevertheless, a small portion of TAZ/YAP negative subtypes, such as Well-differentiated liposarcoma and Clear cell sarcoma, also contained loss of Hippo kinases. This finding suggests T/Y activation is not the primary driver of sarcomagenesis in these subtypes, and highlights potential Hippo tumor suppressor activity on other oncogenic targets.

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<th>T/Y Activation</th>
<th>Proportion of Samples</th>
<th>Loss of MST1</th>
<th>Loss of MST2</th>
<th>Loss of LATS1</th>
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Figure 8: Quantification of MST1/2 and LATS1/2 Expression at a Protein Level in Sarcoma Cell Lines

A. Determining a cell-based mesenchymal control to compare decreased kinase expression in cell lines (GCT: MCF10a and several sarcoma cell lines).

B. Expression of Hippo kinases MST1/2, and LATS1/2 was determined at a protein level by western blot.

C. Quantification of MST1/2 western blot results, using a 2-fold decrease as the threshold for significance.

D. Quantification of LATS1/2 western results, using a 2-fold decrease as the threshold for significance.
Figure 9: Evaluation of MST1/2 Transcriptional Expression by Quantitative RT-PCR

A. RPOL2 and B-Actin reference genes were selected as they had the least variability in expression for the majority of sarcoma cell lines.

B. MST1/2 mRNA expression showed a 50% decrease in MST1, and 58% decrease in MST2, using a 2-fold decrease as the threshold for significance.

C. LATS1 expression was decreased in 17% of cell lines, and LATS2 showed no decrease. A 2-fold threshold was used to determine significance.
**Figure 10: Proteasomal Degradation Partially Explains Loss of MST2 in Sarcoma Cell Lines**

**A.** Addition of 10µM MG132 vs DMSO-treated controls. Protein accumulation visualized by western blot with MG132 addition signifies blocking of degradation by the proteasome. *Note: Data for LATS2 not shown- confirmation of antibody specificity is necessary.

**B.** Quantification of MST2 expression in 10µM MG132 treated cells vs DMSO-treated controls. A 2-fold increase was used as the threshold for significance, with 3/12 cell lines (SK-LMS-1, Saos-2, SJCRH30) showing significant protein accumulation.
Figure 11: Promoter Hypermethylation and Histone Deacetylation in Hippo Pathway Silencing
A. TCGA data correlating CpG promoter hypermethylation to MST2 mRNA expression, r=-0.31.
B. Schematic illustration showing the interaction of methylation and deacetylation through the DNMT, HDAC, MeCP2 complex. Transcriptional repressor MeCP2 binds methylated CpG islands, and subsequently recruits Histone deacetylase (HDAC) to inhibit gene expression.
Figure 12: Individual and Combined Contributions of Hypermethylation and Deacetylation to MST1 Expression

A. Individual treatment with 10µM 5-Azacytidine only increased MST1 expression by 2-fold in RD cells.  
B. TSA treatment alone using 0.125-0.25µM concentration significantly increased MST1 expression (at least 2-fold) in 8/12 cell lines.  
C. MST1 mRNA expression in 0.125-0.25µM TSA only-treated cells (blue bars), 5µM 5-Azacytidine-only-treated cells (gray bars), and cells treated with a combination of 0.125-0.25µM TSA+ 1-5µM 5-Aza (black bars).
Figure 13: Assessment of the Contributions of DNA Hypermethylation and Histone Deacetylation to MST2 Expression

A. A 2-fold increase in MST2 expression was observed in 2/12 cell lines with 10µM 5-Azacytidine treatment

B. Treatment of cells with 0.125-0.25µM TSA alone increased MST2 expression in a majority of cell lines (8/12) by at least 2-fold.

