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Identifying and Targeting Molecular Deregulations in Uterine Leiomyosarcoma: A Role for mTOR Inhibition-Based Combination Therapies

Kari Jo Brewer Savannah

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**Identifying and Targeting Molecular Deregulations in Uterine
Leiomyosarcoma: A Role for mTOR Inhibition-Based Combination Therapies**

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**Identifying and Targeting Molecular Deregulations in Uterine
Leiomyosarcoma: A Role for mTOR Inhibition-Based Combination Therapies**

A
DISSERTATION

Presented to the Faculty of The University of Texas Health Science Center at
Houston and The University of Texas MD Anderson Cancer Center Graduate
School of Biomedical Sciences in Partial Fulfillment of the Requirements for the
Degree of

DOCTOR OF PHILOSOPHY

By

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Houston, Texas

May 2012

Dedication

For my grandmother, Karen Ann Brewer, who always told me that I should write a book about my “science stuff,” and for my parents, who helped to fuel my insatiable desire for knowledge by taking the time to answer all of my “why?” questions as a little girl.

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To my Grandparents, Bob and Karen Brewer and Glenn and Charlotte Perdew, you have been the most amazing and inspiring grandparents. I am so blessed to have grown up with and learned from all four of you. Thank you for always being supportive, for encouraging me to pursue science, and for teaching me that all things are possible through Christ, a little motivation, a great family for support. Granna and Grandpa Brewer – Thank you for showing me that a beer with friends on a bad day can make things much better.

To my husband and best friend, Khefren Savannah, words cannot describe how grateful I am for the tremendous amount of love, support, encouragement, and most of all, the patience that you have shown to me over the last several years. I am truly blessed to have such an amazing man standing beside me for the rest of our lives! Thank you for being my rock.

To my mother, father and sisters, Kimberly, Melanie and Mandy; you have been such an integral part of my life from the very beginning. Thank you for your support and encouragement always.

To Dr. Lee Ann Baron, your support over the last 10 years has meant the world to me and your mentorship has helped shape me into the scientist and the woman that I am today. God truly works through you to work within people.

There is a story that describes good friends as those who will bail you out of trouble, but great friends as those in trouble with you. To my partners in crime Danielle Conley, Kristen Holmes, Brittany Parker, and Catie Spellicy, I could not have completed my Ph.D. without you. Thank you for many nights of laughter and tears – you have made this journey a memorable one.

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**Identifying and Targeting Molecular Deregulations in Uterine
Leiomyosarcoma: A Role for mTOR Inhibition-Based Combination Therapies**

Publication No. _____

Kari Brewer Savannah

Supervisory Professor: Dina Lev, M.D.

Uterine leiomyosarcoma (ULMS) is an aggressive malignancy characterized by marked chemoresistance, frequent relapses, and poor outcome. Despite efforts to improve survival over the past several decades, only minimal advances have been made. Hence, there is an urgent and unmet need for better understanding of the molecular deregulations that underlay ULMS and development of more effective therapeutic strategies.

This work identified several common deregulations in a large (n=208) tissue microarray of ULMS compared to GI smooth muscle, myometrium, and leiomyoma controls. Our results suggest that significant loss of smooth muscle and gynecological differentiation markers is common in ULMS, a finding that could help render improved ULMS diagnosis, especially for advanced disease. Similarly to reports in other malignancies, we found that several cancer-related proteins were differentially expressed; these could be useful together as biomarkers for ULMS.

Notably, we identified significant upregulation and overexpression of the mTOR pathway in ULMS, examined the possible contribution of tyrosine kinase receptor deregulation promoting mTOR activation, and unraveled a role for pS6RP and p4EBP1 as molecular disease prognosticators. The significance of mTOR activation in ULMS and its potential as a therapeutic target were further investigated. Rapamycin abrogated ULMS cell growth and cell cycle progression *in vitro* but induced only slight growth delay *in vivo*. Given that effective mTOR therapies likely require combination mTOR blockade with inhibition of other targets, coupled with recent observations suggesting that Aurora A kinase (Aurk A) deregulations commonly occur in ULMS, the preclinical impact of dually targeting both pathways was evaluated. Combined therapy with rapamycin (an mTORC1 inhibitor) and MLN8237 (an investigational Aurk A inhibitor) profoundly and synergistically abrogated ULMS growth *in vitro*. Interestingly, the superior effects were noted only when MLN8237 was pre-administered. This novel therapeutic combination and scheduling regimen resulted in marked tumor growth inhibition *in vivo*. Together, these data support further exploration of dual mTOR and Aurk A blockade for the treatment of human ULMS.

TABLE OF CONTENTS

Dedication.....	iii
Acknowledgements.....	iv
Abstract.....	vi
Table of Contents.....	viii
List of Figures.....	ix
List of Tables.....	xi
List of Abbreviations.....	xii
Chapter 1: Introduction.....	1
Chapter 2: Materials and methods.....	52
Chapter 3: Identifying molecular deregulations in human ULMS.....	70
Chapter 4: Evidence of targetable pathways in ULMS: the mTOR pathway.....	90
Chapter 5: Creation and development of bioresources.....	106
Chapter 6: Targeting mTOR <i>in vitro</i> in ULMS.....	111
Chapter 7: Aurora A kinase deregulation in ULMS.....	122
Chapter 8: Dual blockade of mTOR and Aurora A kinase.....	129
Chapter 9: Discussion.....	137
Chapter 10: Future Directions.....	170
Appendix.....	174
References.....	190
Vita.....	274

LIST OF FIGURES

Figure 1 – Chapter 1: mTORC1/2 complexes and mTOR functional domains.....	22
Figure 2 – Chapter 1: mTORC1 downstream signaling pathway.....	24
Figure 3 – Chapter 1: mTOR pathway.....	27
Figure 4 – Chapter 1: Structure of rapamycin.....	34
Figure 5 – Chapter 1: Aurora A kinase functional domains.....	43
Figure 6 – Chapter 1: Molecular structure of Aurk A inhibitor MLN8237.....	49
Figure 7 – Chapter 3: ULMS tissue microarray construction.....	71
Figure 8 – Chapter 3: Expression of differentiation markers in ULMS.....	73
Figure 9 – Chapter 3: Expression of proliferation and survival markers	80
Figure 10 – Chapter 3: Expression of invasion and angiogenesis markers.....	82
Figure 11 – Chapter 3: Expression of oncoproteins and tumor suppressors.....	83
Figure 12 – Chapter 3: Expression of signaling molecule β -catenin.....	85
Figure 13 – Chapter 4: Heatmap of AKT/mTOR pathway activation in ULMS.....	90
Figure 14 – Chapter 4: Expression of mTOR pathway proteins & PTEN.....	92
Figure 15 – Chapter 4: Expression of EGFR and IGF-1R in ULMS.....	96
Figure 16 – Chapter 4: Expression of PDGF family receptors/ligands in ULMS.....	98
Figure 17 – Chapter 4: Expression of c-KIT, MET, and AXL in ULMS.....	100
Figure 18 – Chapter 4: Heatmap of mTOR and RTK expression in ULMS.....	102
Figure 19 – Chapter 5: Characterization of cell strains.....	108
Figure 20 – Chapter 6: mTOR pathway activation <i>in vitro</i>	112
Figure 21 – Chapter 6: mTOR pathway inhibition via rapamycin.....	113
Figure 22 – Chapter 6: Rapamycin treatment reduces cell proliferation.....	114

Figure 23 – Chapter 6: Rapamycin effects on cell cycle and apoptosis.....	115
Figure 24 – Chapter 6: Rapamycin is not a sufficient monotherapy <i>in vivo</i>	118
Figure 25 – Chapter 6: Rapamycin and doxorubicin in combination therapy.....	120
Figure 26 – Chapter 7: Gene array identification of Aurk A	123
Figure 27 – Chapter 7: Aurk A expression in ULMS and SiRNA knockdown.....	124
Figure 28 – Chapter 7: MLN8237 abrogates proliferation <i>in vitro</i>	126
Figure 29 – Chapter 7: MLN8237 effects on cell cycle and apoptosis.....	127
Figure 30 – Chapter 8: Combination mTOR/Aurk A blockade is synergic <i>in vitro</i> ..	130
Figure 31 – Chapter 8: Effects of combined mTOR/Aurk A inhibition <i>in vitro</i>	132
Figure 32 – Chapter 8: Combination therapy <i>in vivo</i> is highly effective.....	134
Figure 33 – Chapter 8: Characterization of dual inhibition <i>in vivo</i>	135

LIST OF TABLES

Table 1 – Chapter 1: FIGO staging for uterine leiomyosarcoma.....	6
Table 2 – Chapter 1: Monotherapies in ULMS.....	10
Table 3 – Chapter 1: Combination therapies in ULMS.....	13
Table 4 – Chapter 1: A literature review of clinical outcomes in ULMS.....	15
Table 5 – Chapter 1: Aurora kinases are conserved in several species.....	43
Table 6 – Chapter 1: Aurora A kinase substrates.....	46
Table 7 – Chapter 1: Aurora A kinase inhibitors	49
Table 8 – Chapter 3: ULMS differentiation markers' correlations to disease status.....	76
Table 9 – Chapter 3: ULMS differentiation markers' correlations to outcomes.....	77
Table 10 – Chapter 3: Cancer-related markers' correlations to disease status.....	87
Table 11 – Chapter 3: Cancer-related markers' correlations to tumor status.....	88
Table 12 – Chapter 3: Cancer-related markers' correlations to outcomes.....	89
Table 13 – Chapter 4: mTOR protein correlation between markers.....	94
Table 14 – Chapter 4: mTOR and RTK correlation to disease status.....	103
Table 15 – Chapter 4: mTOR and RTK correlation to tumor status.....	104
Table 16 – Chapter 4: mTOR and RTK correlation to outcomes.....	105
Table 17 – Chapter 5: Cell strains created from primary ULMS cultures.....	107

ABBREVIATIONS

UTMDACC	The University of Texas MD Anderson Cancer Center
STS	soft tissue sarcoma
LMS	leiomyosarcoma
ULMS	uterine leiomyosarcoma
RFS	recurrence-free survival
MFS	metastasis-free survival
DSS	disease specific survival
TMA	tissue microarray
MDM2	murine double minute 2
Rb	retinoblastoma (protein)
mTOR	mammalian target of rapamycin
PI3K	phosphoinositide-3 kinase
PTEN	phosphatase and tensin homolog
DMEM	Dulbecco's modified eagle medium
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with tween-20
FBS	fetal bovine serum
STR	short tandem repeats
PCR	polymerase chain reaction
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
PI	propidium iodide
FACS	fluorescence assisted cell sorting

Introduction

Soft tissue sarcoma

Soft tissue sarcomas (STS) are a complex group of rare malignancies (about 1% of all tumors) that are diagnosed in 9,500-10,520 individuals per year.¹⁻³ Arising from cells of mesenchymal origin, STS can originate from almost any anatomic locus within the body in several tissue types including smooth and striated muscle, fat, and nerves, irrespective of age.^{4,5} Classification of STS is based on the presumed cell of origin and hence, subtype names are reflective of cell of origin. For example, the subtype name “leiomyosarcoma” can be broken down into “leio” – meaning smooth, “myo” – meaning muscle, and “sarcoma” – meaning mesenchymal-derived malignancy; as their name implies, leiomyosarcoma are tumors of smooth muscle origin. There are currently more than 50 recognized histological subtypes of STS that can be divided into 2 groups: simple or complex karyotype STS.^{4,6} STS with simple karyotypes have specific genetic alterations (i.e. translocations or point mutations) that are a major contributor to sarcomagenesis while complex karyotype STS have a wide array of multiple genetic alterations.⁷ STS frequently metastasize and are generally chemoresistant, resulting in 5-year survival rates of only 50%.^{5,8-10}

Leiomyosarcoma (LMS)

Leiomyosarcoma account for about 1/7 to 1/4 of all STS¹¹⁻¹³ and are a more common soft tissue sarcoma subtype, followed in frequency by malignant fibrous histiocytoma and liposarcoma.¹² As their name suggests, leiomyosarcomas are malignancies of smooth muscle origin and can occur throughout the body wherever smooth muscle is present. The most common sites for leiomyosarcoma are the

retroperitoneum, extremities, and uterus; as much as 40% of leiomyosarcoma in women occur within the uterus corpus.^{4,12} Many studies report a strong predilection for the development of leiomyosarcoma in African Americans versus Caucasians. The African American:Caucasian rate ratio is 1.2 for all leiomyosarcoma; in African American versus Caucasian women, this ratio is increased to 1.7.¹² Extra-uterine leiomyosarcoma incidence increases with age in a linear fashion whereas uterine leiomyosarcoma incidence is greatest around pre- to menopausal ages (40-60 years of age).¹² As a whole, leiomyosarcoma are aggressive, chemoresistant, and have a propensity to recur and metastasize.

Uterine Leiomyosarcoma (ULMS)

Uterine leiomyosarcoma (ULMS) arise from smooth muscle walls of the uterus and represent the most common subtype of uterine sarcoma, a group of infrequent but highly devastating diseases.^{14,15} Although ULMS account for only 1-2% of all uterine malignancies, they are the cause of >25% of all uterine cancer-related deaths.¹⁶ The disease most frequently manifests during the fourth to sixth decade of life with slight predilection towards African American women.¹⁴ ULMS are highly aggressive and recur or metastasizes in as many as 73% of patients.¹ Even in patients with early stage (I or II) ULMS, a recurrence rate of nearly 40% is observed (14.3% with isolated pelvic recurrence, 65.7% with distant metastases, and 20.0% with disease in both loci).^{17,18} Metastatic lesions are most commonly found in the lungs, liver, bone, extremity tissues and skin (especially scalp).¹⁹ In addition, case report studies

have identified ULMS metastases in the brain, thyroid, breast, pancreas, and heart.²⁰⁻²⁴

Symptoms, diagnosis, and staging

Women with ULMS typically present asymptotically in early stages, but abnormal vaginal bleeding, pain, and/or a palpable pelvic mass may be present.¹⁵ Such symptoms closely mimic those of leiomyomas (uterine fibroids), common and benign uterine growths that occur in nearly 2/3 of women and are frequently observed as multiple lesions lining the uterus.²⁵ For this reason, it is often hard to distinguish a single ULMS lesion within a uterus containing multiple fibroids. Fibroids are usually treated by observation alone until they become symptomatic, at which time hormone-based therapies, myomectomy, or total abdominal hysterectomies are utilized.^{16,26-29}

The diagnosis of ULMS can be difficult to establish due to many contributing factors. In women who have previously opted for surgical resection of presumed leiomyomas, diagnosis of ULMS is often made incidentally upon surgical specimen review by pathologists post-resection.³⁰ About 0.1% of leiomyomas that are presumed benign are actually found to be ULMS and extensive studies have shown that leiomyoma is a distinct entity which does not degenerate into ULMS.²⁸ Another confounding factor in ULMS diagnosis is the “watch and wait” policy for the treatment of leiomyoma, which often delays ULMS diagnosis.^{28,30}

Diagnosis of ULMS may be indicated or supported by several imaging techniques including ultrasound, computerized tomography (CT) or positron emission tomography/CT (PET/CT).³¹ ULMS are usually visualized via ultrasound as large, oval shaped lesions; bizarre and inhomogeneous internal echo patterns; necrotic tumor centers are often noted. PET imaging uses radio labeled 18-fluorodeoxyglucose ($[^{18}\text{F}]\text{FDG}$) that is taken up in areas of high metabolic activity within the body, especially in tumors. It is often combined with CT to obtain both anatomical and morphological features of the study area.^{32,33} Although the technique cannot readily detect very small or low-grade lesions, PET/CT or CT are regularly performed during diagnosis and follow-up to aid in the diagnosis of recurrent and metastatic ULMS in the lungs and abdomen/retroperitoneum.³⁰

The next step towards confirming a diagnosis of ULMS is by obtaining biopsies and/or resection specimens, which are sent to pathology for histological evaluation and confirmation of ULMS diagnosis. ULMS generally appear as large oval-shaped nodules that are firm to the touch and yellow or tan in color.³⁰ Centralized necrosis and hemorrhaging is frequently observed.^{30,34} Several studies have shown that large size (>15-10cm), extrauterine extension, and infiltrating borders are common features of ULMS.^{15,35,36} ULMS are comprised of spindle shaped cells with elongated nuclei and are characterized by large numbers of atypical nuclei, high mitotic indices, alterations in chromosome copy number, and complex karyotype.^{30,37,38} Ferenczy et al. performed a comparative ultrastructural microscopy-based study ULMS and found they have very large, irregularly shaped

nuclei; multiple nuclei were often observed within the same cell and randomly dispersed chromatin and dense nucleoli were present.³⁹ Noted increases in mitotic activity and nuclear pleomorphism were observed in ULMS as well as reduced collagenous stroma and myofilament bundles, which were also increasingly disorganized.³⁹

Once radiological, then histological and pathological data have been compiled, physicians can classify each patient into stages. ULMS staging is performed as per The International Federation of Gynecology and Obstetrics (FIGO) classification system incorporating tumor, lymph node, and metastatic status in a four-stage schema (**Table 1**). Staging helps physicians to stratify tumors based on the criteria above and provides a starting point for rational therapeutic interventions.

Treatment

Therapeutic interventions for ULMS are varied and first/second line therapies differ depending on tumor stage classification. For all resectable primary ULMS, surgery is regarded as the mainstay of treatment; aggressive wide margin resection is standard. Most commonly, complete hysterectomy with bilateral salpingo-oophorectomy is performed.⁴⁰ However, several studies indicate that ovarian preservation does not negatively affect overall survival, and therefore, it is sometimes considered in women of childbearing age to avoid premature onset of menopause.^{30,40} Additionally, the risk of ovarian metastasis is low (about 4%), with many of these being low-grade, hormone-sensitive LMS.^{30,41-44}

Table 1. FIGO staging for ULMS

TNM	FIGO Stage	Definition		
Primary Tumor (T)				
TX		Primary tumor cannot be assessed		
T0		No evidence of primary tumor		
T1	I	Tumor confined to uterus		
T1a	IA	Tumor <5cm		
T1b	IB	Tumor>5cm		
T2	II	Tumor confined to uterus and pelvis		
T2a	IIA	Tumor involves adnexa		
T2b	IIB	Tumor involves other pelvic tissues		
T3	III	Tumor infiltrates abdominal cavity		
T3a	IIIA	Infiltration at one site		
T3b	IIIB	Infiltration at more than one site		
T4	IV	Tumor growth distant from uterus		
T4a	IVA	Tumor invades bladder or rectum		
Regional Lymph Nodes (N)				
NX		Regional lymph nodes not assessed		
N0		No regional lymph node metastasis		
N1	IIIC	Regional lymph node metastasis		
Metastases (M)				
M0		No distant metastasis		
M1	IVB	Distant metastasis (excluding pelvic and abdominal)		
Overall Diagnosis (TNM combined staging)				
Stage I	T1	N0	M0	
Stage IA	T1a	N0	M0	
Stage IB	T1b	N0	M0	
Stage II	T2	N0	M0	
Stage IIIA	T3a	N0	M0	
StageIIIB	T3b	N0	M0	
Stage IIIC	T1, T2, T3	N1	M0	
Stage IVA	T4	Any N	M0	
Stage IVB	Any T	Any N	M1	

Adapted from American Joint Committee on Cancer 2010.

Several comprehensive studies have shown that unlike epithelial-derived malignancies, sarcomas do not have a propensity for lymph node involvement.⁴⁵ In a large cohort study of 1396 ULMS patients Kapp et al. demonstrated lymphatic spread in only ~3-7% of ULMS.³⁶ While lymphatic involvement is more frequent in advanced stage tumors, ULMS metastases regularly occur in its absence.³⁶ Neither adnexectomy or lymphadenectomy were found to be prognostic factors favoring survival, and fewer than 25% of ULMS patients therefore undergo regional lymphadenectomy.³⁶

Early stage ULMS treatment

For early stage tumors (stages I and II), surgery is regarded as the first-line therapy. Following surgery with clear margins, patients are generally placed on a “watch and wait” policy and do not undergo chemotherapeutic treatment, as it shows no clear clinical benefit.^{17,30,40} If clear margins are not obtained or an aggressive chemotherapy approach is warranted, patients may consider radio- or chemotherapy.

The use of adjuvant radiation therapy to the pelvis may be considered, as some studies suggest it may reduced the risk of local recurrence.⁴⁶ In a 103 patient trial, Reed et al. administered pelvic radiation to stage I and II ULMS patients over the course of 5 weeks; no significant benefits were observed.⁴⁷ However, the efficacy of radiation therapy in this setting remains controversial and most studies agree that there is little to no therapeutic benefit.⁴⁶⁻⁴⁸ Hormonal agents such as tamoxifen are largely ineffective versus aggressive ULMS and may even accelerate

sarcomagenesis.^{49,50} Chemotherapeutic agent doxorubicin also offered no overall or recurrence-free survival benefit.¹⁸ However, adjuvant gemcitabine and docetaxel combination therapy yielded significant improvement in survival over historical rates. In a study conducted by Hensley et al., 59% of stage I or II ULMS patients were progression-free at 2 years; the median progression-free survival while on gemcitabine/docetaxel combination therapy was 39 months.⁵¹ In early stage disease, most studies agree that radiation and chemotherapy have minimal to no added benefit. Additionally, radio- or chemotherapy may be considered.

Locally advanced and recurrent ULMS treatment

Many ULMS patients will be diagnosed with locally advanced (stage III) disease, where the tumor extends beyond the uterus. Additionally, numerous others will experience relapse and recurrent tumors will emerge. Surgery is generally regarded as the only curative modality in both subsets of patients, but achieving clear margins is often difficult or not possible. In such cases, additional therapies may be used to reduce tumor size, which may sometimes allow for surgery.³⁵

Radiation therapy is sometimes employed in locally advanced or recurrent ULMS,⁴⁶ but appears to have little to no impact on progression-free or overall survival.^{46,48} Despite having little to no curative effects, radiation therapy can be used effectively in palliative care setting for large tumors in terminal patients.⁴² Doxorubicin (adriamycin) is one of the best-studied and clinically administered chemotherapies in ULMS. It acts by inhibiting topoisomerase II and thus, DNA replication.⁵²

Doxorubicin has been modestly effective against ULMS with response rates of about 25% and may decrease the likelihood of recurrence (61% in placebo-dosed patients compared with 41% in doxorubicin-treated patients), however, it did not significantly effect progression-free or overall survival.^{18,53-55} Several other monotherapies have shown minimal success in ULMS therapy (**Table 2**). Given the overall lack of response to monotherapies, combination therapies have been examined in locally advanced/recurrent ULMS including ifosfamide/doxorubicin and docetaxel/gemcitabine. Studies in advanced recurrent ULMS (and metastatic ULMS) using a docetaxel/gemcitabine combination yielded the most efficacious results (36% response) in the treatment of recurrent ULMS and are discussed as part of the following section.^{56,57}

Metastatic ULMS treatment

Metastatic ULMS are highly invasive, rapidly proliferating, and aggressive lesions that most frequently develop in the lungs, where patients often develop multiple bilateral lesions.¹⁸ In these lesions surgery, when possible, represents the first-line therapeutic approach. Burt et al. report that aggressive metastasectomies significantly extend overall survival, however, they do not extend disease-free survival and more than 80% of women with pulmonary metastasectomies experienced recurrence.⁵⁸ The study concluded that long-term survival could be achieved through repeated and aggressive pulmonary metastasectomy for patients with recurring ULMS pulmonary metastases.⁵⁸ Surgical resection for extrapulmonary metastases is also advantageous and can enhance the probability

Table 2. Monotherapy regimens for ULMS treatment

Author (year)	Therapy/Regimen	Study	Cohort	Disease Stabilization	Partial/Complete Response	(n)
Sutton (1992)	Ifosfamide (1.5mg/m ² for 5 days)	Uterine sarcoma & ULMS, Phase II	ULMS/uterine sarcomas without prior chemotherapy	-	17%	35
Sutton (1999)	Paclitaxel (175mg/m ² every 3 weeks)	ULMS, Phase II	Recurrent or advanced ULMS without prior chemotherapy	24%	9%	33
Gallup (2003)	Paclitaxel (175mg/m ² every 3 weeks)	ULMS, Phase II	Recurrent or advanced ULMS without prior chemotherapy	23%	8%	48
Look (2004)	Gemcitabine (1000mg/m ² every 3 weeks on days 1, 8, 15)	ULMS, Phase II	Recurrent or persistent ULMS after progression on chemotherapy	16%	21%	42
Anderson (2005)	Temozolomide (50-75mg/m ² daily for 6 of 8 weeks)	ULMS	Recurrent or metastatic, unresectable ULMS	33%	8-14%	13
Wade (1990)	Tamoxifen	ULMS	All stages of ULMS	-	4%	28
Amant (2009)	Trabectedin (1.5mg/m ² every 3 weeks)	Uterine sarcoma	Metastatic high grade uterine sarcoma after progression on chemotherapy	64%	45%	11
Omura (1985)	Doxorubicin (60mg/m ² every 3 weeks)	Uterine sarcoma	Stage I or II uterine sarcoma	no difference in survival	no difference in recurrence	28
Sutton (2005)	Liposomal Doxorubicin (50mg/m ² every 4 weeks)	ULMS, Phase II	Recurrent or advanced ULMS after progression on local therapy	32%	16%	32

of long term survival in ULMS patients.⁵⁹ Resection is regarded as the only curative therapy; moderate successes have been achieved with chemotherapies. Radiation therapy can also be effective in palliative care for metastatic ULMS, but offers no clear curative benefits.⁴⁶

While surgical resection is the only curative treatment in metastatic ULMS, it is not practical or even possible for most patients; so aggressive chemotherapy regimens often represent the best and primary course of therapy. Whether alone or in combinations, chemotherapeutic regimens can be mildly successful in extending survival for ULMS patients with metastatic lesions. Several single-agent regimens such as docetaxel, temazolamide, and cisplatin, have been utilized in ULMS therapy, albeit with very little success (**Table 2**).^{18,55,60-68} Notably, single-agent trabectedin is sometimes used in cases of advanced and metastatic ULMS. While trabectedin offers minimal response rates of 16%, several studies indicate that it can significantly stabilize disease for progression-free responses in about 53% of patients for three months and about 35% of patients for six or more months, suggesting that trabectedin may offer additional benefit in selected patients.⁶⁹⁻⁷¹

Given the overall lack of appreciable response to monotherapies, a number of combined agent approaches in ULMS have emerged (**Table 3**).^{56,57,72-76} For example, combined doxorubicin/ifosfamide therapy yielded a 30.3% overall response rate; a low, but considerably better result than that of either therapy

alone.⁶² Currently, the most successful therapy for advanced and metastatic ULMS utilizes a combined gemcitabine/docetaxel approach.^{57,75} As a first-line approach, gemcitabine/docetaxel yielded complete response in only 4.8% of patients and partial response in 31% of patients, resulting in an overall response rate of 35.8%. In addition, 26.2% of the other study patients experienced disease stabilization while receiving the combined therapy and the median overall survival was extended by >16 months (range 4-41 months).⁵⁷ A similar study utilizing gemcitabine/docetaxel combination therapy as a second-line therapeutic approach for women with metastatic ULMS who previously experienced tumor progression on cytotoxic therapies (mostly doxorubicin-based) showed slightly less favorable, albeit measureable effects. Complete response was observed in 6.3% of patients and partial response in 20.8% of patients; the overall response rate was 27% (n=48 evaluable cases). Notably, 73% of patients had a progression-free interval of at least 12 weeks and 52% were progression-free for at least 24 weeks.⁵⁶ Overall, gemcitabine/docetaxel combination therapy currently represents the most effective chemotherapy regimen for ULMS. While the minimally improved response rates that are observed with administration of this therapy are superior to those of other clinically approved chemotherapeutic agents, it is critical to emphasize that only about one of every three women with ULMS will show any positive response to this therapy; for the remaining two-thirds, efficacious chemotherapies have not yet been identified.

Table 3. Combination therapies for the treatment of ULMS

Author (year)	Therapy/Regimen	Study	Cohort	Stable Disease	Partial/Complete Response	(n)
Hensley (2002)	gemcitabine/docetaxel	LMS, Phase II	Unresectable LMS after progression with ≥ 2 prior chemotherapies.	21%	53%	34
Hensley (2008)	gemcitabine/docetaxel	ULMS, Phase II	Advanced ULMS with no prior chemotherapy	26%	36%	39
Hensley (2008)	gemcitabine/docetaxel	ULMS, Phase II	Unresectable metastatic ULMS after progression on first line chemotherapy	50%	27%	48
Blum (1993)	doxorubicin/ifosfamide	STS, Phase III	Metastatic STS	-	34%	88
Edmonson (1993)	doxorubicin/mitomycin/cisplatin	STS, Phase III	Advanced (recurrent, residual, or metastatic) STS	-	32%	84
Santoro (1995)	doxorubicin/ifosfamide	STS, Phase III	Advanced STS with no prior chemotherapy	-	28%	221
Patel (1998)	doxorubicin/ifosfamide	STS	Primary STS or Metastatic STS	-	66%	33
Maki (2007)	Gemcitabine/docetaxel	STS	Metastatic STS	-	16%	119

ULMS Outcome

Women diagnosed with ULMS often face an ongoing battle and eventually, a grim fate (**Table 4**). High rates of recurrence and metastasis, regardless of adjuvant treatment, coupled with short disease free time intervals (usually <six months to one year), make ULMS a very deadly disease.¹⁷ Kapp et al. reported 5-year disease specific survival for women diagnosed by stage as: stage I - 75.8%, stage II – 60.1%, stage III – 44.9% and stage IV – 28.7%.³⁶ Others cite 5-year survival rates as low as 8% for women diagnosed with stage II-IV ULMS.⁴⁴ A 1971 report indicates a 5-year survival rate of 20.7% with a high level of recurrence.¹⁴ More than 35 years later, reports indicate 5-year overall survival rates at only 20-60% regardless of stage at diagnosis. While the long term survival rate approaches 90% for the much more common endometrial adenocarcinoma,⁷⁷ the ULMS 5-year survival rates are far less encouraging despite several decades of research efforts towards identifying molecular underpinnings of the disease.^{78,79}

In summary, complete wide margin surgical resection in ULMS often confers significant postoperative morbidity and chemotherapy/radiation-based interventions offer very low response rates in uterine leiomyosarcoma and bring clinical benefit to only a small subset of patients. Several chemotherapy monotherapies and combined treatment regimens have been evaluated with inconsistent resultant efficacy and ^{18,47,60,61,63-68,80-82} despite research efforts over the past several decades, very little improvement in ULMS survival rates has

Table 4. Recurrence-free, metastasis-free, and disease specific survival in ULMS.

Author (year)	Stage (n=)	Recurrence Rate	Metastasis Rate	5-yr survival
Salazar (1978)	Stage I Stages II-IV	-	-	(Overall) 56% 7%
George (1986)	All stages (n=81)	(2-year) 23% (19/81)	(2-year) 38% (31/81)	(Overall) ~35%
Larson (1990)	Stage I (n=97) Stage II (n=11) Stage III (n=27) Stage IV (n=8)	(5-year, all stages) 63.6% (49/77)	(5-year, all stages) 54.5% (42/77)	(Overall) 39% ~18% ~7% 0%
Major (1993)	Stage I (n=49) Stage II (n=2) Stage III (n=5) Stage IV (n=3)	(Overall, all stages) 13.5% (8/59)	(Overall, all stages) 64.4% (38/59)	(Overall, all stages) 31%
Gadducci (1996)	Stage I/II (n=126) Stage III (n=16) Stage IV (n=6)	(5-year) 13% (12/90) 56.3% (9/16) 33.3% (2/6)	(5-year) 33.3% (30/90) 62.5% (10/16) 83.3% (5/6)	(DSS) 61.1% 0% 0%
Nordal (1997)	Stage I Stage IV Total (n=475)	-	-	(Overall) 65% 0%
Blom (1998)	Stage I/II (n=29) Stage III/IV (n=20)	68% (19/29) -	- -	(DSS) 42% 0%
Mayerhofer (1999)	Stage I (n=49) Stage II (n=5) Stages III/IV (n=17)	-	-	(Overall) ~78% ~0% ~12%
Ferrer (1999)	All stages (n=43)	-	-	(DSS) 55.4%
Pautier (2000)	Stage I (n=45) Stage II (n=10) Stage III (n=7) Stage IV (n=14)	(5-year, all stages) ~80%		(Overall) ~35%
Giuntoli (2003)	Stage I (n=130) Stage II (n=13) Stage III (n=18) Stage IV (n=41)	-	-	(DSS) ~60% ~47% ~34%

				~20%
Livi (2003)	All Stages (n=72)	-	-	(Overall) 18.8%
Wu (2006)	Stage I (n=51) Stage III/IV (n=10)	(5-year) 29.3% 50%	(5-year) 66.7% 60%	(Overall) 73.6% 43%
Kapp (2008)	Stage I (n= 951) Stage II (n=43) Stage III (n=99) Stage IV (n=303)	-	-	(DSS) 75.8% 60.1% 44.9% 28.7%

been achieved. It is clear that the development of novel, efficacious therapies through a better understanding of molecular deregulations and identification of potential drug targets is essential towards improving survival in ULMS patients.

Molecular underpinnings of ULMS

Over the past several decades, a number of studies have been conducted to examine the molecular underpinnings of ULMS. To develop more effective anti-ULMS therapies, a better understanding of molecular drivers and genetic/epigenetic aberrations in the disease must be acquired.

Genetic/epigenetic

Several genetic/epigenetic aberrations and molecular alterations have been identified in small ULMS patient cohort studies. Many ULMS have regions of amplifications or loss that may contribute to their malignant phenotype. Studies, including comparative genomic hybridization (CGH) arrays, have revealed amplifications or deletions in 1q21, 1p12-13, 1p31-32, 5p14, 8q, 10, 12q13-15, 12q31, 13q, 17p11, 19p13, and 20q13 regions.⁸³⁻⁸⁵ A few genes of interest in these regions include amplification of

Ras oncogenes (*RAB3B* - 1p31-21, *RAP1A* - 1p12-13) and the *MET* protooncogene.^{86,87} In one study, specific gains and losses of gene copy number were evaluated by CGH array in seven ULMS samples and averages of 4.86% gain and 15.1% loss were identified.⁸³ The study reports substantial gain in chromosomes seven and twelve at 7q36.3, 7q33–q35, 12q13–12q15, and 12q23.3. Homozygous loss was identified in chromosomes one, two, six, nine, and fourteen at 1p21.1, 2p22.2, 6p11.2, 9p21.1, 9p21.3, 9p22.1, 14q32.33, and 14q32.33 qter.⁸³ Genes associated with regions of gain were *HMGIC*, *SAS*, *MDM2*, *PDGF-β*, *KLK2*, and *TIM1* genes. Those associated with regions of loss included: *LEU1*, *ERCC5*, *THBS1*, *DCC*, *MBD2*, *SCCA1*, *FVT1*, *CYB5*, and *ETS2/E2*.⁸³ Another CGH array by Packenham et al. compared ULMS (n=8) versus leiomyoma (n=14).⁸⁸ The study showed that only 2/14 leiomyoma showed genetic alterations whereas all ULMS had multiple regions of gains and losses. Gain on chromosome 1 (both arms) was most common in ULMS and occurred in 5/8 samples.⁸⁸ Additional studies have suggested a 291kb region on 16q24 and a 2.5Mb region on 1p36.32 loss and gain of regions in chromosomes 1, 7, and 15. Regions gained were associated with three genes implicated in tumorigenesis: *TCF12*, *ABL2*, and *MET*.⁸⁹ Other genes of potential interest in this region include *CBFA2T2* and *FANCA*, both of which are implicated in acute myeloid leukemia.^{90,91} Amplification of 17p11 and 7q31.2 were also identified and contain *MYCOCD* and *MAP2K4* genes and the gene for a protein that may activate the AKT pathway named calveolin, respectively.⁸⁹ A region of was also identified in 13q14.2, which contains the *RB1* gene encoding the Rb tumor suppressor protein.⁸⁹ Overall, a study in recurrent ULMS confirmed findings from several groups and overall, found that well over half of ULMS samples had clonal chromosomal abnormalities.⁸⁷

Genetic mutations could also lead to a tumorigenic phenotype and have been identified in ULMS. For example, p53, a critical tumor suppressor, was found to be mutated in more than one-third of leiomyosarcoma.⁹² Fumarate hydratase mutations have also been identified in and may predispose patients to ULMS.⁹³ Additionally, mutations in several key proteins such as Jak1, MDM2 and TSC2 have been observed in ULMS.⁹⁴⁻⁹⁶ A combination of amplification in oncogenic regions, loss in tumor suppressor regions, and genetic mutations may contribute to ULMS.

Epigenetic regulation of ULMS has also been minimally evaluated. Several genes encoding tumor suppressor proteins can be epigenetically downregulated in cancer and are implicated in tumorigenesis. For example, Xing et al. showed that BRCA1 downregulation in ULMS is likely due to BRCA1 promoter methylation.⁹⁷ Also, loss of PTEN has been shown to activate the AKT/mTOR pathway in leiomyosarcoma and is suggested to be a driving force of tumorigenesis.⁹⁸ Methylation of the PTEN promoter region has been previously implicated in PTEN loss in other cancer and could be a contributing cause of PTEN loss in ULMS.⁹⁹ However, a single LMS study did not show any significant PTEN promoter methylation, suggesting that PTEN methylation may not be as prevalent in LMS/ULMS as in other cancers.⁹⁹ The p16 protein and tumor suppressor is reported to be reduced or lost in some ULMS. Xu et al. examined the methylation status of *INK4A* (the gene that encodes p16 protein) compared to p16 expression in a series of 38 LMS compared against 10 leiomyomas and 5 normal smooth muscle samples.¹⁰⁰ In total, almost one-quarter of LMS had hypermethylated *INK4A* promoter regions; samples with hypermethylated promoter regions also had absent or faint positive p16 staining, suggesting that 5' CPG methylation to the

promoter region of *INK4A* may be a significant cause of diminished expression of p16 protein in LMS.¹⁰⁰ A number of epigenetic deregulations could occur in ULMS and may contribute to leiomyosarcomagenesis, however, additional studies are required to confirm existing reports and identify novel epigenetic regulations.

Gene Alterations

Studies have been conducted to identify genes differentially expressed in ULMS/LMS compared with myometrium and leiomyoma. Skubitz et al. examined genes associated with malignant transformation in ULMS/LMS compared against myometrium and leiomyoma.¹⁰¹ Several differentially expressed genes were identified, including many that were overexpressed in ULMS/LMS compared to leiomyoma and myometrium. Genes that had increased expression by ≥ 10 -fold were: *interleukin-17B*, *proteolipid 1*, *doublecortin*, *osteopontin*, *pituitary tumor transforming 1*, *calpain 6*, and *ubiquitin conjugating enzyme E2C*. A number of additional genes were enriched five to ten-fold in ULMS/LMS compared to other tissues including those encoding for CDC2 associated protein CKS2, cellular retinoic acid binding protein 2, cyclin-dependent kinase inhibitor 2A (CDKIN2A), diaphanous (Drosophila homolog) 3, forkhead box M1B, popeye protein 2, protein regulator of cytokinesis (PRC-1), suppression of tumorigenicity 5 (STS 5) and topoisomerase II- α .¹⁰¹

Other potential deregulations

In addition to genomic/epigenetic expression profiling, a handful of studies have examined several proteins that are potentially over- or under-expressed in ULMS compared with myometrium or leiomyoma controls including c-Myc, MET, IGF and

IGFR, PTEN, Rb, MMP1 and MMP2, and PDGFR- α .^{38,84,85,89,102-105} Together, evidence suggests that ULMS has deregulations in critical pathways.¹⁰⁶ Small cohort studies have identified several potential genetic, epigenetic, and molecular aberrations that may contribute to the aggressive ULMS phenotype; however, few large scale studies have been conducted to date, so global conclusions cannot be drawn at this time. Comprehensive, large-cohort studies could confirm previous findings and provide insight into the molecular deregulations that contribute to and underlay ULMS.

The mTOR Pathway in ULMS

Molecular aberrations can play a role in pathway deregulation and contribute to tumorigenesis. Several groups have reported mTOR pathway activation in cancer, sarcoma, and even LMS, suggesting that it may be a critical pathway in cancer and lending evidence to suggest that it could be deregulated in ULMS.^{98,107}

mTOR : biochemistry and function

The protein mTOR (mammalian target of rapamycin) lies at the crux of the PI3K/AKT/mTOR signaling cascade and controls cell survival, growth, migration, and cell cycle progression through regulation of mRNA translation.^{108,109} mTOR, also known as FRAP1, is a 289kDa serine/threonine kinase that functions mainly in two unique complexes: mTORC1 and mTORC2 (**Figure 1A**).^{102,103,110-112} The primary differences between mTORC1 and mTORC2 are that mTORC1 complexes with raptor and is sensitive to mTOR inhibitor rapamycin, while mTORC2 binds in a complex with rictor and is rapamycin insensitive.^{113,114} Several domains within the mTOR protein have been identified including HEAT repeat, FAT, FRB, FATC, and Ser/Thr kinase domains (**Figure 1B**).¹¹⁰ mTOR is a member of the TOR (target of rapamycin) family; only one TOR protein exists in mammals compared to two in yeast and one in *Drosophila*.^{115,116} The TOR proteins are evolutionarily conserved to some extent and share approximately 40-60% homology between mammals, yeast, worms, and *drosophila*.¹¹⁶⁻¹¹⁹ This pronounced homology preservation among several different species further indicates that the protein confers essential functions in cell

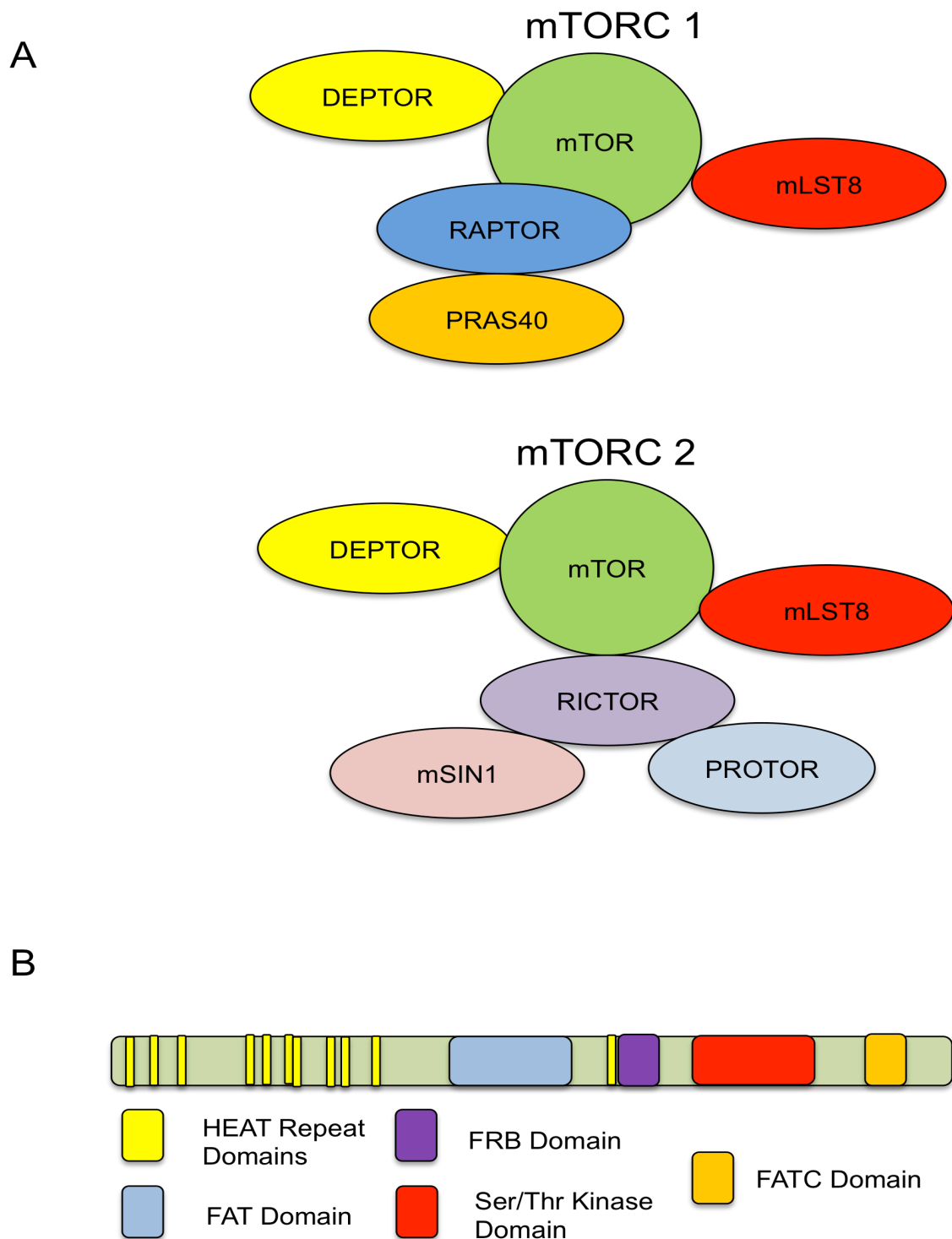


Figure 1. mTOR is a critical signaling molecule. A) mTOR functions in two distinct complexes, mTORC1 and mTORC2. B) mTOR has several domains that lend towards its functionality. (Adapted and reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Neuroscience] Bove, J. et al., Fighting neurodegeneration with rapamycin: mechanistic insights, copyright 2011).¹¹⁰

growth. Moreover, mTOR knockdown results in embryonic lethality just after implantation due to developmental abnormalities and points further to the critical role of mTOR in growth regulation.^{120,121}

mTOR has four predominant phosphorylation sites, Ser1261, Thr2446, Ser2448, and Ser2481.¹²² Phosphorylation at Ser1261 is thought to be the only site that directly regulates mTOR activity.¹²² Additionally, Ser2481 is shown to be autophosphorylated.¹²³ The status of this autophosphorylation has been linked to mTOR activity, although the mechanisms by which this occurs are largely unknown.^{124,125} Activated AKT is responsible for phosphorylation of residues Thr2446 and Ser2448, presumably through AKT activation induced by a S6 kinase feedback loop that is described later in this chapter. The function(s) of these latter phosphorylations remain unclear.^{126,127}

mTORC1 plays a critical role in protein translation and regulation of the AKT/mTOR pathway. Consisting of mTOR, raptor (regulatory-associated protein of mTOR) and GβL/LST8, the complex relays PI3K/AKT signals downstream and regulates translational effects from such signaling. As part of mTORC1, mTOR phosphorylates two main downstream targets, ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor binding protein 1 (4E-PB1; **Figure 2**).¹²⁸ Phosphorylation of S6K occurs at Thr389 by mTORC 1 and Thr229 by PDK1, thereby activating the protein,