C. MST2 mRNA expression in TSA only-treated cells (blue bars), 5-Azacytidine-only-treated cells (gray bars), and cells treated with a combination of 0.125-0.25µM TSA+ 1-5µM 5-Aza (black bars).
Figure 14: Effects of DNA Methyltransferase Inhibitor Treatment on MST1/2 Protein Expression

Western blot correlating protein response with 5μM and 10μM 5-Aza treatment in RD and A204 cells. While 5μM 5-Aza treatment did not significantly increase protein expression of MST1 or MST2, cells treated with 10μM 5-Aza had significantly increased MST1 protein.
Table 14: Hippo Kinase Protein and mRNA Expression and Mechanism of Loss in Sarcoma Cell Lines

2-fold decreases in MST1/2 protein and mRNA expression occurred in a majority of cell lines. Presence of hypermethylation or deacetylation based on individual 10µM 5-Azacytidine treatment and individual 0.25µM Trichostatin A treatment data. Clear associations between promoter hypermethylation, histone acetylation, and loss of MST1/2 occurred in RD, and protein degradation for MST2 in SK-LMS-1. Histone deacetylation appeared to be the primary contributor to decreased expression for MST1 in SK-LMS-1, HT1080, A204 and SW982 lines and MST2 in SW684, HT1080 and A204 lines. Protein or mRNA loss could not be attributed to any of the three mechanisms investigated for all kinases in cell lines, indicating other mechanisms may lead to dysregulation of the Hippo pathway.

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CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Sarcomas are mesenchymal cancers which represent 1% of all cancers, and 15% of cancers in children\(^1\). However, the low 5-year survival rates of those affected by metastatic disease (~16%) as well as the limited availability of effective treatments makes their study an absolute necessity\(^2\). Previous work by this lab and others has implicated the constitutive activation of transcriptional coactivators TAZ and YAP in sarcomagenesis\(^74\). It is thought that a principle mechanism by which their activation occurs is through dysregulation of the Hippo pathway; a primary negative regulator of TAZ/YAP. In the pursuit of novel therapeutic options, a thorough understanding of Hippo kinase dysregulation in multiple sarcomas is necessary; however, at present, little is known regarding the extent of dysregulation and how it occurs. To address this knowledge gap, one of the aims of this thesis project was to characterize Hippo kinase expression within several sarcoma cell lines and patient tumors.

A novel component of this project was to assess TAZ/YAP activation, as well as expression of key Hippo pathway components MST1/2 and LATS1/2 within the same subset of patient clinical samples. This unique approach allows correlation between initiating events in tumorigenesis like the loss of Hippo regulation and TAZ/YAP activation and patient outcomes. Overall, it was discovered that TAZ/YAP were activated in a majority of sarcomas, and MST1/2 expression was decreased in a majority of cell lines; this was also true for a significant portion of patient samples as well. This decrease was observed at transcriptional and translational levels. Unexpectedly, LATS1/2 did not exhibit significantly decreased expression in cell lines at a transcriptional level, although LATS2 protein expression was decreased in 58% of sarcoma cell lines evaluated, which suggests proteasomal degradation as the major mechanism governing
loss of LATS in sarcoma cell lines. As for how TAZ/YAP become constitutively active, exon DNA sequencing ruled out mutations in Hippo-targeted serines within TAZ/YAP, which implicates deregulation of the Hippo kinases themselves.

In assessing the mechanisms by which MST1/2 are dysregulated, it was found that degradation by the proteasome accounted for significantly decreased MST2 in 3/12 sarcoma cells. However, MST2 appears to be the exception, as MST1 and LATS1 protein did not accumulate in the 12 sarcoma lines with proteasome inhibitor MG132 treatment.

In addition, we endeavored to determine whether their downstream transcriptional co-activators (TAZ and YAP) can be therapeutically targeted in sarcomas. Verteporfin (VP) is a previously identified drug which is known to inhibit the YAP-TEAD4 interaction in breast cancer and melanomas\(^6^1\). It was found that the TAZ/YAP-TEAD4 interaction could be directly inhibited in sarcomas via VP, and that VP treatment reduced proliferation, colony formation, \textit{CTGF} and \textit{CYR61} expression in HT1080 and SK-LMS-1 cells. In terms of therapeutically targeting the mechanisms of MST1/2 dysregulation, treatment with 5-Azacytidine, a DNA methyltransferase inhibitor, increased MST1/2 expression in a number of cell lines. These results corroborated previous findings which show MST1/2 promoter hypermethylation in sarcomas\(^6^7\). Although promoter hypermethylation of MST1 was implicated as a mechanism of decreased expression in RD, and MST2 in RD, and SW872 cells, the remaining cell lines showed only a modest increase in kinase expression, suggesting another mechanism of epigenetic control could be at play.

Our research also identified histone modifications as another epigenetic mechanism of Hippo pathway suppression; an aspect which to our knowledge, was not previously investigated in sarcomas or other cancers. Treatment of cells with HDAC inhibitor Trichostatin A (TSA) alone increased MST1/2 expression at least 2-fold in 8/12 cell lines, and TSA + 5-Aza combination therapy synergistically increased expression to an even greater extent in A204 cells for MST2,
and RD cells for MST1. This synergism can be explained by the fact that histone deacetylase exists in a complex with transcriptional repressor MeCP2, DNMT1, and promoter hypermethylation to suppress transcription\textsuperscript{86, 88, 87}. To our knowledge, the role of histone deacetylation has not been previously determined in sarcomas, nor in the dysregulation of Hippo kinases.

This research project addressed several unknowns in the field of Hippo pathway research, and by doing so, has provided a clearer understanding of the dysregulation of core Hippo kinases MST1/2 and LATS1/2, and their unique contributions to sarcomas.

**Future Directions**

Although the previous thesis work determined three mechanisms which contribute to Hippo kinase dysregulation, there is still much to be done. In order to gain a complete understanding of the mechanisms of sarcomagenesis, further experiments will address previously unexplored mechanisms of Hippo dysregulation which could contribute to TAZ/YAP activation in cell lines, patient samples, and \textit{in-vivo}. Importantly, a goal of future experiments would be to therapeutically target TAZ/YAP activity and Hippo pathway components to restore Hippo regulation and decrease sarcomagenesis/tumor progression.

1. Delineate whether \textit{CTGF} and \textit{CYR61} expression is predominantly TAZ-dependent or YAP-dependent. TAZ and YAP will be individually knocked down in SK-LMS-1 and HT1080 via shRNA, and mRNA expression of \textit{CYR61} and \textit{CTGF} assessed.

2. Sequence kinase domains of MST1/2 for mutations which would render them inactive and unable to negatively regulate TAZ/YAP, thus leading to TAZ/YAP constitutive activation.
3. Identify copy number changes in MST1/2 and LATS1/2, as previously reported TCGA data showed 24% of soft-tissue sarcomas within the data set have copy number variations.

4. Bisulfite-based amplicon sequencing in patient tumor samples to assess the prevalence of promoter hypermethylation of Hippo kinases MST1/2.

5. Determine whether other epigenetic mechanisms, such as microRNA interference play a role in Hippo pathway suppression, using miRNA arrays/ChIP.

6. Evaluate whether post-translational modifications like phosphorylation play a role in MST/LATS dysregulation in cells where TAZ/YAP are constitutively activated. MST/LATS phosphorylation status will be determined by western blot and probing with phospho-specific antibodies.

7. Investigate the effects of MST1/2 re-expression on sarcomagenesis in-vivo. To achieve this, MST1/2 will be re-expressed through transduction in human sarcoma lines which they are lost, and grafted into NSG mice to assess tumorigenesis vs parental cell line xenografts.

8. Treat sarcoma line mouse xenografts with bortezomib (MG132 analog), valproic acid (Trichostatin A analog), or decitabine (5-Azacytidine analog), to determine effects of therapeutically targeting Hippo kinase proteasomal degradation, histone deacylation, or kinase promoter hypermethylation in sarcomagenesis.

9. Develop a MST1/2 flox/flox p53 flox/flox transgenic mouse model to recapitulate sarcomagenesis, and determine effects of therapeutically targeting Hippo kinase proteasomal degradation, histone deacylation, or promoter hypermethylation in sarcomagenesis via bortezomib, valproic acid, and decitabine (as above).
REFERENCES


Hisaoaka, M., Tanaka, A. & Hashimoto, H. Molecular alterations of h-warts/LATS1 tumor suppressor in human soft tissue sarcoma. Laboratory investigation; a journal of technical methods and pathology 82, 1427-1435 (2002).