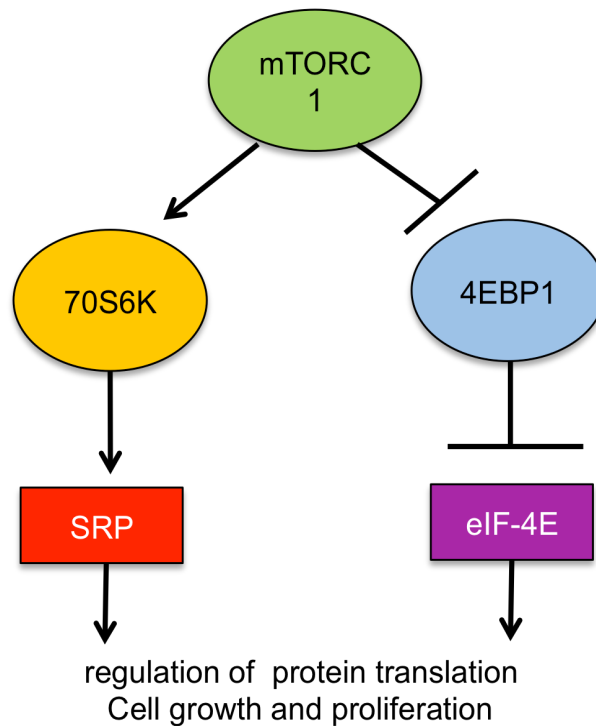


Figure 2. mTORC1 downstream signaling. mTORC1 phosphorylates at Thr389 to activate the 70S6K; further activation by PDK1 at Thr229 fully activates the protein. Activated 70S6K phosphorylates and activates S6RP, which signals downstream to regulate translation of 5'TOP-containing mRNAs and ultimately, enhancement of cell growth and metabolism. Phosphorylation of 4EBP1 is inhibitory and results in abrogation of 4EBP1-binding to eIF4E translation initiation factor, resulting in unbound eIF4E and enhanced translation.

which in turn activates 4eIF4B, a molecule involved in translation, and S6RP.^{127,129-132}

Through this pathway, S6K regulates translation of 5'TOP-containing mRNAs.¹³³

Cumulatively, S6K activation via phosphorylation results in enhancement of cell growth and metabolism. mTOR also phosphorylates 4E-BP1 in an inhibitory manner, which results in abrogation of 4EBP1 binding to translation initiation factor eIF4E and promotes translation initiation.¹³⁴ Together, the effects of mTOR inhibition on downstream targets are prevention of the translation of mRNA critical in cell cycle advancement from G1 to S phase, thereby trapping cells in G1.^{38,109,134,135}

mTORC1 regulates protein translation by several complex mechanisms. Eukaryotic translation is a three-step process involving temporally oriented initiation, elongation, and termination steps. Many initiation and elongation steps in protein translation are regulated by mTOR and its downstream effectors. For example, translation initiation factors (eIFs) help to mediate recruitment of mRNA to the 40S ribosome unit and also help to bring methionyl-tRNA molecules to the sites of translation, which recognize the start codon and initiate translation.¹³⁶ One particularly important eIF, eIF4E, binds to the 5' of the mRNA and coupled with the activities of several additional proteins such as scaffold protein eIF4G and DNA helicase eIF4A, eIF4E forms a complex that binds to mRNA and initiates translation. The binding site for eIF4G located within eIF4E also serves as a binding site for 4E-BP1.¹³⁶ When bound to eIF4E, 4E-BP1 occupies the binding site for eIF4G, thereby abrogating binding and assembly of the translation complex and subsequent initiation of mRNA translation.¹³⁶ Activated mTOR (via the mTORC1 complex) phosphorylates 4E-BP1, inactivating 4E-BP1 which results in eIF4E and eIF4G binding, complex formation, and initiation of translation. Interestingly, mTORC1, but not mTORC2, phosphorylates 4E-BP1 at Thr37 and Thr46, which are not sufficient to inhibit the molecule, but are required to prime 4E-BP1 for additional phosphorylations at Ser65 and Thr70.¹³⁶ These latter phosphorylations are near the eIF4E binding site on 4E-BP1. Thus, when 4E-BP1 is hyperphosphorylated, it abrogates binding to eIF4E and blocks the inhibitory effects that 4E-BP1 exerts on eIF4E.¹³⁶ Functioning eIF4E forms a complex and initiates translation of many pro-tumorigenic molecules such as c-myc, HIF-1 α , VEGF, and cyclin D1.^{129,137} In addition to shorter term increases in mRNA translation initiated by

growth/hormone signaling molecules via the mTOR pathway, the pathway can also mediate longer term translation capabilities of the cell by upregulating transcription of translational machinery.^{138,139}

mTOR has additional functions beyond regulation of mRNA translation, including cell cycle regulation, exerting anti-apoptotic effects, and promoting angiogenesis. Inhibition or sequestering of mTOR can also induce autophagy in several models, including mice and human cells.^{140,141} For example, mTOR controls G1 to S cell cycle progression by inhibiting cyclin D1 turnover as well as enhancing p27 degradation.^{139,142,143} It can also regulate apoptosis by activation of S6K, which has been shown to localize at the mitochondrial membrane. There, it phosphorylates BAD, a pro-apoptotic protein that is inhibited via phosphorylation. Through this interaction, mTOR promotes cell survival through inhibition of pro-apoptotic signaling molecules. Additionally, mTOR also enhances intracellular levels of pro-survival molecule survivin.¹⁴⁴ Although mTOR functions primarily in the cytoplasm, there is evidence to suggest that it also has functions as a cytoplasmic-nuclear shuttling protein.¹⁴⁵

mTOR regulation

The mTOR pathway is regulated predominantly via extracellular signaling molecules (i.e. hormones and mitogens) through PI3K (**Figure 3**).

PI3K/PTEN

PI3K is an important intracellular signaling molecule that phosphorylates phosphatidylinositol-4,5-bis-phosphate (PIP2) to PIP3 at the cellular membrane.¹⁴⁶

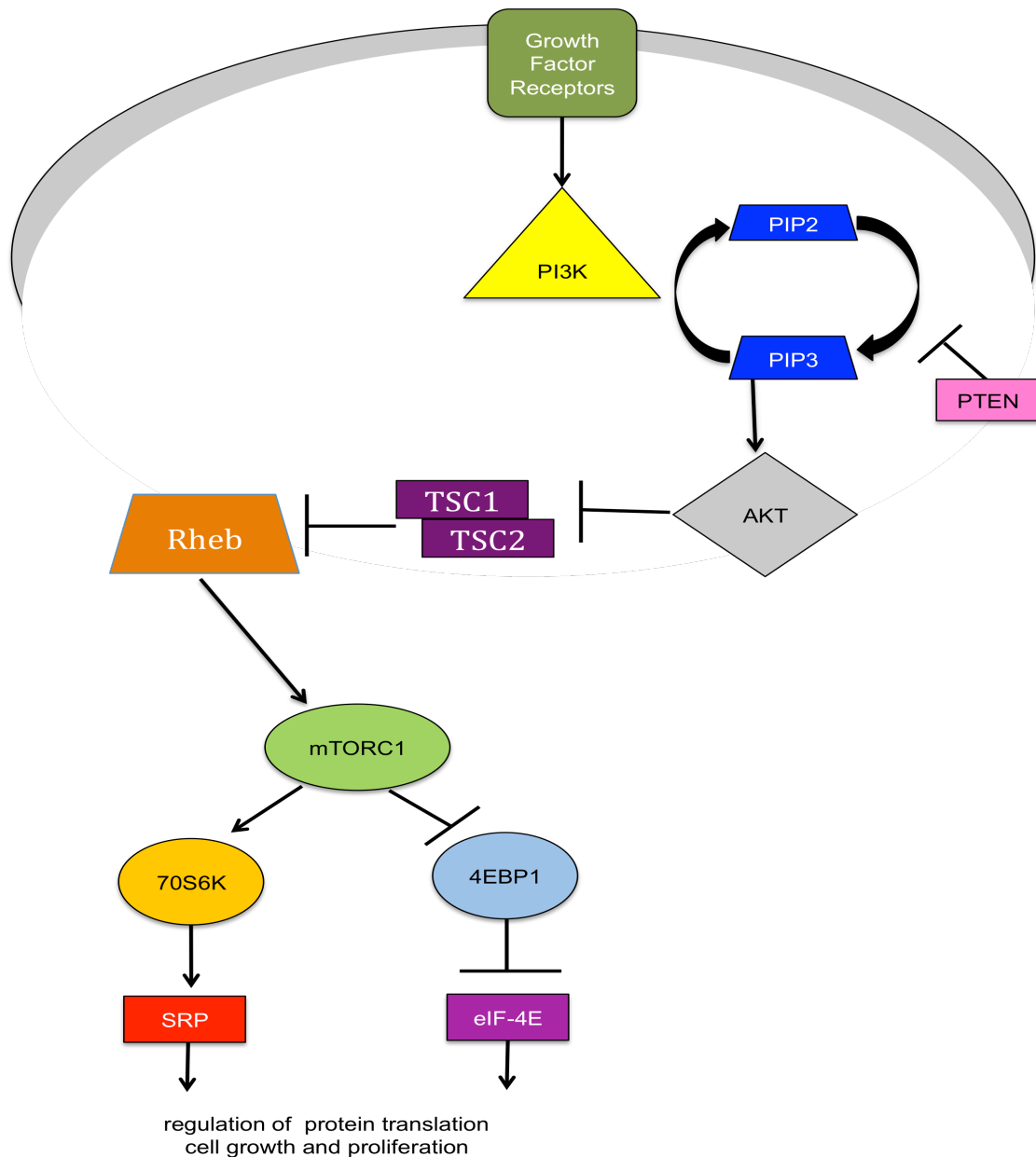


Figure 3. The mTOR pathway. A schema of the canonical PI3K/AKT/mTOR pathway. PI3K is a cell signaling molecule that is activated by a series of tyrosine kinase receptors on the cellular membrane. Once activated, PI3K causes the conversion of PIP2 to PIP3, which recruits AKT to the cellular surface. PTEN tumor suppressor acts by converting PIP3 to PIP2, thereby reducing PI3K signaling. AKT is later fully activated by the mTORC2 complex by phosphorylation at a second site. Activated AKT phosphorylates TSC2, thereby activating Rheb and subsequently, mTOR. mTORC1 has two main downstream targets, S6K and 4EBP1. Phosphorylation of these targets leads to enhanced translation, cell proliferation, and promotion of cell survival.

PI3K serves as a primary signaling relay molecule, receiving growth signals from activated tyrosine kinase receptors (IGF-1R, c-KIT, PDGFR, EGFR, MET, etc.) on the cell surface and transmitting them to intracellular downstream components.¹⁴⁷ Through interaction with their pleckstrin homology domains, PIP3 brings 3-phosphoinositide-dependent protein kinase 1 (PDK1) and AKT to the cellular membrane. Upon interaction with PIP3, PDK1 is activated and phosphorylates AKT at Thr308.^{148,149} Phosphorylation of AKT at Thr308 results in partial activation of AKT. AKT is phosphorylated further by mTORC2 at Ser473 in the amino terminal domain of AKT to achieve full activation.^{112,150,151} Relative levels of intracellular PIP2 and PIP3 are also regulated by phosphatase and tensin homolog (PTEN), which is an important tumor suppressor and regulator of the mTOR pathway.¹⁵² As a phosphatase, PTEN converts PIP3 to PIP2 via dephosphorylation, thus limiting PI3K signaling.¹⁴⁶

TSC1/2

The tuberous sclerosis proteins (TSC1 and TSC2) are tumor suppressors that regulate the mTOR pathway.¹⁵³ TSC1 and TSC2 heterodimerize to form the TSC1/2 complex, which acts as a GTPase-activating protein to inhibit GTP-binding protein rheb (Ras homolog enriched in brain).¹⁵⁰ In the absence of functional TSC1/2, GTP-bound Rheb can bind to and directly activate mTOR.¹⁵⁴⁻¹⁵⁶ TSC1/2 activity is regulated directly by fully activated AKT, which can phosphorylate TSC2 at Ser939 and Thr1462, thereby inhibiting the complex and activating rheb and subsequently, mTOR.^{150,157,158} TSC2 can also be phosphorylated by other signaling molecules such as MAP kinase pathway protein ERK1/2, which phosphorylates TSC2 at Ser 664 and Ser1798.¹⁵⁹⁻¹⁶¹ Evidence also suggests that S6 kinase can phosphorylate TSC2,

suggesting a positive feedback regulation.¹⁶² Phosphorylation of TSC2, regardless of the responsible kinase, results in inactivation of GTPase-activating activity, enhancement of rheb activation, and promotion of downstream mTOR activation and signaling.^{160,162-165}

Intracellular factors

mTORC1 can also be regulated intracellularly by both amino acid and energy levels. While the mechanisms surrounding regulation via amino acid levels are largely yet to be elucidated, experiments show that these effects are likely indirect.¹⁶⁶⁻¹⁶⁸ Exhaustion of cellular ATP levels can also downregulate mTOR signaling through mechanisms that remain elusive, but may involve LKB1. Evidence suggests that the mechanism likely involves AMPK inactivation via phosphorylation by LKB1, leading to rheb and mTORC1 inhibition.¹⁶⁹ The LKB1 energy sensing pathway is thought to be parallel to the PI3K pathway.¹⁷⁰

Feedback and regulatory loops

The mTOR pathway is a complex pathway and has several feedback and regulatory loops. First, mTORC2, can phosphorylate and partially activate AKT, contributing to full AKT activation and ultimately, activation of the mTORC1 complex.¹⁷¹ This positive feedback loop is triggered upon inhibition of mTORC1. Second, mTOR/S6K are also known to participate in a negative feedback loop with PI3K/AKT via the insulin receptor substrate 1 (IRS1). In this loop, S6K is thought to phosphorylate IRS, thereby inactivating the protein.¹⁷¹

mTOR in cancer

Aberrant mTOR signaling and subsequent cell proliferation and survival effects are thought to be important in tumorigenesis and cancer progression. The PI3K/AKT/mTOR pathway is highly activated and implicated in the pathogenesis of several malignancies, including lung, skin, thyroid, colon, prostate, and endometrial cancers.¹⁷²⁻¹⁸⁰

Notably, several studies have identified high mTOR pathway activity in human soft tissue sarcomas. Iwenofu et al. reported that in a 20 patient cohort with high grade, metastatic sarcomas, more than half (11 patients) had high pS6RP expression before beginning treatment.¹⁸¹ Additionally, Dobashi et al. immunohistochemically evaluated 140 bone and soft tissue sarcomas for AKT/mTOR pathway activation and concluded elevated AKT and mTOR expression was evident in 61% and 66% of tumors, respectively.¹⁸² Staining of a tissue microarrays for proteins in the PI3K/AKT/mTOR pathway showed that the pathway was highly active in human myxoid/round cell liposarcoma, epithelioid sarcoma, and malignant peripheral nerve sheath tumors.¹⁸³
^{184,185}

In vitro, many studies have identified AKT/mTOR pathway upregulation/overexpression in cancers in general and in sarcoma specifically. A study in ovarian carcinoma cells show elevated pAKT levels and targeting of the AKT/mTOR pathway in this model inhibited cell proliferation.¹⁸⁶ Dobashi et al. examined a panel of bone and soft tissue sarcomas using immunoblotting and showed prominent activation of AKT as well as mTOR downstream targets S6K and

4EBP1.¹⁸² In a panel of STS cell lines, Zhu et al. demonstrated that STS cells highly express phosphorylated AKT.¹⁸⁷

Elevated activity of mTOR pathway proteins have been observed, identified as potential prognostic factor(s), and correlated to outcome in multiple cancers, including sarcoma.^{109,188-191} In hepatocellular carcinoma, Feng et al. showed that there is a correlation between phospho-mTOR levels, tumor size, and metastasis potential.¹⁸⁰ In rhabdomyosarcoma, high levels of AKT were associated with poor disease-free and overall survival.¹⁹¹ A patient-based study in sarcoma found that activation status of the AKT/mTOR pathway was significantly associated with poorer survival.¹⁰⁹ Additional studies found that enhanced AKT activation in sarcoma significantly correlated with a higher probability of developing metastases¹⁸² and that enhanced AKT/mTOR pathway activity was associated with shorter recurrence-free and poorer overall-survival in childhood rhabdomyosarcoma.¹⁹¹ Several other studies have shown that intratumoral expression of the mTOR pathway in cancer associated with tumorigenesis, progression, recurrence, metastases, and poor prognosis.^{137,192-195}

mTOR in LMS/ULMS

mTOR pathway hyperactivation has been identified in many cancer types, including sarcoma; however, relatively few studies on the AKT/mTOR pathway have been conducted in LMS and to the best of our knowledge pathway activation/deregulation has not yet been reported in human ULMS. Evidence to suggest that the AKT/mTOR pathway is highly active in LMS stems from a study by Hernando et al., who proposed that elevated activation of the mTOR pathway played a defining role in the

development of LMS.⁹⁸ Using GEM mouse models, researchers conditionally knocked out PTEN, an important tumor suppressor that suppresses the mTOR pathway. PTEN knock out led to rapid development of leiomyosarcoma in the mouse model, likely due to elevated activation of the mTOR pathway, suggesting that mTOR activation was a driving force in leiomyosarcomagenesis in these mice and suggesting potential roles for PTEN deletion and subsequent mTOR pathway activation in the formation of human ULMS.⁹⁸ As part of larger studies in STS, there is also some evidence of AKT/mTOR pathway hyperactivation in LMS.^{107,187,196} As part of a larger *in vitro* STS study, our group reported elevated pAKT in Mes-Sa and SKLMS1 (uterine sarcoma and leiomyosarcoma of gynecological origin, respectively).¹⁸⁷ Very few studies using LMS or ULMS cell lines/strains have been conducted and therefore, little is currently understood.

Causes of mTOR pathway deregulation

There are several possible deregulations that could lead to mTOR pathway activation in cancer. First, overexpression or mutation leading to hyperactivation of receptor tyrosine kinases (RTKs) could potentially induce high mTOR pathway signaling activation. In fact, overexpression/upregulation of several RTKs have been shown in carcinoma and in sarcoma.¹⁹⁷⁻²⁰⁴ Many activating mutations or amplifications within pathway components such as PI3K, AKT, TSC1/2, and mTOR have been identified in cancer and could also significantly contribute to tumorigenesis.²⁰⁵⁻²⁰⁷ Loss or inactivation of tumor suppressors regulating a given pathway could also lead to hyperactivation. For example, Cowden's syndrome is characterized by the loss of

PTEN due to gene mutation.²⁰⁸ As previously mentioned, PTEN expression can also be lost due to methylation of the PTEN promoter region.²⁰⁸

Several scenarios could explain the elevated activity of the mTOR pathway observed in sarcoma and other cancers. While much research focuses on identifying the pathway as highly activated, fewer concentrate on possible explanations. Additional studies to identify deregulation(s) within the mTOR pathway that lead to increased pathway activation are essential to develop novel and more specific targeting therapeutic strategies for anti-cancer treatment, especially in ULMS, where little is currently known.

Targeting mTOR in cancer: rapamycin and rapalogs

mTOR pathway activation has been established in cancer and more specifically, in sarcoma. Many malignancies with PTEN loss of function (and therefore activated mTOR pathways) are very sensitive to mTOR inhibition and respond well to mTOR inhibitors.^{209,210} As such, the pathway represents an attractive axis for therapeutic targeting in anti-cancer therapies and has fueled development of several mTOR inhibitors.

Rapamycin, a lactone-based macrocyclic compound, inhibits mTORC1 and abrogates many tumorigenic effects of the mTOR pathway (**Figure 4**). The potent mTOR inhibitor was originally discovered on Rapa Nui (Polynesian for “Easter Island”), where it was further isolated from *Streptomyces hygroscopicus*.²¹¹ Rapamycin, also known as sirolimus, was initially identified as an anti-fungal agent in the 1970s and nearly

two decades later was found to have significant activity as an immunosuppressant.^{109,211,212} After several years, it was identified as an anti-cancer agent in studies at the National Cancer Institute.^{213,214} Since FDA approval in 1999,

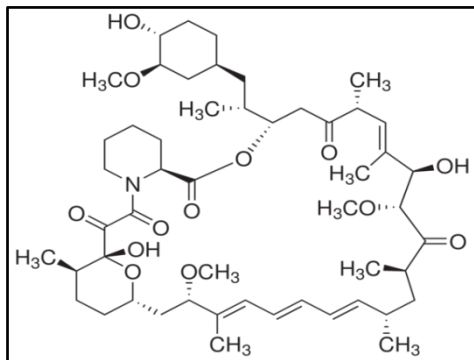


Figure 4. The molecular structure of rapamycin

rapamycin has been administered as an immunosuppressant for transplant patients; and over the past half-decade, in clinical applications for cancer patients.

The mechanism of rapamycin-induced mTOR inhibition has been partially elucidated. Rapamycin binds with immunophilin FKBP12 to form a complex; the complex then binds to the amino terminal domain of mTOR and inhibits the protein.²¹⁵ However, mTOR inhibition via rapamycin appears to be effective in inhibiting only mTORC 1 (but not mTORC 2) complex and therefore does not affect mTORC2 signaling in many models.²¹⁶

Rapamycin has been tested extensively *in vitro* against several types of cancer cells, including sarcoma. Houghton et al. evaluated rapamycin against a panel of pediatric cancer cell lines and found that rapamycin effectively reduced cell proliferation *in vitro*.²¹⁷ Using concentrations ranging from 0.01 – 100nM for cell growth assays, a median EC₅₀ of about 0.7nM was found; almost half of all sarcoma lines tested

responded with $\geq 50\%$ growth inhibition.²¹⁷ Further, solid tumor pediatric sarcoma xenografts were treated with rapamycin to examine its efficacy *in vivo* using mouse models. Seventy five percent (27/36) of solid tumor xenografts achieved event-free survival while on rapamycin therapy.²¹⁷ Preclinical studies using rapamycin in combination with an epidermal growth factor inhibitor (erlotinib) for the treatment of epithelioid sarcoma also resulted in improved therapeutic benefit compared with erlotinib administration alone *in vitro* and *in vivo* using mouse models.¹⁸⁴ Rapamycin was also found to extend survival in an experimental bone metastases model.²¹⁸

Little is known about the role of mTOR inhibition in ULMS; however, several *in vitro* studies in LMS and STS suggest the possibility of a highly activated mTOR pathway in ULMS, which would render rapamycin-based therapy a viable therapeutic option. Human leiomyosarcoma cell lines (SKN [SKLMS1] and SK-UT-1) were used to test the effects of rapamycin *in vitro*.¹⁹⁶ Rapamycin administration induced significantly reduced SKN cell proliferation and western blotting confirmed diminished levels of phosphorylated mTOR, 70S6K, and S6K. Notably, rapamycin did not induce apoptosis.¹⁹⁶

After publishing a single case report demonstrating the superior effects of rapamycin and gemcitabine in a patient with LMS,²¹⁹ Merminsky et al. carried out a series of cell-based experiments combining rapamycin with either common chemotherapy gemcitabine or the c-KIT/PDGFR inhibitor imatinib mesylate using several cancer models, including a cell line made from cervical leiomyosarcoma (SKLMS1).²²⁰ Of note, gemcitabine is a commonly used first-line chemotherapy in LMS and yielded

synergistic response when combined with rapamycin in a pancreatic cancer model.²²¹ However, in leiomyosarcoma, gemcitabine showed neither synergistic nor significant benefit in combination rapamycin versus single agent therapy with the exception of results at very short time points of less than 48h.²²⁰ In the same study, imatinib mesylate was also examined as combination therapy with rapamycin in leiomyosarcoma because of high PDGF expression found in SKLMS1 cells.^{220,222} Further rationale for this combination can be derived from the work of Mohi et al., who showed that rapamycin and imatinib mesylate combination therapy yielded synergistic cell growth reduction effects in leukemia.²²³ Interestingly, monotherapy with imatinib mesylate was not effective in inhibiting SKLMS1 cell growth *in vitro* and combination therapy with rapamycin did not significantly reduce tumor cell growth and yielded results similar to rapamycin treatment alone, indicating that it may not be effective for the clinical treatment of ULMS.²²⁰

At clinically relevant doses (5-10mg/m² daily), rapamycin can induce toxicities including hypertriglyceridemia, thrombocytopenia, leucopenia, fever, and anemia, however, it is relatively well tolerated.²²⁴⁻²²⁷ Several clinical studies have tested rapamycin efficacy versus carcinomas,²²⁸⁻²³¹ however, relatively few studies have been conducted in sarcomas.²³² Extensive human studies have not been carried out with rapamycin given several limitations with the drug. Rapamycin has poor solubility in aqueous formulations for oral administration and shows problematic bioavailability profiles, presumably due to cytochrome P450 3A4/5 metabolism.¹³⁴ To overcome these challenges, derivatives of rapamycin (rapalogs) have been developed.

Currently, three rapamycin analogs (rapalogs) are FDA approved and extensively studied: CCI-779 (temsirolimus), AP23573 (deforolimus), and RAD-001 (everolimus).

Temsirolimus

Rapalog temsirolimus (CCI-779) was developed by Wyeth Pharmaceuticals and was approved by the FDA in 2007. It differs from rapamycin by an additional esterdiol group and is approved by the FDA for the treatment of cancers including renal cell carcinoma. It has been evaluated in several clinical trials for lymphoma, glioblastoma multiforme, endometrial carcinoma, and metastatic breast cancer.²³³⁻²³⁶ In a recent phase II STS clinical trial of 41 total patients including nine with leiomyosarcoma and five with sarcomas of the uterus, pharmacokinetic studies indicated that >80% of patients achieved doses of temsirolimus sufficient to inhibit pS6K.²³⁷ Unfortunately, only 2 patients, including one with LMS, had partial responses while 95% (39/41) of STS patients experienced tumor progression while on temsirolimus therapy.²³⁷

Temsirolimus was also tested against advanced leiomyosarcoma in a small cohort of six patients based on the premise that PTEN loss is a common mTOR pathway-activating deregulation in LMS. In the study, 50% (3/6 patients) experienced disease stabilization while on therapy; the remainder, including the single patient with ULMS, had disease progression.²³⁸ Interestingly, the PTEN status of each patient was tested in this study; five of six patients did not show loss of PTEN as assessed via immunohistochemical methods, and PTEN status was not predictive of response to temsirolimus.²³⁸ A phase I trial of a novel oral temsirolimus formulation was conducted in a cohort of 24 STS patients, two of which had leiomyosarcoma.²³⁹ While,

none of the 19 evaluable patients had partial or complete responses, seven (about 1/3 of study patients) had disease stabilization for several weeks, including one LMS patient.²³⁹ Taken together, these results suggest that temsirolimus may not be a viable monotherapy option in STS, but may possess useful anti-tumor activity against leiomyosarcoma.

Ridaforolimus

Ridaforolimus (AP23573, deforolimus, MK-8669) was developed by ARIAD Pharmaceuticals Inc. in an effort to overcome difficulties with rapamycin solubility for drug formulation. This rapamycin analog is water-soluble and has enhanced *in vivo* stability. Ridaforolimus is the most recently developed rapalog and has been examined briefly in SKLMS1 cells as part of a large cancer cell model panel. Rivera et al. showed inhibition of mTOR signaling in SKLMS1 using ridaforolimus at concentrations of 0.3-1nM, suggesting that leiomyosarcoma may be fairly sensitive to mTOR inhibition.²⁴⁰ In a wide array of cell models, they also showed that loss of PTEN did not correlate with cell sensitivity to ridaforolimus therapy.²⁴⁰

In addition to cell-based models, ridaforolimus has been examined in several clinical trials including sarcoma. Chawla et al. evaluated the efficacy of ridaforolimus in a phase II clinical study in patients with advanced bone and soft tissue sarcomas that did not respond to several previous chemotherapy regimens.²⁴¹ In total, 28.8% (n=61) achieved clinical benefit response for a median progression free survival of just 15.3 weeks.²⁴¹

In October of 2011, ARIAD, in partnership with Merck Pharmaceuticals, filed a FDA New Drug Application for the use of ridaforolimus in metastatic STS and bone sarcomas in patients who responded well to chemotherapy. Ridaforolimus resulted in only mild toxicity in Phase I sarcoma clinical trials and inhibited mTOR downstream target p4EBP1.²⁴² Preliminary results in STS showed that 28% of patients responded (complete response, partial response, or disease stabilization) for at least 16 weeks with ridaforolimus therapy.²⁴¹ In another study, partial response was observed in 2% of patients and disease stabilization was identified in an additional 27%.²⁴³ Studies have also found that patients with high levels of pS6K are more likely to experience disease stabilization while on deforolimus and that pS6RP expression can be used to predict early tumor response to mTOR inhibition-based therapies such as deforolimus.¹⁸¹ Finally, a phase III trial (NCT00538239) is currently underway to determine the efficacy of this drug as long-term maintenance therapy.

Everolimus

Everolimus (RAD001, Zortress, Certican, Afinitor), owned by Novartis, has been minimally examined using animal modeling in LMS. Hernando et al. reported that the mTOR/AKT pathway is overexpressed in and drives leiomyosarcomagenesis in a transgenic mouse model with loss of PTEN.⁹⁸ To determine if mTORC1 inhibition significantly prolonged life, everolimus was administered when tumors were detectable by MRI at 1 month of age. Everolimus had considerable effect on leiomyosarcoma in these models; average lifespan of mice in the control group was 9.4±4.0 weeks compared to >37 weeks (at which time therapy was halted) in the everolimus-treated mice.⁹⁸ Notably, downregulation of pAKT and pS6K was found in

these mice; reduced Ki67 expression and minimal TUNEL positivity compared with controls was also observed, suggesting cytostatic rather than cytotoxic effects.⁹⁸

Everolimus is currently approved by the FDA for the treatment of several cancers including pancreatic neuroendocrine tumors, astrocytomas, endometrial carcinoma, recurrent and metastatic breast cancer, and advanced kidney cancer.²⁴⁴⁻²⁴⁷ Notably, a patient with metastatic renal cell carcinoma achieved disease stabilization for >28 months; a partial response with everolimus was identified after 28 months, supporting the use of mTOR inhibition in long-term maintenance for cancer.²⁴⁷

Few studies have examined the efficacy of everolimus therapy in LMS or ULMS. A pre-clinical and phase I trial combined everolimus mTOR inhibition with figitumumab IGF-1R kinase receptor inhibition in STS, demonstrating relatively favorable efficacy: toxicity ratios.²⁴⁸ In this study, disease stabilization was noted in 71% of patients and only 10% experienced disease progression while receiving combined therapy.²⁴⁸ Nearly a quarter of the 21 patients enrolled in this trial had leiomyosarcoma. Everolimus and figitumumab combination therapy in these leiomyosarcoma patients yielded stable disease in three of four individuals with long-term disease stability in one patient.²⁴⁸

Taken together, studies with mTOR inhibitors suggest that rapamycin and its analogs may have clinical benefit, chiefly by inducing disease stabilization, in certain subtypes of cancer such as LMS. The upregulation/overexpression of the mTOR pathway in LMS and the use of rapamycin or rapalogs in LMS is supported by multiple *in vitro*

and *in vivo* studies. Despite promising results in LMS, very little is known about possible deregulations in the mTOR pathway or the potential efficacy of mTOR inhibitors against human ULMS. Additional preclinical studies are necessary to examine pathway activation, identify potential deregulations, and test the efficacy of mTOR inhibitors in ULMS alone and in combination with other potential ULMS-relevant therapies.

Aurora A kinase

Recently we have identified that ULMS overexpress gene products responsible for regulating centrosome structure and function, highlighting a role for Aurk A as a novel ULMS therapeutic target.^{249,250} Several lines of evidence support a role for Aurk A in cancer: 1) it was found to be amplified and/or over-expressed in multiple cancers including breast, ovarian, and hepatic carcinomas,²⁵¹⁻²⁵³ 2) forced Aurk A expression resulted in NIH3T3 fibroblast oncogenic transformation and induced aneuploidy in a nearly diploid breast cancer cell line,²⁵⁴ and 3) Aurk A interacts with and modifies the function of several key cancer-associated molecules such as BRCA1 and p53.²⁵⁵

Aurora A Kinase

Aurk A kinase is part of a family of serine/threonine kinases (Aurk A, Aurk B, and Aurk C) that are critical in cell cycle regulation, especially mitosis.²⁵⁶ Aurora kinases were originally discovered in *Drosophila*, where absence of this protein led to chromosomal separation failure with the subsequent formation of monopolar spindles. Because the defects occurred at the spindle poles, the proteins were named “Aurora” kinases because of the *aurora borealis* light displays that are occasionally seen at the poles of the Earth.²⁵⁷ The Aurora kinases are evolutionarily conserved in mammals and are also found in several other species (**Table 5**). Aurora kinases in mammals have an N-terminal regulatory domain and a C-terminal catalytic domain.²⁵⁸ Although their regulatory domains are very different, the family members share >70% homology in their catalytically active domains (**Figure 5**).²⁵⁵

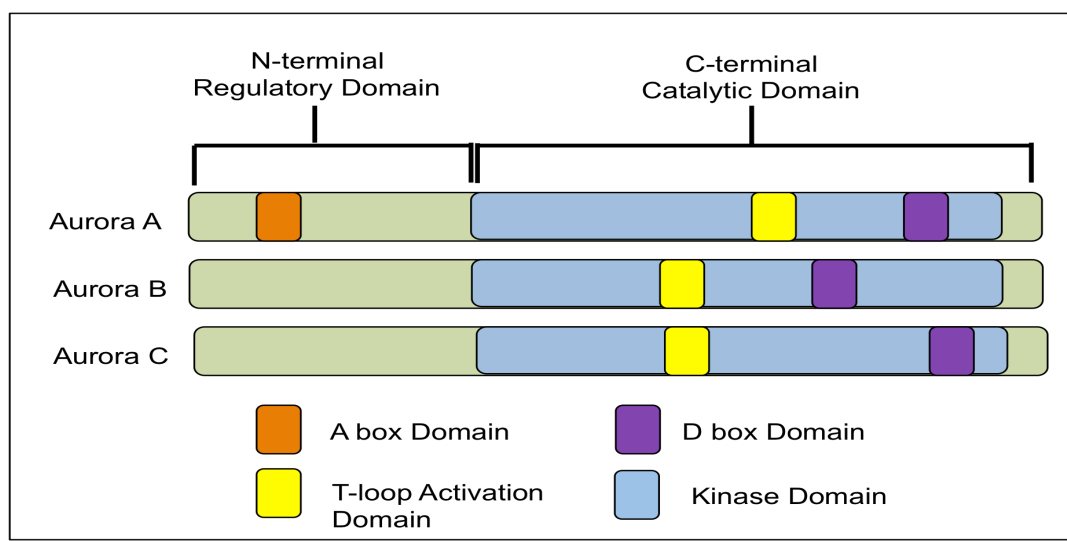


Figure 5. Aurora kinase protein domains. Functional domains in Aurora kinases A, B, and C. (Adapted and reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] Keen et al. Aurora-kinase inhibitors as anticancer agents, 2004).²⁵⁸

Table 5. Aurora kinases are conserved across several species.

Species	Aurk Kinases	Names	Author (Year)
<i>Drosophila</i>	2	Aurk A, Aurk B	Glover (1995)
<i>Saccharomyces cerevisiae</i>	1	Ipl1	Chan (1993)
<i>Caenorhabditis elegans</i>	2	AIR-1, AIR-2	Schumacher JM (1998)
Xenopus	2	Eg2, AIRK2	Adams (2000)
Mammals	3	Aurk A, Aurk B, Aurk C	Nigg (2001)

Aurk A kinase

Aurora A kinase (also called Aurk A kinase, BTAK, STK6, AIK, ARK1, AURA, STK7, STK15, Aurora 2, and MGC34538) is a 46kDa, 403 amino acid serine/threonine kinase that was first discovered in *Drosophila* in 1995; it is functional in cell cycle regulation^{257,259} and is located on human chromosome 10q13.2, a region that is often amplified in cancers.^{254,260} Aurk A kinase activity is directed towards arginine residues within the active loops of Aurk A substrates, making the protein an arginine-directed kinase.²⁵⁵ A threonine residue at amino acid 288 serves as the main site for activation of Aurk A kinase function, which requires phosphorylation of the T288 residue. Since resolution of the crystal structure of Aurk A in 2002 by Cheetham et al., it is now well established that Aurk A has two major domains; an amino terminal regulatory domain and a carboxy terminal catalytic domain.²⁶¹ Similarly to many other serine/threonine kinases, the catalytic domain is comprised of two lobes adjoined by a hinge region.^{261,262} A carboxy terminal D-Box and amino terminal A-box are required for Aurk A degradation via ubiquitinylation by APC/C complex.²⁶³⁻²⁶⁵

Unlike Aurk B and C family members, Aurk A has distinct localization, binding partners, and functions. Aurora A expression emerges in late S and early G2 phases and diminishes by the end of telophase with peak activity observed in G2-M transition.^{266,267} During the S to G2 transition, Aurk A begins to localize at the centrosomes; by metaphase, Aurk A is localized with spindle poles, microtubules and the microtubule organizing centers.²⁶⁸ Aurk A expression diminishes towards the end of mitosis when cyclin B expression is decreased.²⁶⁶

Aurk A kinase substrates

Aurk A kinase is a key regulator of mitosis. To date, Aurk A has several identified substrates (**Table 6**).^{255,259,269-286} Aurk A exerts both kinase and non-kinase functions on these substrates, the majority of which are centrosome or spindle related proteins. Karthigeyan et al. reviews the interactions of all known Aurk A substrates in depth.²⁵⁵

At this time, Aurk A mechanisms of action can be classified as kinase-independent or kinase-dependent.²⁵⁵ While most are kinase-dependent interactions, two kinase-independent mechanisms have been identified, and interactions with several additional proteins remain to be elucidated.²⁵⁵ Centrosomin is a centrosomal protein critical in centrosomal assembly, maturation, and function in both *drosophila melanogaster* and mammalian cells.^{287,288} Terada et al. showed that Aurk A binds to the carboxy terminal domain of centrosomin and are mutually dependent on one another for proper localization to spindle poles.²⁸⁸ The amino terminal domain of centrosomin binds to γ -tubulin, initiating microtubule nucleation and anchoring the tubulin to the centrosome.²⁸⁸ Aurk A also stabilizes n-Myc by inhibiting ubiquitin ligase SCF^{Fbxw7} interaction, thereby allowing stability of n-Myc that is independent of growth factor signaling.^{289,290}

Several protein substrates of Aurk A kinase are phosphorylated by the protein at a consensus site of K/RXT/SI/L/V.²⁹¹ Several of these Aurk A-regulated proteins are involved in transcriptional regulation. BRCA1 is a DNA repair molecule and E3 ubiquitin ligase is phosphorylated by Aurk A at Ser308. Phosphorylation results in

Table 6. A list of known Aurk A binding partners and their functional consequences. (used with permission from Karthigeyan et. al)²⁵⁵

First Author	Protein	Residue Affected	Functional Consequence
Toji (2004)	Lats2	Ser83	Centrosome maturation
Venoux(2008)	ASAP	Ser625	Bioplar spindle assembly
Mori (2007)	NDEL1	Ser251	Microtubule reorganization, centrosome separation/maturation
Sakai (2002)	MBD3	Ser24	Undetermined
LeRoy (2007)	TACC3	Ser558	Regulates XMAP215
Zhang (2008)	MCAK	Ser196, Ser719	Loss of microtubule depolymerase activity
Kemp (2004)	SPD2	Undetermined	Enhanced microtubule depolymerase activity
Geit (1999)	Eg5	Ser543	Recruits SPD5, centrosome maturation
Dutertre (2004)	Cdc25B	Ser353	Microtubule organization
Rong (2007)	RASSF1A	Thr202, Ser203	M phase progression
Wirtz-Peitz (2008)	Par6	Undetermined	NUMB localization in cell division
Du (2004)	P160ROCK	Undetermined	Blocks transactivation
Yu (2008)	CFos	Undetermined	Undetermined
Kunitoku (2003)	CENP-A	Ser7	Kinetochore organization
Huang (2002)	CPEB	Ser174	Oocyte maturation
Seki (2008)	Plk1	Thr210	Centrosomal organization
Yu (2005)	HURP	Ser627, Ser725, Ser757, Ser830	Serum-independent growth acceleration
Ouchi (2004)	BRCA1	Ser308	Microtubule nucleation
Katayama (2003)	p53	Ser215, Ser315	Blocks transactivation, enhances proteosomal degradation
Briassouli (2007)	IκBa	Ser32, Ser36	Enhances NFκB activity
Colon (2011)	GSK-3β	Ser9	Enhanced β-catenin activity
Colon (2011)	N-Myc	Kinase independent	Stabilization of N-Myc
Terada (2003)	Centrosomin	Kinase independent	Centrosome maturation

abrogated ligase activity and this interaction is thought to be critical in G2/M transition as well as centrosome-dependent microtubule nucleation.^{292,293} Aurk A also phosphorylates GSK-3 β , resulting in accumulation of β -catenin in the nucleus. Nuclear β -catenin binds to LEF/TCF; together these proteins initiate transcription of several pro-growth molecules such as c-Myc and Cyclin D1.^{294,295} Tumor suppressor and transcription factor p53 is regarded as the gatekeeper of the human genome and is often mutated in cancers. Aurk A phosphorylates p53 at Ser315 and Ser215. Phosphorylation at Ser315 strengthens p53 and MDM2 interaction, leading to increased degradation of p53.^{296,297} Aurk A phosphorylation at Ser215 abrogates DNA binding ability and eliminates transcriptional activity of p53 including transcription of downstream targets such as p21 and PTEN.²⁹⁸

Overall, Aurk A kinase is required for several centrosome and spindle assembly-related functions in mitosis.²⁶⁸ Mutations in Aurk A can lead to centrosome separation failure and monopolar spindle development, resulting in improper cell division.^{257,299,300} Aurk A overexpression can produce chromosomal misalignment, defective mitotic spindles, and inappropriate chromosomal segregation, which can all contribute to malignant phenotypes.^{260,301}

Aurora A kinase in cancer

Aurk A kinase has been shown to promote many processes significant in cancer formation and progression, and is upregulated in multiple cancer cell lines and malignancies including >50% of ovarian, colorectal, and ovarian tumors and 94% of invasive ductal breast adenocarcinomas.^{254,302-304} We recently reported Aurk A

upregulation and overexpression in ULMS.^{249,250} Aurk A overexpression and chromosomal amplification are characterized in many different tumor types. The protein is encoded on chromosomal region associated with frequent amplification in cancer.^{254,260,266,305,306} In addition to chromosomal amplification, Aurk A mRNA and protein expression is often upregulated in hepatocellular and ovarian carcinomas, colorectal cancer, neuroblastoma, breast cancers, and many other malignancies.^{251,307-310} Many of these reports also indicate that Aurk A protein expression levels correlate well with prognosis or survival and that it may be useful as a prognostic factor.^{251,307,309-311}

Aurk A is implicated in numerous processes with known roles in cancer such as inactivation of tumor suppressor p53 and DNA repair molecule BRCA1, up-regulation of pro-cancer genes, and promoting cell survival and cellular motility/migration (described previously).²⁵⁵ Several functional studies have been performed to determine whether Aurk A overexpression alone is sufficient for malignant transformation; the results are inconclusive. While several groups have shown that overexpression of Aurk A kinase is sufficient to transform NIHT3T and rat fibroblast cell lines, several others demonstrate that Aurk A overexpression alone is not sufficient to induce malignant transformation.^{254,260,312} Forced overexpression of Aurk A helps DNA damaged cells bypass mitotic checkpoints, evade regulatory cell death mechanisms, and develop chemoresistance, all of which lead to genetic instability, a hallmark of cancer.

Targeting Aurk A kinase in cancer

Given the potential usefulness of Aurora A inhibition in cancer therapy, several anti-Aurk A drugs have emerged, have shown pre-clinical efficacy, and are currently undergoing further clinical investigation (**Table 7**). MLN8237 (Alisertib, Millennium Pharmaceuticals, **Figure 6**) is a second-generation small molecule Aurora A kinase inhibitor that blocks Aurk A kinase activity *in vitro* with concentrations as low as 1.2nM (enzyme assay). While it is a specific Aurk A inhibitor, it can also weakly inhibit Aurk B kinase at or above 400nM concentrations.³¹³⁻³¹⁵ Nevertheless, MLN8237 exhibits a

Table 7. Commercially developed Aurk A inhibitors.

Inhibitor Name	Additional Names	Target(s)
ZM447439	-	Aurora A and B kinases
VX-680	tozasertib	Aurora A, B, and C kinases
PHA-680632	-	Aurora A, B, and C kinases
PHA-739358	danusertib	Aurora A, B, and C kinases
MK-5180	-	Aurora A kinase
MLN 8054	-	Aurora A kinase
MLN8237	alisertib	Aurora A kinase

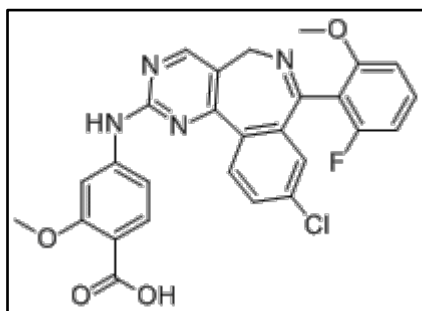


Figure 6. Structure of MLN8237.

200-fold selectivity for Aurk A over Aurk B or C *in vitro*; comprehensive studies indicate that no significant cross-reactivity occurs in a diverse panel of receptors, ion channels, and 205 kinases.³¹⁵ Mechanistically, MLN8237 binds to the active site of the kinase domain in Aurk A, thereby preventing activation. This orally administered and rapidly available investigational drug is thought to promote mitotic spindle abnormality, trapping many cells in mitosis resulting in polyploidy and (ultimately) death via apoptotic pathways.³¹⁴

MLN8237 has been evaluated in a number of cell types. It was found most effective in leukemia and lymphoma cell lines, but also exhibited activity against Ewing's sarcoma and neuroblastoma at >100nM doses. Rhabdomyosarcoma cell lines had IC50s in the 100nM to 10uM range.^{316,317} Preliminary studies in mouse neuroblastoma xenografts as well as human lymphoma and leukemia indicate that MLN8237 effectively inhibits tumor growth at doses of 20mg/kg given twice daily for three weeks. To our knowledge, MLN8237 had not yet been tested in uterine leiomyosarcoma, leiomyosarcoma, or comprehensively in soft tissue sarcomas *in vitro* or in mouse models.

MLN8237 been evaluated in a few clinical trials in cancer therapy to date. Phase I and II clinical trials with MLN8237 in pediatric solid tumors, leukemia, ovarian cancer, non-Hodgkin's lymphoma, peritoneal carcinoma, and hematological malignancies have shown marked improvement over standard treatments.^{313,318} Notably, a phase II clinical trial is currently underway to assess the efficacy of MLN8237 treat recurrent refractory solid tumors or leukemia in young patients. This study is enrolling patients

with sarcoma and is anticipated to be complete in February 2016. Although the results of the phase I clinical trial have not yet been published, a 2010 abstract from the annual American Society of Clinical Oncology (ASCO) meeting reveals preliminary study results; the most effective, toxicity-limiting dosing option is 80mg/m²/d to be given once per day for seven continuous days in a 28 day cycle. Dose limiting toxicities in this study included severe mood alteration, mucositis, neutropenia-thrombocytopenia, myelosuppression, and somnolence.³¹⁹

Chapter 2: Materials and Methods

Patients and clinical database. Medical records from more than 350 patients who were evaluated at The University of Texas MD Anderson Cancer Center (UTMDACC) for ULMS between January 1989 and April 2011 were accessed under the permission of an Institutional Review Board (IRB) approved protocol (Protocol #RCR05-0260). Patients included in the database were required to have documented history of uterine leiomyosarcoma. A clinical database was constructed using ClinicStation to incorporate many parameters including: 1) patient-related variables: date of birth, sex, race, age at admission, history of previous hysterectomy unrelated to ULMS diagnosis, and family history of sarcoma or other cancers; 2) medical history-related variables: first symptom associated with diagnosis of ULMS, age at initial diagnosis of ULMS, stage at diagnosis, number of recurrences prior to evaluation at MD Anderson, development of metastases prior to evaluation at MD Anderson; 3) tumor-associated variables: stage at presentation to UTMDACC, status at presentation to UTMDACC, tumor size, and number of mitoses per 10 high power fields; 4) treatment-related variables: neoadjuvant or adjuvant chemotherapy or radiation interventions, descriptions of surgical interventions, surgical margins obtained, and if lymph nodes were resected and found to contain malignant cells; and 5) outcome-related information: recurrences post-admission at UTMDACC, post-admission development of pulmonary and/or extra-pulmonary metastases and follow up information consisting of date of last follow up and status at that time.

Creation of tissue microarray (TMA). Under an IRB approved protocol at UTMDACC (Protocol #LAB04-0890), our clinical database was searched for patients for which formalin-fixed, paraffin-embedded (FFPE) tissue blocks were available. From our initial cohort of more than 350 patients, more than 200 could not be included on the TMA, mainly due to lack of tissue availability. All patients selected in our TMA cohort had sufficient follow up with the UTMDACC sarcoma center. Blocks were obtained for all cases that matched our criteria and the clinical diagnosis of ULMS was re-confirmed by UTMDACC pathologists (Alexander J. Lazar and Elizabeth G. Demicco, both soft tissue sarcoma experts) by further examination of H&E stained slides. The tissues were then organized, and control tissues were selected for inclusion on the TMA. H&E slides corresponding to each block were marked with a circle around the desired tumor area to indicate from where the punches were to be taken. An automated TMA apparatus (ATA-27, Beecher Instruments) was used to perform 0.6mm core punches in duplicate for each block. These punches were then formatted into a standard 4.5 x 2 x 1 cm recipient block to create ULMS TMA blocks A, B, C, and D as previously described.³²⁰ H&E stained slides of the cut TMA blocks were used to verify viable tumor within the punch sample area throughout the block. As previously described, ClinicStation was used to assemble an annotated clinical database corresponding to each sample that was used for correlative analysis.

TMA immunohistochemistry and scoring criteria.

Antibodies. Once constructed, the TMA was stained immunohistochemically for several potential biomarkers of interest. All primary antibodies and working

concentrations used for immunohistochemistry in this body of work are identified in **Appendix Table A1**.

Immunohistochemical staining. Tissue microarray slides were placed in an oven at 60°C overnight. Next, slides were de-waxed and rehydrated by a series of xylene and ethanol incubations. Antigen retrieval via microwave for 10-20min at 98°C or steam cooker for 45min was carried out. Following endogenous blocking with H₂O₂ as previously described,³²⁰ samples were blocked with a 4% fish gelatin or 5% normal horse serum with 1% normal goat serum in PBS for 20min at room temperature (RT). The primary antibody was then applied overnight at 4°C in a humidity chamber. After several washes, secondary antibody was applied and incubated; a Mach 4 Universal HRP Polymer Detection Kit (BioCare Medical) or goat anti-rabbit/goat anti-mouse HRP (BioCare Medical) was used for detection. Briefly, excess 3,3-diaminobenzidine (DAB, Open Biosystems) was added to cover the tissues completely; and tissues were monitored via brightfield microscopy. When adequate DAB incubation length was reached, samples were washed in deionized water several times followed by 15-20 second staining with Gill's #3 hematoxylin (Sigma Aldrich). Slides were covered with Permount (Fisher Scientific, Inc) and glass cover slips; slides were allowed to dry horizontally before further examination. A BONDMAX automated immunohistochemistry apparatus and protocol was used to stain for some proteins. Specific protocols for each antibody are detailed in **Appendix Table A2**.

Scoring criteria. Following staining for specified proteins; TMA slides were scored by independent observers (Kari Brewer Savannah, Alexander Lazar and Elizabeth

Demicco; the latter two are soft tissue sarcoma pathology experts). Care was taken to exclude samples of insufficient tumor quality or tissue from further analysis. TMA slides were scored for both intensity (0 = absent, 1= weak, 2= moderate, 3= strong) and for distribution (percentage of cells in each tissue sample staining positively for each protein, 0-100%). Distribution was scored as absent (<10% of cells per sample positive), low (10-49% of cells per sample positive) and high (\geq 50% cells per sample positive). Membranous staining for some proteins was also noted. For most markers, intensities of 0 or 1 (absent and weak) were grouped together and considered “absent-weak” intensity while intensities of 2 or 3 (moderate and strong) were combined as “moderate-strong” intensity for statistical analysis. Distribution expression levels were grouped as none (under 10% of cells positive), low (10-49% positive) and high (>50% positive). For most markers, only samples with >50% of cells staining positively were considered as “high” for statistical evaluation. For progesterone receptor, estrogen receptor, Ki67, and cyclin D1, only the percentage of cell nuclei per sample expressing the proteins was evaluated. For statistical calculations, all samples with >10% of cells per sample with positive expression were considered high expression. Rb intensity was scored as absent (score of 0) or strong (score of \geq 1) for statistical analyses. Likewise, the percentage of cells expressing Rb per ULMS sample was scored as absent (<10% cells per sample positive) or high (>10% of cells positive). SMA, SMM, desmin, caldesmon, WT-1, c-KIT, nuclear β -catenin, and membranous β -catenin were scored as absent (-), high (+) or weak/focal, which was counted as high (+) for statistical considerations. All other proteins were scored as per the described standard.

ULMS primary cell cultures. ULMS primary cell cultures were created by our laboratory directly from patient tumors under an IRB approved protocol and with informed consent from the patients (Protocol #LAB06-0581). Surgical specimens were received in ~20mL sterile PBS in a conical vial with approximately 5-30g of tumor. PBS was aspirated and the tumor was transferred to a glass petri dish. 10-20mL of DMEM media was added and the tumor was cut into 1mm portions using a sterile scalpel. Minced tumor was then transferred in DMEM to a 50mL Falcon spinner flask and the petri dish was rinsed 3 times with DMEM to collect any remaining tissue. Media was added to the flask to bring the total volume to 25mL and 2.5mL of 3% collagenase Type 1 (Sigma), 5.0mL of 0.02% DNase I, Type II (Sigma), and 1.0mL of 1.5mg/mL hyaluronidase (Sigma) were added. Concentrations of enzymes were adjusted to reflect initial tumor weight. Spinner flasks were placed on a Bell-Stir at speed 8 at 37°C for 2-4h or until tumor digestion occurred. The sample was then strained through a sterile mesh screen into a new 50mL tube and centrifuged at 1500rpm at room RT for 5min. Undigested tissue was discarded. Following centrifugation, media was aspirated and PBS was added for a total volume of 40mL to wash the cell pellet. The sample was centrifuged a second time at 1500rpm at RT for 10min and PBS was aspirated. The pellet was then resuspended in 20mL of PBS and histopaque tubes were set up for every 1mL of cell pellet. 10mL of 100% histopaque (Sigma) was added to each 50mL conical tube and 15mL of 75% histopaque in PBS was added carefully on top of the 100% histopaque. The cell suspension was added slowly to the top of the histopaque, taking care that the solutions were not mixed. Tubes were sealed and centrifuged at 1800rpm for 30min at 4°C. The first interface (tumor cells) was carefully pipetted out of the tube without disrupting the histopaque

layer or second interface (lymphocytes). 50mL of PBS was added to the extracted cells and the solution was centrifuged at 1800rpm at RT for 5min. PBS was aspirated and cells were resuspended in DMEM with high glucose, 10% FBS, and 1% antibiotics (penicillin/streptomycin), placed in a cell culture flask, and incubated at 37°C. All tumor handling was done with sterile technique to prevent possible contamination.

Short Tandem Repeat (STR). DNA fingerprinting via sequencing of short tandem repeats (STR) was used to validate cell lines and the tumors from which they originated at increasing passages. DNA from cell strains/lines was obtained using a Qiagen Blood & Cell Culture DNA Maxi kit as per manufacturer's recommendations. An AmpFISTR Identifier PCR Amplification kit (Applied Biosystems) was used to amplify tumor DNA for fingerprinting. The kit was used as directed by Applied Biosystems and results in amplification of the amelogenin gender determining marker and 15 tetranucleotide repeat loci using multiple primers. Cell strain fingerprints were compared with fingerprints of the original tumors when available; if not available, fingerprints were compared with those from the earliest available passage of cells. Cell line fingerprints were compared with known fingerprints given by the American Type Culture Collection (ATCC) or other published records. These markers/loci and methodology are consistent with worldwide database recommendations for tumor and cell identity validation.

p53 mutational analysis. p53 mutational analyses were conducted on cell strains/lines as previously described.³²¹ Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen Sciences) as per kit instructions. A NanoDrop spectrophotometer was employed to evaluate the integrity and concentration of extracted DNA. Primers specifically designed to recognize and bind intronic sequences flanking exons five through nine of the *p53* gene were obtained (Sigma Genosys). Briefly, 100ng genomic DNA underwent polymerase chain reaction (PCR) amplification for exons 5-9 of the p53 gene in an Eppendorf Mastercycler Pro Thermal Cycler (Eppendorf AG). The PCR product was then sequenced via an Applied Biosystems 373 automated DNA sequencing instrument and sequence analysis was performed with Sequence Scanner (version 1.0, Applied Biosystems).

GEIMSA. 500,000 cells were counted and suspended in 10mL of media. 10µg/mL of colcemid was added for a final concentration of 0.05µg/mL in media and incubated at 37°C for 2h in a 15mL conical tube. The cells were then spun down at 1200rpm for 5min and supernatant was aspirated. The pellet was washed once with 5mL of PBS and spun down again, discarding the supernatant. 1mL of 75mM potassium chloride was added to the cell pellet and incubated at RT for 30min to lyse the cells. Slides were washed with cold ethanol and chilled at -20°C for a minimum of 45min and fixing solution of methanol: glacial acetic acid (3:1) was prepared. The fixing solution was added to KCl/cell suspension for a final volume of 10mL and incubated for 10min at RT. Cells were then spun down at 1200rpm for 5min, supernatant was aspirated, the pellet was resuspended in 1mL of fixing solution, and 1-3 drops of cell suspension was dropped onto each cold slide. Slides were dried at 68°C for 2h and stained with

GIEMSA stain for 1-2h. Following several washes in deionized water to remove excess staining solution, slides were cover slipped and examined in immersion oil at 100x.

Cell culture. Human SKLMS1, Mes-Sa, and normal smooth muscle cells (NSMC) were obtained from the American Type Culture Collection (ATCC, SKLMS1 and Mes-Sa) and ScienCell Research Laboratories (Carlesbad, CA, NSMC). Leiomyosarcoma cell strains Leio012, Leio016, Leio196, Leio285, Leio485, Leio505, and Leio987B were created in our laboratory. Cells were cultured in DMEM/F-12 50/50 with the exception of Mes-Sa and normal smooth muscle cells, which were cultured in McCoy's 5A Modified Medium and Smooth Muscle Cell Medium, respectively. Media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All media and supplements were purchased from Cell Gro, Inc. Short Tandem Repeat (STR) DNA fingerprint testing was performed on cell lines and strains every 10-15 passages to confirm cell identity.

Reagents for experimental procedures. Commercially available antibodies were employed during western blotting, immunohistochemistry, and immunofluorescence. Antibodies directed against desmin, pS6K, S6K, p4EBP1, 4EBP1, pAKT, AKT, PTEN, cyclin D1, and Aurora A kinase were purchased from Cell Signaling (Danvers, MA). Cleaved PARP was purchased from Abcam (Cambridge, MA) and p21, p53, vimentin, and β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CD31 for immunofluorescence studies was purchased from BD Pharmagen (San Diego, CA) and Ki67 was obtained from Thermo/Lab Vision (Kalamazoo, MI).

Additional details regarding antibodies, including working concentrations, are provided in **Appendix Table A3**. Goat anti-rabbit IgG and goat anti-mouse IgG antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used as secondary antibodies. The Dead End Fluorometric TUNEL Kit (Promega, Madison, WI) was employed and used as directed for terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL).

Rapamycin for *in vitro* studies was purchased as a powder and reconstituted in DMSO. A liquid, orally ingested formulation of rapamycin, Rapamune (Pfizer), was purchased from the UTMDACC pharmacy and used for *in vivo* experiments. Doxorubicin was purchased from (APP Pharmaceuticals), diluted with sterile water, aliquoted, and stored in an opaque container at -20°C until use. MLN8237, an investigational Aurk A inhibitor, was kindly provided by Millennium Pharmaceuticals (Cambridge, MA). For animal studies, MLN8237 powder (95.94% pure) was suspended in an equivolume solution of MLN8237 dissolved in 20% 2-hydroxypropyl- β -cyclodextrin in sterile water and a solution of 2% sodium bicarbonate in sterile water for final concentrations of 10% 2-hydroxypropyl- β -cyclodextrin and 1% sodium bicarbonate. The two solutions were prepared separately within 14 days of use and mixed together each morning for daily use. The 95.94% purity of MLN8237 in powder form was accounted for in animal calculations so that each mouse received 30mg/kg/daily of active drug.

Western blotting. Western blot analyses were used to evaluate levels of protein expression and phosphorylation and were conducted as previously described.¹⁸⁷

Briefly, protein lysates were made following treatment and kept at 20°C until use. A Bradford assay was performed to formulate a standard curve using bovine serum albumin (BSA) and to determine concentration of protein in lysate by fitting absorbance values to the BSA standard curve. Aliquots of 40µg protein were prepared with a final concentration of 40µg protein and 1x loading buffer in 10% β-mercaptoethanol in 40µl total volume. The protein was loaded into 8, 10, or 12% bis-acrylamide gels and 10µl of a protein molecular weight marker was added to the first well of each gel for protein molecular weight determination. Proteins were run at 100volts for 90min or until desired separation of proteins occurred. Nitrocellulose membranes were cut to size and dipped in 100% methanol then soaked in transfer buffer before use. The transfer was run at 100volts for 60min and membranes were washed with deionized water thoroughly following transfer. All western blots were blocked in either 5% bovine serum albumin or milk in PBS-T for 1h and incubated overnight at 4°C with 1:1000 primary antibody, except β-actin, which was incubated 1:5000 for 45min at RT. Membranes were then washed 3 times for 10min with PBS-T and secondary antibody was applied for 1h at RT. Goat anti-rabbit IgG and goat anti-mouse IgG antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc) were used as secondary antibodies (1:3000). Blots were then washed 3 times for 10min with PBS-T and developed in an x-ray apparatus using a Western Lightning ECL kit (Perkin-Elmer, Inc.). β-actin expression was used to ensure normalized protein levels in each sample.

Cell growth analysis. Cell growth was measured via MTS assay using a CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega) as previously

described.¹⁸⁵ Cells were plated in 96 well plates with 100 μ L of media per well. The following day, all media was replaced with 200 μ L of drug-containing media or media with DMSO as controls. Media was replaced as described every 48h for the duration of the experiment. Media alone or media containing the highest concentration(s) of drug(s) were added to empty wells containing no cells as controls at the time of the final media change. 20 μ L MTS (Sigma Aldrich) was added to each well and allowed to incubate at 37°C for 4h. Following incubation, absorbances of each well were read using a Beckman absorbance detector (Beckman Coulter, Inc.) with a 490nm wavelength filter. Absorbances of media alone or drug containing media in wells without cells was subtracted from control and drug treated wells, respectively. Drug treated wells were then normalized to non-treated controls for analysis of cell proliferation effects. Combination treatments were performed with 24h pre-treatment of rapamycin or MLN8237, followed by combination therapy for 72h, or co-treatment of rapamycin and MLN8237 for 96h. Drug concentrations required to inhibit cell growth by 50% (GI₅₀) were determined by interpolation of dose-response curves. Averages of at least three wells in triplicate experiments were averaged to obtain final values.

Clonogenic assays were employed to assess cell proliferation with both pre- and continuous- dosing treatments and were conducted as previously described.¹⁸⁵ Briefly, for pre-treatment, cells were plated in culture dishes at 80% confluency, allowed to attach, and treated for 24h. The following day, cells were trypsinized, counted, and 200 viable cells were re-plated in 6 well plates and allowed to normal medium. After 10 (Leio285, SKLMS1) or 14 (Mes-Sa) days, media was aspirated and

colonies were stained with a 5% glutaraldehyde and 0.1% crystal violet in 20% methanol for 2h at RT. Plates were then rinsed well with water and dried. Images were captured digitally and the number of large colonies in each well was counted. Alternatively, for continuous treatment, 200 viable cells per well were first plated and then treated with drugs at doses indicated, following the procedure described above. Drug/media was replenished for both pre- and continuous-treatments every 48h for the duration of the experiment.

Cell cycle analysis. Determination of cell cycle progression was conducted as previously described.¹⁸⁷ Following drug treatment for 48h, cells were trypsinized, pelleted at 1500rpm for 5min, and washed with PBS. After 2 washes, cells were resuspended in 70% ethanol in water and fixed at -20°C overnight. Cells were spun down, washed twice with PBS, and resuspended in 5% propidium iodide (PI) solution with 10µg/mL RNase. After 30min incubation on ice in the absence of light, cells were analyzed for DNA content using fluorescence-activated cell sorting (FACS) with an Epics XL-MCL flowcytometer (Beckman-Coulter, Miami, FL) and Multicycle software (Phoenix Flow Systems, San Diego, CA).

FACS analysis for detection of apoptosis. FITC-conjugated Annexin V was used in conjunction with PI to assess levels of apoptotic cells through FACS analyses as previously described.¹⁸⁷ Briefly, cells were plated at ~60 or 80% confluence (96h and 48h treatments, respectively) and allowed to attach overnight. The following day, drug was added and replaced every 48h for the duration of treatment. Cells were then trypsinized, collected, and neutralized with fresh media. Care was taken to collect

dead and floating cells. Next, cells were pelleted by centrifugation at 1500rpm for 5min and washed with PBS. After 2 washes, cells were resuspended in 100 μ L 1x binding buffer and PI and Annexin V-FITC were added as directed (Annexin V:FITC Apoptosis Detection Kit 1; BD Pharmagen). The cells were incubated for 30min on ice in the absence of light and an additional 200 μ L of binding buffer was added just before FACS analysis using an Epics XL-MCL flowcytometer (Beckman-Coulter, Miami, FL). Analyses were performed with System II software (Beckman-Coulter, Miami, FL).

SiRNA knockdown. 2×10^5 cells/well were plated in 2mL of media with 10% FBS and no antibiotics in a 6 well plate and allowed to attach overnight. The following day, media was replaced and serum-free media (mock), non-targeting SiRNA with 20 μ L Extreme Gene transfection reagent in serum-free media, or Aurk A targeting SiRNA with 20 μ L of Extreme Gene transfection reagent in serum-free media were added at 4 μ g/100 μ L to each well. SiRNA was incubated with the cells for 24h and replaced with FBS and antibiotic-supplemented media. Cells were harvested at 48h after removal of SiRNA, care was taken to preserve floating apoptotic cells for FACS analysis. Cells were also lysed for western blot confirmation of knockdown.

Animal experiments. All animal procedures/care were approved by UTMDACC Institutional Animal Care and Usage Committee (Protocol #07-95-06336). Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." Animal models were utilized as previously described.³²² Viable SKLMS1 cells were confirmed using trypan blue staining, and

2×10^6 cells/0.1mL RPMI/mouse were used. Cell suspensions were injected subcutaneously into the flank of 6-8 week old female hairless SCID mice ($n= 7-8$ per group) and growth was measured twice weekly; after establishment of palpable lesions (average diameter of $\sim 4-5$ mm), mice were assigned to one of the following treatment groups: 1) vehicle control; 2) rapamycin (3.75 mg/kg/d, per gavage, 5 days per week); 3) MLN8237 (15mg/kg/bid, per gavage) or 4) combination of both agents. Rapamycin dose followed previously published studies³²³; MLN8237 dose was selected based on the company's recommendation and previously published data demonstrating that the maximal tolerated dose of the compound in most mouse strains (continuous dosing for ~ 21 days) is approximately 20mg/kg/bid (i.e. a total of 40mg/kg/d) and anti-tumor efficacy is observed with a total dose of 30mg/kg/d.^{315,324} Of note, MLN8237 was administered alone on day one of treatment while rapamycin treatment was initiated on day two. Mice were followed for tumor size, well-being, and body weight, and sacrificed when control group tumors reached an average of 1.5cm in largest dimension (21 days of treatment). Tumors were resected, weighed, and frozen or fixed in formalin and paraffin-embedded for immunohistochemical studies.

Immunohistochemistry and immunofluorescence for xenograft experiments.

IHC and immunofluorescence studies were conducted as previously described.^{322,325} Slides prepared from tumor xenografts were warmed for ~ 45 min on a plate warmer at 60°C . Next, they were de-waxed and re-hydrated through a series of xylene and ethanol incubations. Commercially available antibodies against phospho-S6 ribosomal protein (pS6RP, used as a surrogate for phosphorylated S6 Kinase; Ser235/236, #2211S, Cell Signaling, Danvers, MA; 1:200; 10mM sodium citrate buffer at pH6.0

with microwave retrieval for 10min at 98°C), phospho-4EBP1 (Thr70, #9455, Cell Signaling; 1:400; 10mM sodium citrate buffer at pH6.0 with microwave retrieval for 10min at 98°C) were used for immunohistochemistry. Appropriate biotinylated secondary antibodies and horseradish peroxidase-labeled streptavidin (4 plus system Biocare Medical, Concord, CA) were used for immunostaining, with 3,3-diaminobenzidine serving as chromagen. For xenograft tissues with primary antibodies made in mouse, fragment blocking was performed prior to administration of primary antibody. Briefly, 1:10 mouse IgG f(ab) was mixed suspended in normal horse (1:10) and normal goat (1:10) serum in PBS, applied to the tissues, and incubated at 4°C overnight. The following day, tissues were washed well, reincubated with protein block for 10min, and primary antibody was added.

Frozen sections were used for fluorescent CD31 and TUNEL detection. Frozen tissues were fixed with cold acetone and chloroform, washed in PBS, and blocked with 4% fish gelatin in PBS for 20min. Next, rat anti-mouse CD31 (BD Pharmagen, 1:500) in 4% fish gelatin solution was added and incubated overnight at 4°C. Secondary goat-anti-rat IgG antibody conjugated to Alexa Fluor 594 (Invitrogen) was incubated for 1h at RT under limited light exposure. Slides were then washed, stained with Hoechst dye (Molecular Probes; 1:10,000 in PBS) and cover slipped using fluorescent mounting medium. Terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) experiments to evaluate levels of apoptosis were performed on frozen tissues as directed by the Dead End Fluorometric TUNEL Kit purchased from Promega.

Ki67, TUNEL, and CD31 counts were calculated as the average number per high power field (200x) in five separate fields of two to three independent tumors from each group. The percentage of proliferating cells as indicated by Ki67 expression was calculated by dividing the number of Ki67 positive cells by the total number of nuclei stained by Gills #3 hematoxylin per field. The percentage of apoptotic cells was calculated by dividing the number of apoptotic cells by the total number of Hoescht-stained nuclei present in each field.

Statistical analysis. Spearman Rank Correlation was used to determine the correlation between biomarkers' expression and disease status/tumor status. For markers scored as – or +, Fisher's exact test was employed. Correlation between the different biomarkers was also evaluated using Spearman's correlation coefficient analyses; only one tissue sample per lesion was included for analysis. To evaluate the correlation of TMA biomarker expression and patient outcome (recurrence-free survival [RFS], metastasis-free survival [MFS], and disease specific survival [DSS]) each independent variable was examined separately in a univariable Cox proportional hazards model. Local recurrence was defined as pelvic, abdominal, or retroperitoneal recurrence where tumor did not infiltrate other organs. Metastases were characterized as tumor presence outside of the retroperitoneal, abdominal or pelvic regions such as lung or brain, and tumor infiltration into retroperitoneal/abdominal/pelvic organs such as kidney, bone, and liver. All tumor recurrence or metastases was confirmed via clinical/radiological evidence. All time intervals to recurrence, metastases, or death were defined as the interval of time between the diagnosis of the tumor with which the patient first presented to UTMDACC to the date when the recurrence/metastases

occurred or the patient died, respectively. Only local tumors were considered for outcome analysis; one tissue specimen from the earliest occurring tumor available was selected per patient. A p-value of $p \leq 0.05$ was set as the cutoff. All computations were performed using SAS for Windows (release 9.2; SAS Institute, Cary, NC).

To score each gene expression profile of ULMS or normal myometrium for similarity to a predefined gene transcription “signature” of the PI3K/AKT/mTOR pathway, we derived a “t score” for the sample profile in relation to the signature patterns as previously described.³²⁶⁻³²⁸ In brief, the PI3K mRNA t score was defined as the two-sided t statistic comparing the average of the PI3K-induced genes with that of the repressed genes within each tumor (after normalizing the log-transformed values to standard deviations from the median across samples). The mapping of transcripts or genes between the two array datasets was made on the Entrez Gene identifier; where multiple human array probe sets referenced the same gene, one probe set with the highest variation represented the gene.

Cell culture-based assays were repeated at least twice; mean \pm SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, two-sample t-tests were used to assess differences. To determine whether the cytotoxic interactions of rapamycin and MLN8237 in SKLMS1 cells were synergistic, additive, or antagonistic, drug effects were examined using the combination index (CI) method of Chou and Talalay.³²⁹ Briefly, the fraction affected (Fa) was calculated from cell viability assays, and CIs were generated using

CalcuSyn software (Biosoft, Cambridge, UK). Differences in xenograft growth *in vivo* were assessed using a two-tailed Student's t-test. Significance was set at $p \leq 0.05$.

Chapter 3: Identifying molecular deregulations in human ULMS

ULMS tissue microarray. A large tissue microarray (TMA) was constructed to facilitate identification of molecular deregulations in ULMS in a streamlined and high-throughput manner (**Figure 7**). Eight tumor samples were not considered for further evaluation after thorough examination of tissues and patient history revealed tumors may not be ULMS. In total, two hundred and forty three tumor blocks (representing 208 lesions retrieved from 109 patients) containing sufficient viable tumor tissue adequate for analytic purposes were selected for TMA construction. These included 18 primary lesions, 66 recurrent lesions, and 124 metastatic lesions. In addition, FFPE blocks of 10 healthy gastrointestinal smooth muscle specimens, 15 healthy myometrium samples, and 10 benign leiomyomas were identified as controls. ULMS patient clinical information including demographic, therapeutic, tumor, and clinical outcome variables were retrieved from institutional medical records and tabulated for correlative analyses. Blocks were organized and sent to the MD Anderson Pathology Core for physical construction of the TMA as previously described.³²⁰ The completed tissue microarray consisted of 4 blocks; 3 blocks were composed of 70 samples and 1 was comprised of 76 samples. Tumor samples from each block are included in duplicate to ensure staining quality and consistency. There are also 5 blocks with large tumors included on each of the four master TMA blocks for quality control between TMA



Figure 7. Uterine Leiomyosarcoma tissue microarray. ULMS tissue microarray paraffin blocks and corresponding slides. Four blocks in total contain 243 tumor samples and represent 208 lesions from 109 patients.

slides. Two-hundred and eight different lesions were identified on the TMA for inclusion in biomarker analyses; localized tumors from 57 independent patients were utilized for outcome analyses.

Evaluation smooth muscle and gynecological differentiation markers.

In many tumor types, loss of differentiation markers can confound already difficult diagnoses. Recurrent and metastatic ULMS diagnosis can be complicated in part due to their frequent variability in morphology. Potential loss of differentiation markers in ULMS could contribute significantly to difficulty with diagnosis and awareness of the frequency of loss of differentiation markers in ULMS could aid pathologists in diagnosis of more complicated ULMS cases. Towards this goal, we evaluated the

expression of several markers of both smooth muscle and gynecological differentiation in our large ULMS TMA.

Smooth muscle differentiation markers

Cells originating from smooth muscle stain positively for smooth muscle markers such as smooth muscle actin (SMA), smooth muscle myosin (SMM), desmin, and caldesmon (**Figure 8A and Table 8A**). SMA expression was observed diffusely in 88% (n=182), focally in 7% (n=14) of ULMS samples; expression was not found in 6% (n=12) of ULMS specimens. Diffuse expression of SMM was found in 72% (n=146) of ULMS, focal expression was found in 7% (n=15) of ULMS and 21% (n=43) did not express SMM. Desmin expression was observed diffusely in 66% (n=135) of ULMS, focally in 21% (n=43) of ULMS and was not expressed in 14% (n=28) of ULMS samples. Lastly, diffuse expression of caldesmon was found in 70% (n=143), focal expression in 12% (n=24), and no expression in 19% (n=38) of ULMS samples on our TMA. No GI smooth muscle, myometrium, or leiomyoma controls exhibited loss of any smooth muscle differentiation markers (**Appendix Table A4A**). Three of the four muscle markers examined in this study were significantly lost in ULMS compared with controls (SMA, $p=0.0823$; SMM, $p=0.0020$; desmin, $p=0.0130$; caldesmon, $p=0.0039$).

In our series, we found significant loss of smooth muscle differentiation markers; 31% of ULMS lost at least one of the four muscle markers, 18% lost at least 2, 9% lost expression of at least 3 markers, and 2% of confirmed ULMS lost expression of all 4

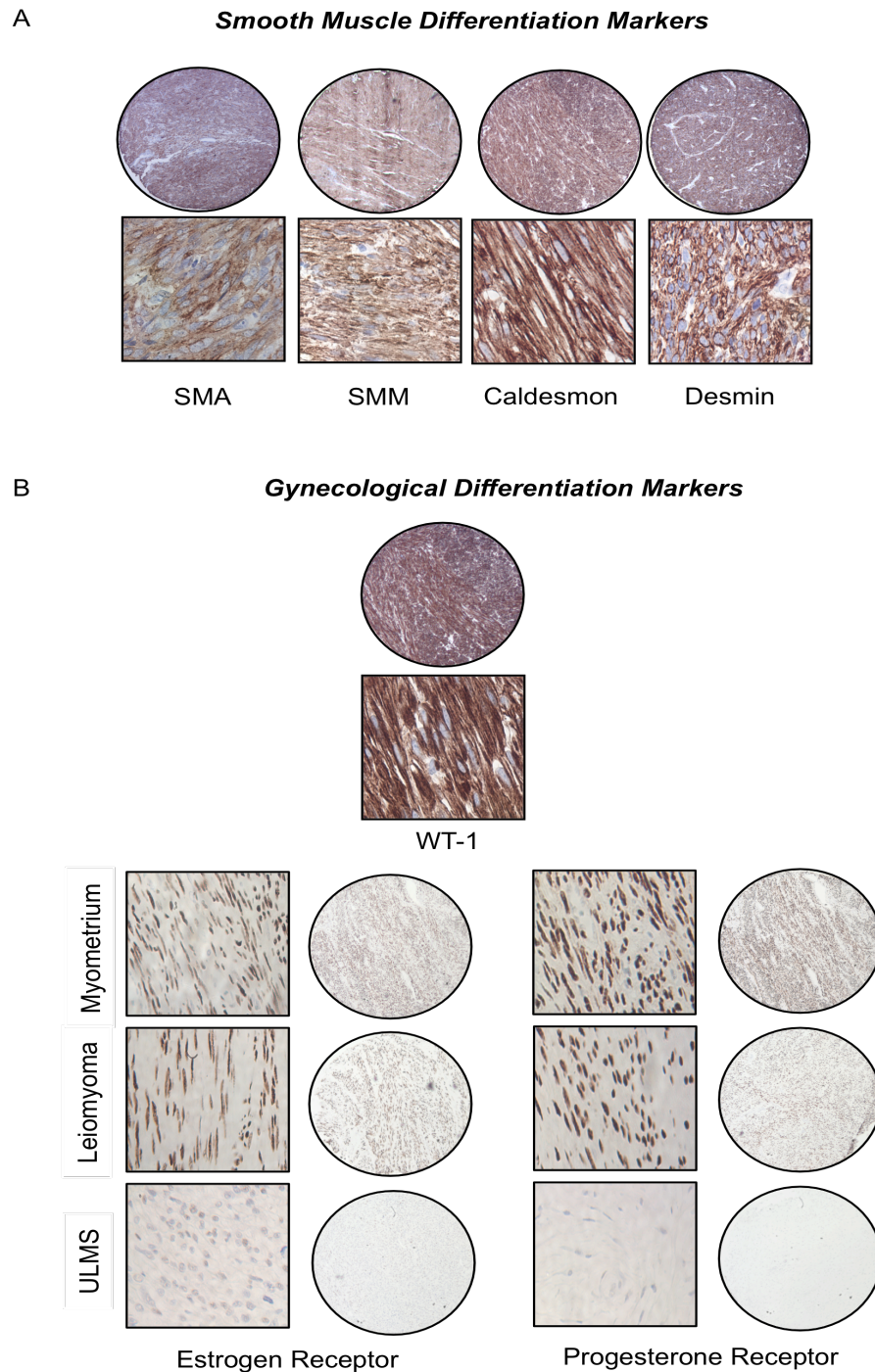


Figure 8. Differentiation markers in controls compared to ULMS. Photographic representations of A) smooth muscle differentiation markers and B) gynecological differentiation markers in ULMS versus controls. Loss of differentiation markers was observed for all markers in ULMS but not in controls.³³⁰

muscle markers examined. No normal smooth muscle controls or leiomyoma exhibited any loss in muscle marker expression. Loss of 2 or more muscle markers examined was found more frequently in ULMS; this difference was statistically significant ($p=0.0046$). A marked increase in loss of muscle markers was observed within tumor groups; recurrent and metastatic tumors had a loss of muscle markers nearly 3 fold higher than primary tumors and the only group exhibiting loss of all 4 muscle markers were metastatic tumors (**Supplementary Table S4B**).

Gynecological differentiation markers

Next, we examined the expression of three gynecological differentiation markers in ULMS: Wilms Tumor 1 (WT-1), estrogen receptor (ER), and progesterone receptor (PR; **Figure 8B, Table 8B**). These markers are high in tissues of gynecological origin thus, we wanted to examine whether they were largely retained or lost in ULMS. GI smooth muscle was not used as a control for gynecological differentiation markers because it does not originate from gynecological tissues and therefore, is not expected to express differentiation markers specific to tissues of that origin. Nuclear WT-1 expression was found in 96% ($n=23$) of myometrium and leiomyoma controls compared with only 55% ($n=111$) of ULMS samples ($p<0.0001$; **Table 8B**). Further, WT-1 loss was found within ULMS tumor progression groups; more advanced tumors exhibited increased WT-1 loss (**Appendix Table A4B**).

High levels of hormone receptors such as estrogen and progesterone receptors are found commonly in gynecologic tissues. Elevated levels of hormone receptors have also been suggested to drive gynecological tumors. To determine expression levels of

estrogen and progesterone receptors in ULMS, the TMA was utilized. We found a marked reduction of estrogen and progesterone receptors in ULMS compared with myometrium and leiomyoma controls ($p < 0.0001$ and < 0.0001 , respectively; **Table 8B**). Estrogen receptor expression was high in 32% ($n=67$), weak in 13% ($n=26$) and absent in 55% ($n=114$) of ULMS samples included on the TMA. Similarly, progesterone receptor was highly expressed in 45% ($n=89$), weakly expressed in 17% ($n=33$) and was not expressed in 39% ($n=78$) of ULMS tissues (**Appendix Table A4B**). An average of 38% ($\pm 35\%$) and 28% ($\pm 36\%$) of ULMS cells per sample exhibited positive staining for ER and PR, respectively (**Appendix Table A4B**). For both receptors, marked loss of expression was observed in ULMS.

Smooth muscle and gynecological differentiation marker's correlations to outcome.

Expression of smooth muscle and gynecological differentiation markers were examined for correlation with recurrent-free, metastasis-free, and disease specific survival. Loss of smooth muscle myosin and caldesmon were both significantly associated with recurrence-free survival ($p=0.0073$ [HR=0.28, 95% CI: 0.11-0.71] for both proteins). Loss of two or more of the four muscle markers we evaluated also correlated with RFS ($p=0.0073$ [HR=0.28, 95% CI: 0.11-0.71]). We also found that reduced estrogen receptor expression correlated to longer recurrence-free survival ($p=0.0432$ [HR=0.99, 95% CI: 0.98-1.00]). No muscle or gynecological differentiation markers correlated significantly with metastasis-free or disease specific survival (**Table 9**).

Table 8. Smooth muscle and gynecological differentiation markers' expressions in ULMS versus control.

	Smooth muscle		Leiomyoma		ULMS		
Marker	Total n=	Loss	Total n=	Loss	Total n=	Loss	<i>P</i>
A. Smooth muscle differentiation markers							
SMA	25	0 (0%)	7	0 (0%)	208	12 (6%)	0.0823
SMM	25	0 (0%)	8	0 (0%)	204	43 (21%)	0.0020
Desmin	25	0 (0%)	7	0 (0%)	206	28 (14%)	0.0130
Caldesmon	25	0 (0%)	8	0 (0%)	205	38 (18%)	0.0039
Loss ≥50% markers	25	0 (0%)	7	0 (0%)	199	35 (18%)	0.0046
B. Gynecological differentiation markers							<i>P</i>*
	Myometrium		Leiomyoma		ULMS		
Nuclear WT-1	14	1 (7%)	10	0 (0%)	202	91 (45%)	<0.0001
Marker	Total n=	high	Total n=	high	Total n=	high	
ER	15	15 (100%)	8	6 (75%)	207	67 (32%)	<0.0001
PR	14	14 (100%)	7	5 (71%)	200	89 (45%)	<0.0001

p compares all controls (GI smooth muscle, myometrium, and leiomyoma) to ULMS for all smooth muscle differentiation markers

*p** compares controls (myometrium and leiomyoma only) to ULMS for all gynecological differentiation markers

Table 9. Differentiation markers' correlations to outcome in ULMS.

A. Biomarker intensity expression univariate correlation to recurrence-free (RFS) and metastasis-free (MFS) survival				
Marker	RFS (univariable)		MFS (univariable)	
	<i>P</i>	Hazard Ratio (95% CI)	<i>P</i>	Hazard Ratio (95% CI)
Loss of SMA	0.4318	-	0.9583	-
Loss of SMM	0.0073	0.28 (0.11-0.71)	0.5454	-
Loss of Desmin	0.6304	-	0.4833	-
Loss of Caldesmon	0.0073	0.28 (0.11-0.71)	0.5454	-
Loss of ≥ 50% muscle markers	0.0073	0.28 (0.11-0.71)	0.5454	-
Loss of WT-1	0.9216	-	0.2544	-
Loss of ER	0.0432	0.99 (0.98-1.00)	0.1460	-
Loss of PR	0.0771	-	0.2820	-
B. Biomarker intensity expression univariate and multivariate correlation to disease specific survival				
DSS (univariable)				
Marker	<i>P</i>	Hazard Ratio (95% CI)		
Loss of SMA	0.1720	-		
Loss of SMM	0.0649	-		
Loss of Desmin	0.8608	-		
Loss of Caldesmon	0.0649	-		
Loss of ≥ 50% muscle markers	0.0649	-		
Loss of WT-1	0.8483	-		
Loss of ER	0.0839	-		
Loss of PR	0.6371	-		

Evaluation of cancer-related biomarkers.

An array of studies have presented clinical prognosticators for ULMS, however, most have not examined potential molecular biomarkers. To our knowledge, this study represents one of the largest clinical databases and undoubtedly provides the most comprehensive evaluation of potential markers in ULMS published to-date. We constructed a TMA to examine intensity expression profiles of several potentially critical proteins in ULMS compared to healthy smooth muscle and benign leiomyoma (**Table 10**). The percentages of cells expressing each protein at low, moderate, and high levels are given for controls and ULMS in **Appendix Tables A5B and A6B**, respectively; only differences in distribution correlating with outcome are noted in this text. Tumor status was also examined; primary, recurrent, and metastatic ULMS groups were compared to identify biomarkers that were differentially expressed (**Table 11** and **Appendix Table A6A**). One sample per ULMS lesion within the TMA was utilized for biomarker analysis (n=208). Further, we examined biomarker correlation to outcomes (**Table 12 intensity, Appendix Table A7 distribution**). For outcomes, only one local lesion per patient was evaluated (n=57).

Proliferation

Ki67 and cyclin D1 were evaluated as markers of proliferation and found to have significantly increased expression compared to controls (**Figure 9**). About one quarter (n=50) of ULMS samples expressed no Ki67 while 63% (n=129) had low expression and 13% (n=13) had high expression of Ki67. Cyclin D1 was expressed in 25% of ULMS; 20% (n=41) had low expression and 5% (n=11) had high expression. An average of 22% ($\pm 18\%$) and 8% ($\pm 17\%$) of cells per ULMS sample expressed Ki67

and cyclin D1, respectively. Elevated cyclin D1, but not Ki67, expression was observed in leiomyoma compared with other controls (25% [n=2 of 10] and 8% [n=2 of 25], respectively). In sum, both Ki67 and cyclin D1 expression were increased significantly in tumor versus controls ($p<0.0001$ and $p=0.0099$).

Survival

Next, markers of survival were evaluated (**Figure 9**). Bcl-2 expression was slightly reduced in ULMS samples compared with controls, but this difference was not significant ($p=0.3556$). All but one ULMS expressed Bcl-2: 34% ($n=69$) had weak expression, 47% ($n=95$) had moderate expression and 19% of ULMS samples had strong expression of Bcl-2. An average of 43% ($\pm 33\%$) of cells per ULMS sample expressed Bcl-2. Between controls, Bcl-2 expression was higher in myometrium and leiomyoma compared with GI smooth muscle (**Appendix Table A5A**). Survivin expression was assessed for both nuclear and cytoplasmic intensities; increased expression was observed in both locations, but only increased nuclear expression was significant in ULMS versus controls ($p<0.0001$). Both nuclear and cytoplasmic survivin expression was observed in all ULMS samples. Strong nuclear expression of survivin was observed in only one case of ULMS (0.5%); strong cytoplasmic expression was not observed. Further, 67% ($n=135$) and 30% ($n=60$) of ULMS moderately expressed cytoplasmic and nuclear survivin, respectively. Weak cytoplasmic expression was noted in 33% ($n=66$) and weak nuclear expression was observed in 70% ($n=140$) of ULMS. Averages of 69% ($\pm 18\%$) and 39% ($\pm 19\%$) cells

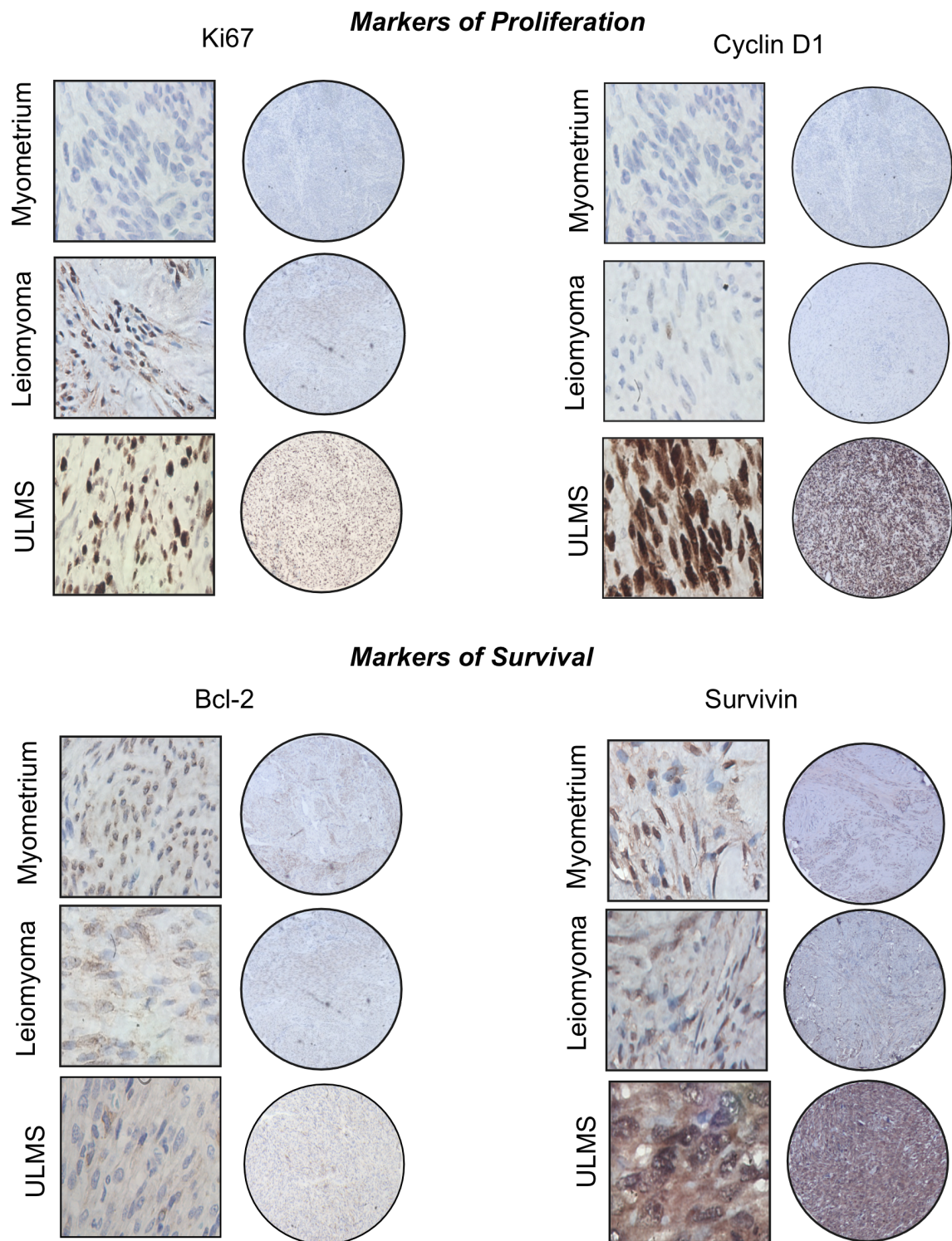


Figure 9. Markers of proliferation and survival. Photographic representation of markers of proliferation and markers of survival in ULMS compared with myometrium and leiomyoma controls.³³⁰

per ULMS sample were observed to express cytoplasmic and nuclear survivin, respectively.

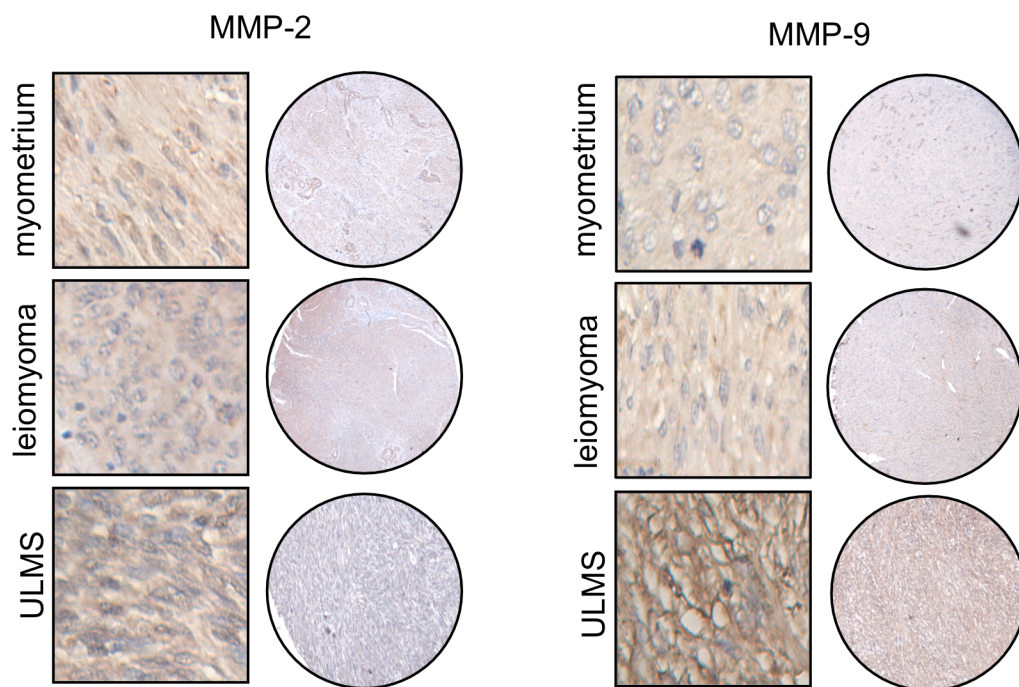
Invasion and angiogenesis

Expressions of invasion-associated proteins MMP-2 and MMP-9 were investigated (**Figure 10**). MMP-2 expression was observed infrequently in ULMS; 94% (n=189) samples did not express MMP-2, 2% (n=5) had weak expression, 2% (n=4) had moderate expression, and only 1% (n=3) strongly expressed MMP-2. Conversely, all but one ULMS expressed MMP-9; 41% (n=83) had weak expression, 36% (n=72) had moderate expression, and 23% (n=46) had strong MMP-9 expression. While both MMP-2 and MMP-9 showed slightly increased expression in tumor versus normal tissues, the differences were not significant ($p=0.4893$ and $p=0.2521$, respectively). Averages of 5% ($\pm 19\%$) and 77% ($\pm 23\%$) of ULMS cells showed expression for MMP-2 and MMP-9, respectively. Additionally, we evaluated the expression of vascular endothelial growth factor (VEGF), a critical and driving protein in angiogenesis (**Figure 10**). VEGF expression was noted in all but one ULMS sample. High expression was observed in 19% (n=39), moderate expression in 47% (n=95), and low expression in 34% (n=69) of ULMS examined. VEGF expression increased significantly in tumor versus controls ($p=0.0287$) with an average of 63% ($\pm 23\%$) of cells per ULMS sample staining positively.

Oncoproteins and tumor suppressors

Utilizing the TMA, expression levels of select tumor oncogenes and suppressors were also evaluated (**Figure 11**). MDM2, p53, and p16 levels all showed significantly

Markers of Invasion



Markers of angiogenesis

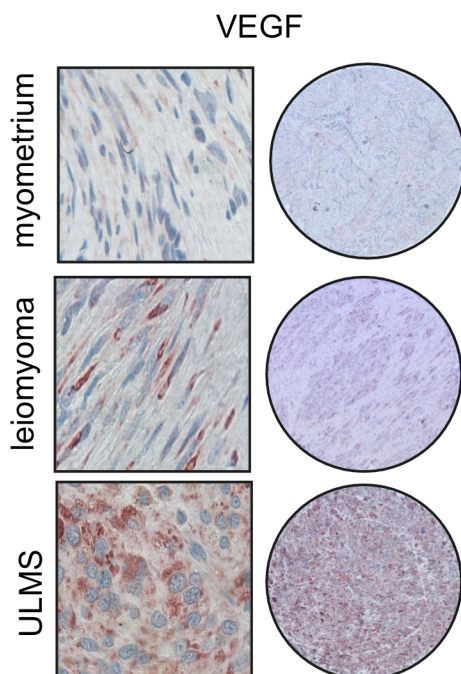


Figure 10. Markers of invasion and angiogenesis. Images of markers of invasion (MMP-2 and MMP-9) and angiogenesis (VEGF) in ULMS compared to controls.

Oncoproteins and Tumor Suppressors

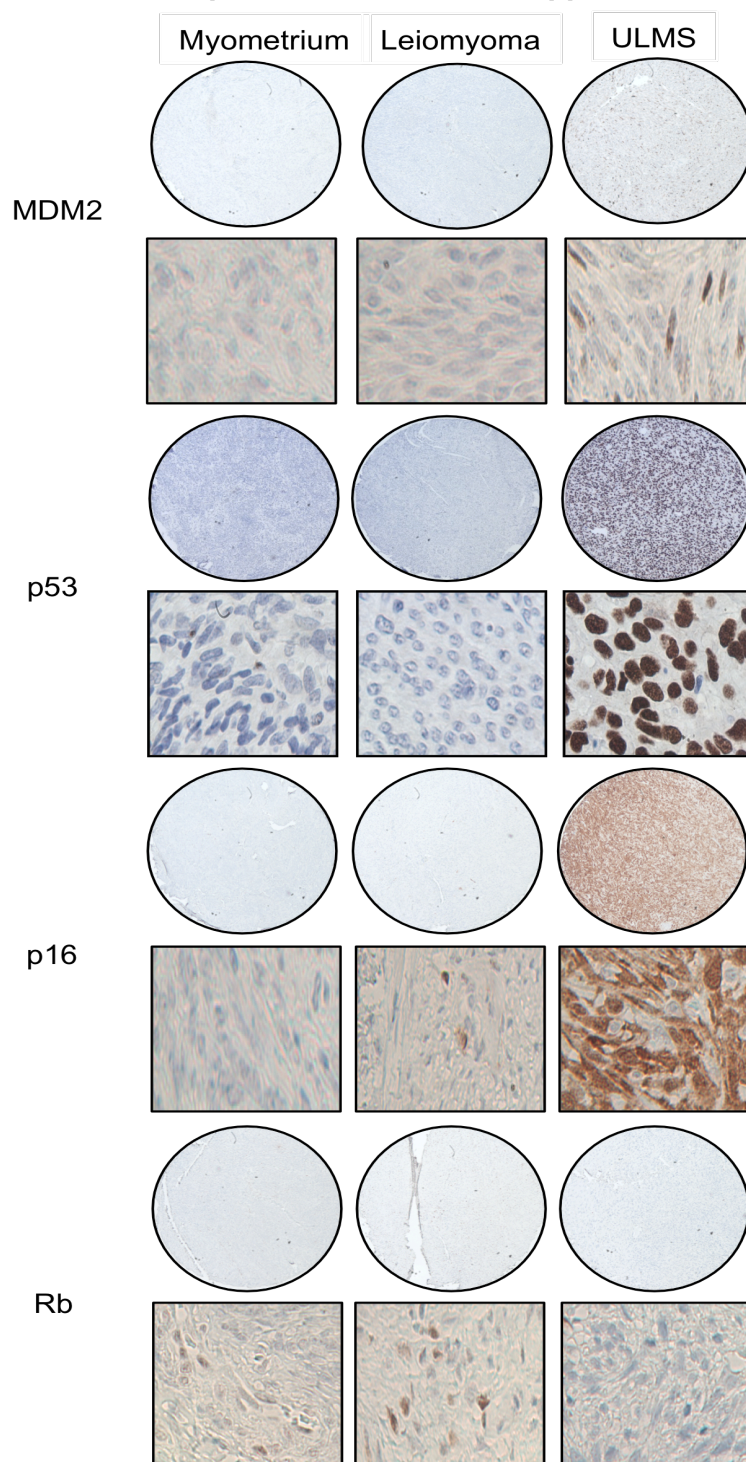


Figure 11. Oncoprotein and tumor suppressor expression in myometrium, leiomyoma, and ULMS. Photographic representation of MDM2, p53, p16, and Rb expression in ULMS compared to myometrium and leiomyoma controls.³³⁰

increased expression in tumor compared with controls ($p=0.0228$, $p=0.0018$, and $p<0.0001$, respectively). MDM2 expression was absent from 88% ($n=176$) of ULMS while 3% ($n=6$) had weak expression, 14% ($n=27$) had moderate expression and 2% ($n=4$) had strong expression. Additionally, 43% of ULMS did not stain positively for p53 and 25% ($n=52$) stained weakly for p53. Moderate p53 expression was observed in 31% ($n=64$) and strong expression in one additional ULMS sample compared with no moderate or strong staining in controls. Rb expression was not found in 59% ($n=117$) of ULMS. Weak expression of Rb was detected in 14% ($n=27$) and strong expression was seen in 27% ($n=53$) of ULMS compared to none in controls. Expression of p16 was observed in nearly all ULMS samples. Weak expression was found in 14% ($n=29$), moderate expression in 31% ($n=62$) and strong expression in 49% ($n=98$) of ULMS. Only 6% ($n=13$) of ULMS did not express p16 compared with more than half of all controls. An average of 2% ($\pm 10\%$) of ULMS cells per sample expressed MDM2 while 26% ($\pm 32\%$) expressed p53 and 66% ($\pm 32\%$) had positive expression for p16. Within controls, GI smooth muscle showed reduced expression of p53 compared to myometrium and leiomyoma (**Appendix Table A5**). Additionally, Rb expression decreased significantly ($p=0.0239$) in ULMS; an average of 10% ($\pm 20\%$) of ULMS cells in each sample expressed Rb.

Signaling molecule β -catenin

Given its potential implications in cancer cell signaling, we evaluated expression levels of β -catenin (**Figure 12**). Expression was noted in nearly all ULMS samples; 34% ($n=69$) had low expression, 42% ($n=85$) had moderate expression, and 22% ($n=45$) had strong expression of cytoplasmic β -catenin. Only 1% ($n=3$) of ULMS did

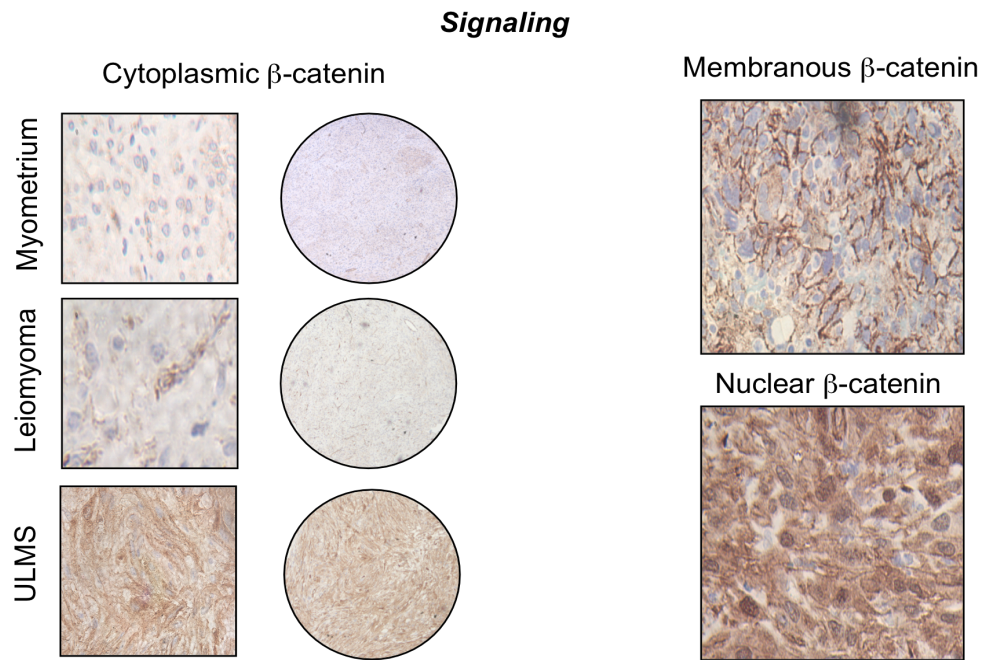


Figure 12. β -catenin expression in ULMS compared to controls. Photographic representation of β -catenin expression in ULMS. Notably, nuclear expression and membranous expression of β -catenin was also observed in ULMS.³³⁰

not express cytoplasmic β -catenin and we found that expression was increased significantly in ULMS compared with controls ($p < 0.0001$). It was expressed in an average of 62% ($\pm 27\%$) cells per ULMS sample. Interestingly, nuclear and membranous β -catenin expression were found in 7% ($n=14$) and 20% ($n=41$) of ULMS samples, respectively; nuclear or membranous expression was not found in any controls ($p=0.0012$ and 0.26 , respectively; **Appendix Table A5A**)

Biomarker correlations: tumor status and outcome

Interestingly, expressions of several markers were significantly different between primary, recurrent, and metastatic lesions (**Table 11**). VEGF expression was significantly decreased in local (primary and recurrent) versus metastatic lesions

($p=0.0358$). Conversely, MDM2 expression is significantly increased in local versus metastatic lesions ($p=0.0148$). p53 expression is significantly higher in advanced disease (recurrent and metastatic) as compared with primary ULMS ($p=0.0074$) and similarly, nuclear expression of β -catenin was found elevated in advanced ULMS compared with primary lesions ($p=0.0012$). Next, the correlation between expression levels of each biomarker and patient outcome was evaluated by univariable Cox proportions model (**Table 12**). In these analyses, only localized ULMS samples were included ($n=57$). Increased cytoplasmic and nuclear survivin correlated with shorter recurrence-free survival ($p=0.0020$ [HR=3.78, 95% CI: 1.63-8.81] and $p=0.0144$ [HR=2.35, 95% CI: 1.19-4.66], respectively). Enhanced nuclear distribution expression of survivin also correlated to decreased RFS ($p=0.007$ [HR=1.03, 95% CI: 1.01-1.05]). Both nuclear and cytoplasmic β -catenin high intensity expression ($p=0.0369$ [HR=1.58, 95% CI: 1.03-2.42] and $p=0.0010$ [HR=5.81], respectively) and high distribution expression ($p=0.0045$ [HR=1.02, 95% CI: 1.01-1.04] and $p=0.0001$ [HR=5.81, 95% CI: 2.03-16.60], respectively) were also associated with shorter RFS. Interestingly, high MMP-9 intensity expression correlated significantly with increased MFS ($p=0.0251$ [HR=0.57, 95% CI: 0.35-0.93]). Only high Bcl-2 intensity and distribution intensities correlated to longer DSS ($p=0.0381$ [HR=0.61, 95% CI: 0.38-0.97] and $p=0.0142$ [HR=0.98, 95% CI: 0.97-1.00], respectively). Taken together, these data identify several biomarkers for ULMS although no significant biomarkers correlate well to DSS. Additionally, this work presents a number of potential therapeutic targets for further evaluation towards new therapies for ULMS in the clinical setting.

Table 10. Cancer-related biomarkers' correlation to disease status.

Spearman Rank Correlation with Disease Status							
Marker	Smooth muscle		Leiomyoma		ULMS		P
	Total n=	high expression (%)	Total n=	high expression (%)	Total n=	high expression (%)	
Proliferation							
Ki67	24	0 (0%)	6	0 (0%)	205	155 (76%)	<0.0001
Cyclin D1	25	0 (0%)	8	0 (0%)	207	52 (25%)	0.0099
Marker	Total n=	moderate-strong intensity (%)	Total n=	moderate-strong intensity (%)	Total n=	moderate-strong intensity (%)	
Survival							
Bcl-2	24	14 (58%)	8	3 (38%)	204	85 (42%)	0.3556
Cyt. survivin	25	13 (52%)	8	5 (63%)	201	135 (67%)	0.1723
Nuc. survivin	25	1 (4%)	8	0 (0%)	201	61 (30%)	<0.0001
Invasion							
MMP2	25	0 (0%)	8	0 (0%)	201	7 (3%)	0.4893
MMP9	25	18 (72%)	8	4 (50%)	202	118 (58%)	0.2521
Angiogenesis							
VEGF	25	15 (60%)	8	4 (50%)	204	134 (66%)	0.0287
Oncoproteins and tumor suppressors							
MDM2	25	0 (0%)	5	0 (0%)	200	18 (9%)	0.0228
p53	25	0 (0%)	7	0 (0%)	204	65 (32%)	0.0018
p16	25	7 (28%)	7	3 (43%)	202	160 (79%)	<0.0001
Rb	24	19 (79%)	6	6 (100%)	197	80 (41%)	0.0239
Signaling							
Cyt. β-catenin	25	0 (0%)	8	0 (0%)	202	130 (64%)	<0.0001
Nuc. β-catenin	25	0 (0%)	8	0 (0%)	203	14 (7%)	0.0012*
Memb. β-catenin	25	0 (0%)	8	0 (0%)	203	41 (20%)	0.26*

Total n= indicates the number of evaluable samples in each dataset

@ is defined as having a score of 0 on a scale of 0-3 (defined in *) and indicates complete loss of protein expression

*p values were calculated by chi-squared test, except where 50% of the cells had expected counts less than 5%, in which case Fisher's exact test was applied

All p-values compared control (myometrium, GI smooth muscle, leiomyoma) versus ULMS; bolded values indicate statistical significance ($p < 0.05$)

Table 11. Cancer-related biomarkers' correlation to tumor status.

Spearman Rank Correlation with Tumor Status							
Marker	Primary ULMS		Recurrent ULMS		Metastatic ULMS		P
	Total n=	high expression (%)	Total n=	high expression (%)	Total n=	high expression (%)	
Proliferation							
Ki67	18	9 (50%)	64	55 (86%)	123	91 (74%)	0.1210
Cyclin D1	18	6 (34%)	65	18 (28%)	124	28 (23%)	0.0663
Marker	Total n=	moderate-strong intensity (%)	Total n=	moderate-strong intensity (%)	Total n=	moderate-strong intensity (%)	
Survival							
Bcl-2	18	10 (55%)	64	26 (41%)	122	49 (40%)	0.1991
Cyt. survivin	18	7 (39%)	63	45 (71%)	120	83 (69%)	0.1033
Nuc. survivin	18	2 (11%)	63	21 (34%)	120	38 (32%)	0.2089
Invasion							
MMP2	18	0 (0%)	65	2 (3%)	118	5 (5%)	0.3892
MMP9	18	11 (61%)	66	42 (64%)	118	65 (55%)	0.2324
Angiogenesis							
VEGF	18	9 (50%)	65	36 (55%)	121	89 (74%)	0.0358
Oncoproteins and tumor suppressors							
MDM2	18	2 (11%)	65	11 (17%)	117	5 (5%)	0.0148
p53	17	5 (29%)	65	27 (42%)	122	22 (27%)	<0.0001
p16	18	10 (56%)	64	55 (86%)	120	95 (80%)	0.4723
Rb	18	9 (50%)	62	24 (39%)	117	47 (40%)	0.2596
Signaling							
Cyt. β-catenin	18	8 (44%)	64	43 (68%)	120	79 (66%)	0.1961
Nuc. β-catenin	18	0 (0%)	64	11 (17%)	121	3 (2%)	0.0026*
Memb. β-catenin	18	1 (6%)	64	13 (20%)	121	27 (22%)	0.36*

Total n= indicates the number of evaluable samples in each dataset

@ is defined as having a score of 0 on a scale of 0-3 (defined in *) and indicates complete loss of protein expression

*p values were calculated by chi-squared test, except where 50% of the cells had expected counts less than 5%, in which case Fisher's exact test was applied

All p-values compared control (myometrium, GI smooth muscle, leiomyoma) versus ULMS; bolded values indicate statistical significance (p<0.05)

Table 12. Expression of cancer biomarkers' correlation to outcomes.

A. Biomarker intensity expression univariate correlation to recurrence-free (RFS) and metastasis-free (MFS) survival				
Marker	RFS (univariable)		MFS (univariable)	
	<i>P</i>	Hazard Ratio (95% CI)	<i>P</i>	Hazard Ratio (95% CI)
Ki67	0.1909	-	0.2044	-
Cyclin D1	0.5493	-	0.5559	-
Bcl-2	0.4256	-	0.9340	-
Cyt. survivin	0.0020	3.78 (1.63-8.81)	0.5778	-
Nuc. survivin	0.0144	2.35 (1.19-4.66)	0.5060	-
MMP2	0.9553	-	0.9925	-
MMP9	0.1493	-	0.0251	0.57 (0.35-0.93)
VEGF	0.1134	-	0.4774	-
MDM2	0.6971	-	0.6054	-
p53	0.1987	-	0.6027	-
p16	0.8916	-	0.4812	-
Rb	0.5758	-	0.8143	-
Cyt. β -catenin	0.0369	1.58 (1.03-2.42)	0.2165	-
Nuc. β -catenin	0.0010	5.81 (2.03-16.60)	0.6238	-
Memb. β -catenin	0.1519		0.5537	-
B. Biomarker intensity expression univariate and multivariate correlation to disease specific survival				
Marker	DSS (univariable)			
	<i>P</i>	Hazard Ratio (95% CI)		
Ki67	0.1240	-		
Cyclin D1	0.4388	-		
Bcl-2	0.0381	0.61 (0.38-0.97)		
Cyt. survivin	0.5612	-		
Nuc. survivin	0.7355	-		
MMP2	0.8270	-		
MMP9	0.9404	-		
VEGF	0.8332	-		
MDM2	0.8361	-		
p53	0.2507	-		
p16	0.6224	-		
Rb	0.3709	-		
Cyt. β -catenin	0.3446	-		
Nuc. β -catenin	0.3173	-		
Memb. β -catenin	0.5741	-		
Outcomes for all markers were calculated by Cox analysis.				

Chapter 4: Evidence of Targetable Pathways in ULMS: The mTOR Pathway

Several studies have identified aberrant mTOR signaling in soft tissue sarcoma, including LMS^{107,187,331}; however, to the best of our knowledge, it has not yet been determined if this pathway is activated in human ULMS specifically. Therefore, recently obtained gene expression profiles of 12 ULMS specimens (FIGO stage I) and 10 healthy myometrium samples²⁵⁰ were compared bioinformatically to three previously reported different PI3K/AKT/mTOR-related gene expression signatures: 1) the CMap signature, which identified commonly deregulated genes using expression profiles generated from several different cell lines treated with PI3K inhibitors³²⁷; 2) the Saal profile, based on gene expression in breast cancers exhibiting PTEN loss (and thus exhibiting a highly active mTOR pathway)³²⁸; and 3) the Majumder gene set which examines gene regulation in mice over expressing AKT1.³²⁶ Each profiled ULMS or myometrium sample was scored for PI3K pathway activation based

Figure 13. mTOR pathway is commonly activated in human ULMS. A PI3K/AKT/mTOR pathway is more active in ULMS, based on gene expression profiling data. Three previously described gene transcription signatures for the PI3K/AKT/mTOR pathway were applied to expression profiles of ULMS samples; each sample was scored for relative signature activity (heatmap is depicted: yellow = more active, blue = less active).²⁴⁹

on pathway-specific transcriptional targets. For each signature evaluated, ULMS tumors had higher PI3K/AKT/mTOR pathway activation scores versus myometrium ($p < 0.01$, each signature, two-sided t-test) (**Figure 13**), providing initial evidence of AKT/mTOR deregulation in ULMS.²⁴⁹

mTOR pathway proteins are overexpressed in human ULMS.

Next, we evaluated expression levels of the activated (phosphorylated) mTOR downstream effectors S6RP and 4EBP1 in a large human ULMS TMA. (**Figure 14, Figure 17, Table 14A**). Only samples representing different lesions were included in the final analysis ($n=208$). All ULMS specimens expressed pS6RP: low levels were observed in 28.5% ($n=55$), moderate expression was noted in 48.5% ($n=97$), and high expression was found in 24% ($n=48$). An average of 60.7% (± 20) of tumor cells per sample exhibited positive pS6RP staining. While pS6RP expression was observed in all controls, staining intensity was found to be significantly lower ($p < 0.0001$). No differences in the expression of this marker were identified between normal myometrium and leiomyoma. p4EBP1 was scored for both cytoplasmic and nuclear expression intensity. All ULMS samples expressed cytoplasmic p4EBP1: low levels were observed in 22% ($n=43$), moderate expression was noted in 59% ($n=114$), and high expression was found in 19% ($n=36$). An average of 72.5% (± 14.4) of tumor cells per sample exhibited positive cytoplasmic p4EBP1 staining. Similarly, nuclear p4EBP1 expression was found in all ULMS samples: low levels were observed in 39% ($n=75$), moderate expression was noted in 45% ($n=87$), and high expression was

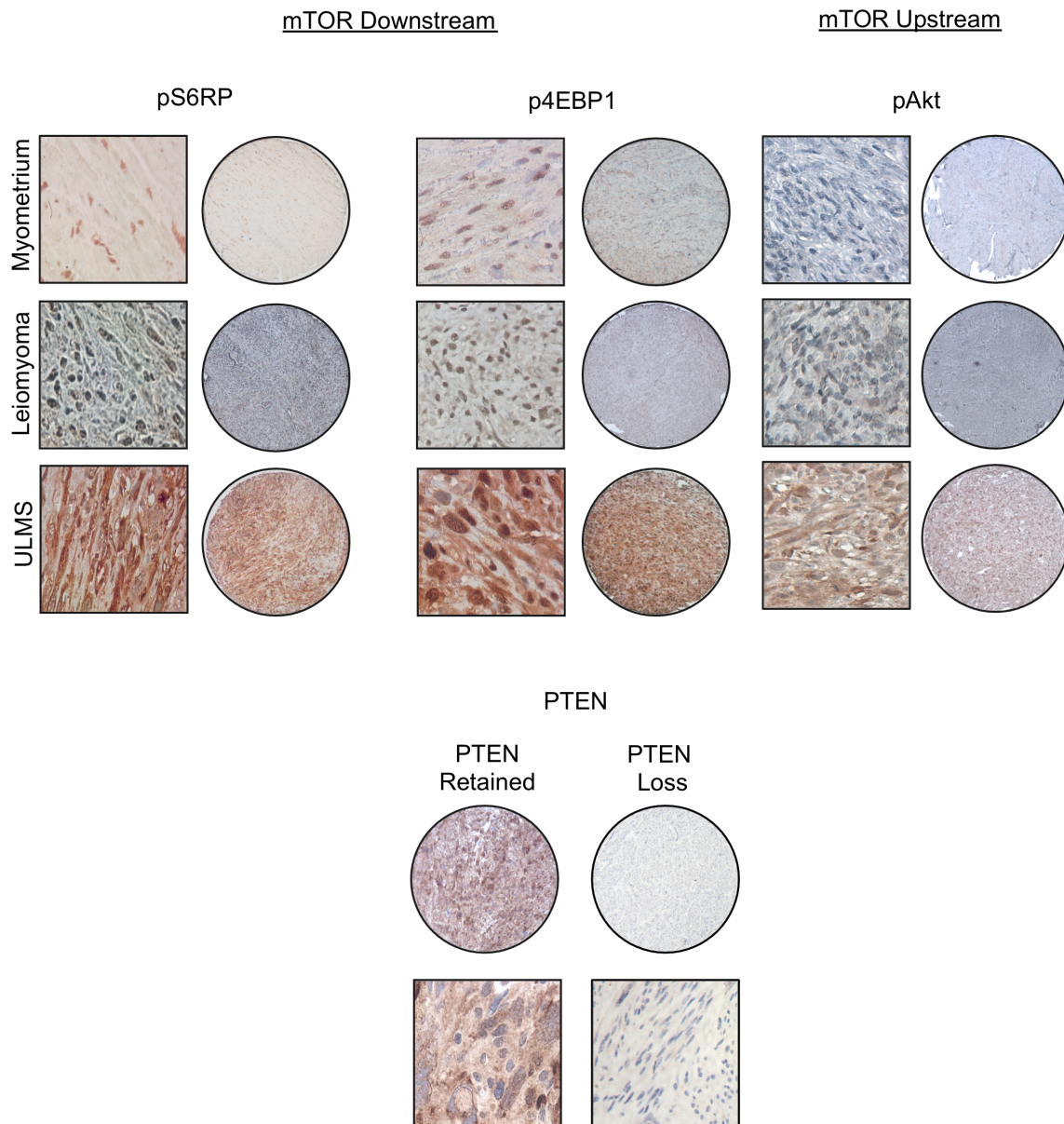


Figure 14. The mTOR pathway is commonly activated in human ULMS. Representative photographs of ULMS tissue microarray pS6RP, p4EBP1, and pAKT immunostaining (original images were captured at 20x (circles) or 400x magnification (squares) depicting differences in expression between tumor and normal smooth muscle. Staining for PTEN is also shown and includes an example of PTEN expression loss as compared to positive staining – the latter was observed in most evaluable samples.²⁴⁹

found in 16% (n=30). An average of 70.8% (± 13.7) of tumor cells per sample exhibited positive nuclear p4EBP1 staining. All controls exhibited cytoplasmic and nuclear p4EBP1 staining, albeit at markedly lower levels compared to ULMS (p=0.0011 and p=0.0001, respectively; **Appendix Table A8B**).

Expression of pAKT, the major upstream regulator of mTOR, was next evaluated (**Figure 14, Figure 17, and Table 14A**). All ULMS exhibited pAKT expression at varying degrees: low levels were observed in 41% (n=76), moderate in 47% (n=87), and high in 12% (n=23; **Appendix Table A9A**). An average of 66.4% (± 20.4) of tumor cells per sample exhibited positive pAKT staining. In contrast, all control samples exhibited only low levels of pAKT expression (p<0.0001; **Appendix Table A8A**). Loss of the PTEN tumor suppressor was suggested as a potential common molecular LMS deregulation, contributing to mTOR activation.⁹⁸ Interestingly, immunohistochemical analysis identified loss of PTEN expression in only 7% of ULMS samples (n=15); 9% of controls (two normal smooth muscle and one leiomyoma) exhibited no PTEN expression. Distribution scores for each marker in controls and ULMS are given in **Appendix Table A8B** and **Appendix Table A9B**, respectively.

Next, we evaluated the association of biomarker expression to tumor status (primary, recurrent, or metastatic; **Table 15A**). pS6RP expression levels were identified in recurrent and metastatic ULMS samples compared to primary lesions (p=0.0259). pS6RP, cytoplasmic and nuclear p4EBP1 expression levels also associated significantly with recurrent and metastatic lesions compared to primary ULMS when a simple Fisher's Test was applied (p=0.0005, p=0.05, and p=0.0088, respectively).

Further, Spearman Rank Correlation test was used to examine correlations between biomarkers. We found that pS6RP, cytoplasmic and nuclear p4EBP1, and pAKT expression all statistically correlated with one another but none significantly correlated with PTEN expression status (**Table13**). No difference between pAKT expression levels was noted between primary, recurrent, and/or metastatic ULMS specimens (**Table 15A**). Taken together, these data indicate that the mTOR pathway is highly activated in ULMS, but that the driver of this activation is not loss of the PTEN tumor suppressor per se, so there must be due to other contributing mechanisms.

Table 13. Correlation between biomarkers by Spearman's Rank test.²⁴⁹

	pS6RP	cyt. p4EBP1	nuc. p4EBP1	pAKT	PTEN
pS6RP		r=2.13 p=0.002 (n=186)	r=0.193 p=0.004 (n=186)	r=0.145 p=0.027 (n=180)	r=0.056 p=0.217 (n=197)
cyt. p4EBP1	r=0.213 p=0.002 (n=186)		r=0.416 p<0.001 (n=193)	r=0.247 p<0.001 (n=185)	r=0.053 p=0.236 (n=189)
nuc. p4EBP1	r=0.193 p=0.004 (n=186)	r=0.416 (p<0.001, n=193)		r=0.216 p=0.002 (n=185)	r=0.036 p=0.310 (n=189)
pAKT	r=0.145 p=0.026 (n=180)	r=0.247 p<0.001 (n=185)	r=0.216 p=0.002 (n=185)		r=0.099 p=0.091 (n=183)
PTEN	r=0.056 p=0.217 (n=197)	r=0.053 p=0.236 (n=189)	r=0.036 p=0.310 (n=189)	r=0.099 p=0.091 (n=183)	
total n= indicates the number of evaluable samples in each dataset bolded values indicate statistical significance (p<0.05)					

Outcome analysis

A univariable Cox model was utilized to determine whether the expression intensity and distribution (i.e., % positive cells) of any of the mTOR-related biomarkers was associated with clinical outcome; only localized ULMS specimens were included (n=57; **Table 16**). Only distribution of nuclear p4EBP1 correlated with any outcome factors; high distribution expression of nuclear p4EBP1 correlated with DSS (p=0.0494 [HR=1.03, 95% CI: 1.00-1.06]; **Appendix Table A10**). None of the mTOR-related biomarkers correlated with RFS or MFS.

Taken together, these data demonstrate mTOR deregulation as commonly occurring in human ULMS; their expression however, does not correlate well with outcome, indicating that mTOR pathway-related markers may serve as good biomarkers, but may not be viable prognostic markers. Further evaluation is needed to determine whether mTOR is a good potential ULMS therapeutic target. Identified mTOR overexpression provides rationale for further examination of factors promoting mTOR pathway deregulation.

Tyrosine kinase Receptors may potentially contribute to deregulation of the mTOR pathway

The mTOR pathway is activated by a large family of receptor tyrosine kinases (RTKs) that internalize cellular signals from extracellular molecules such as vascular endothelial growth factor (VEGF) and platelet derived growth factors α and β (PDGF- α and PDGF- β). To explore molecular deregulations contributing to mTOR deregulation, a number of signaling molecules, including seven tyrosine kinase

receptors and two RTK ligands were evaluated for enhanced expression that may lend to pro-tumorigenic phenotypes.

EGFR and IGF1R

First, expressions of epidermal growth factor receptor (EGFR) and insulin growth factor receptor 1 (IGF-1R) were evaluated (**Table 14B, Figure 15**). EGFR expression was present in over two-thirds of ULMS; 44% (n=88) had weak expression, 30% (n=60) had moderate expression, and 6% (n=12) had strong expression of EGFR (**Appendix Table A9B**). Weak IGF-1R expression was observed in 32% (n=64) of ULMS while moderate and strong expression was found in 14% (n=27) and 2% (n=4), respectively. IGF-1R expression was absent in only 52% (n=104) of ULMS compared

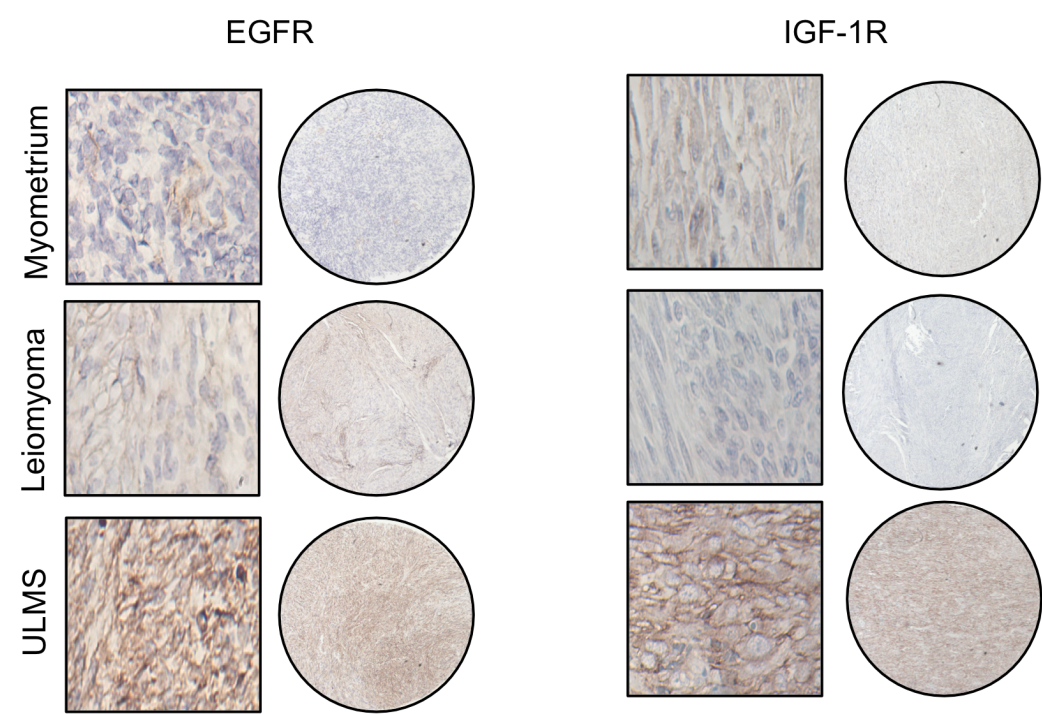


Figure 15. EGFR and IGF-1R receptor tyrosine kinase expression in ULMS and controls. Images of EGFR and IGF-1R expression in myometrium, leiomyoma, and ULMS samples. A marked increase is found in ULMS compared to controls.³³⁰

to more than three-quarters of controls. EGFR and IGF-1R expressions were both increased in tumor versus controls, but only IGF-1R elevated expression was found to be significant ($p=0.2239$ and $p=0.0003$, respectively). The average of cells with positive expression per ULMS sample were EGFR - 73% ($\pm 14\%$) and IGF-1R - 23% ($\pm 32\%$; **Appendix Figure A9B**). Expression and distribution between controls is given in **Appendix Figure A8B**).

PDGF- α and - β receptors and ligands

Expression levels of platelet derived growth factor receptors alpha and beta (PDGFR- α and PDGFR- β) and their respective ligands (PDGF- α and PDGF- β) were examined (**Figure 16, Table 14B**). PDGFR- α expression was strong in 42% ($n=83$), weak in 50% ($n=100$) and absent in 9% ($n=18$) of ULMS (**Appendix Table A9B**). Furthermore, PDGF- α was strongly expressed in 8% ($n=17$), weakly expressed in 64% ($n=129$) and not expressed in 28% ($n=57$) of ULMS. PDGFR- α expression significantly increased while PDGF- α ligand expression was significantly reduced in ULMS compared with controls ($p<0.0001$ and $p<0.0001$) with averages of 43% ($\pm 27\%$) and 23% ($\pm 25\%$) cells per ULMS sample with positive expression, respectively. Conversely, PDGFR- β and PDGF- β expression was strong in only 1% ($n=1$ and $n=2$, respectively) of ULMS samples; moderate expression was noted in 21% ($n=42$) for PDGFR- β and 27% ($n=54$) for PDGF- β of samples. PDGFR- β was only weakly expressed in 74% ($n=145$) and was not expressed in 4% ($n=8$) of ULMS. Similarly, PDGF- β was weakly expressed in 72% ($n=146$) and absent in one ULMS sample. PDGFR- β and PDGF- β expression levels were both significantly increased in

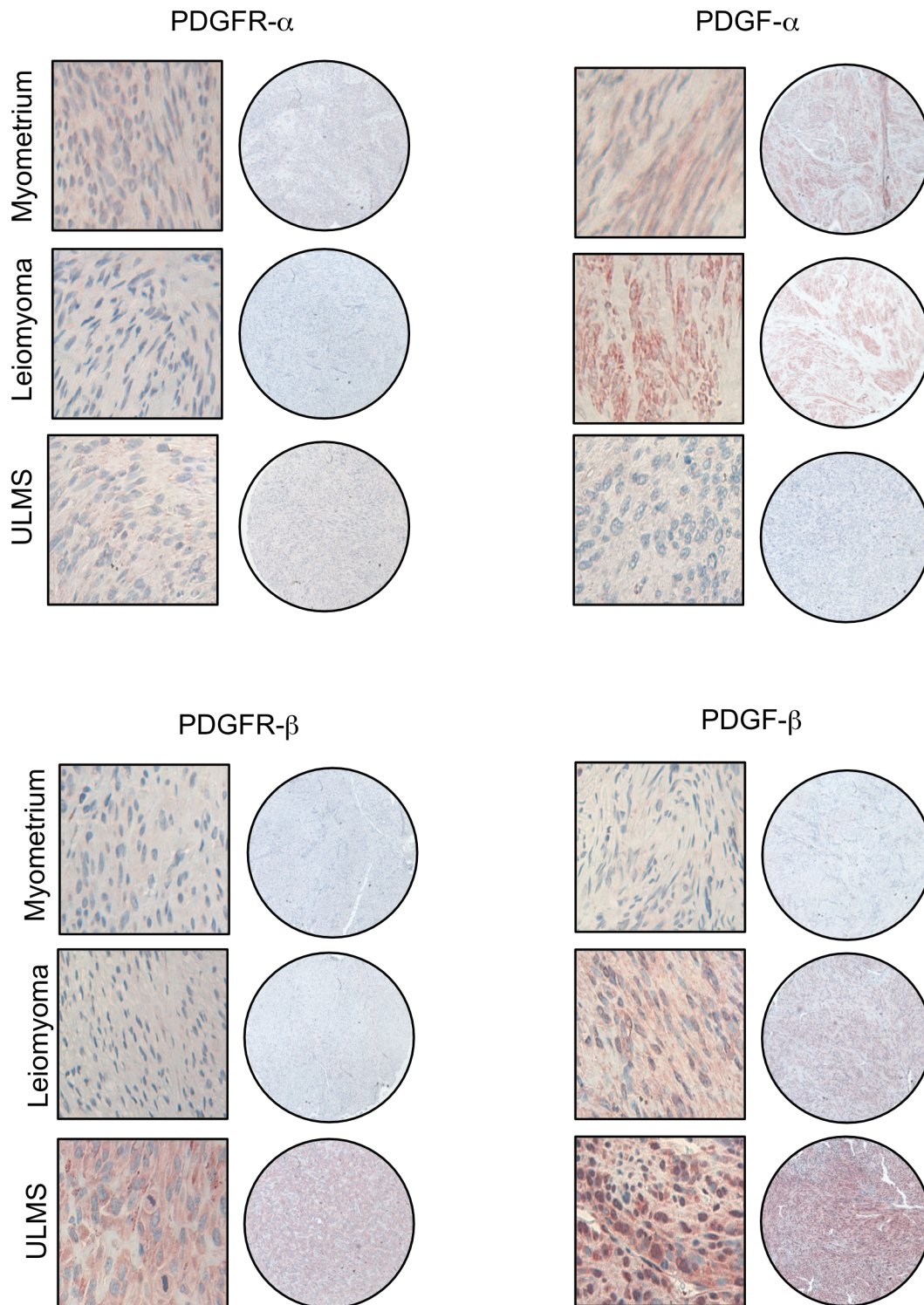


Figure 16. Expression of PDGF family receptors and ligands in ULMS compared to controls. Photographic representation of PDGF receptors $-\alpha$ and $-\beta$ show increased expression in ULMS compared to myometrium and leiomyoma controls. Further, PDGF- α ligand expression decreases in ULMS while PDGF- β ligand expression is enhanced.³³⁰

ULMS compared with controls ($p<0.0001$ and $p=0.0009$) and had positive expression in 57% ($\pm 24\%$) and 57% (± 24), respectively. Expression and distribution of controls is given in **Appendix Figure A8B**.

c-KIT, MET and AXL

Expression levels of c-KIT, MET, and AXL were also evaluated (**Table 14B, Figure 17**). c-KIT expression was not common in ULMS; 98% ($n=202$) of all ULMS samples had no expression. Further, only 2% ($n=5$) of ULMS had weak c-KIT expression and no samples had strong expression (**Appendix Figure A8B**). Similarly, all controls were negative for c-KIT expression; there was no significant difference between ULMS and controls ($p=0.46$). c-KIT expression was absent in most samples and was therefore scored as present or absent; an average percentage of cells expressing c-KIT could not be calculated. MET was expressed in all ULMS; 7% ($n=14$) had strong expression, 57% ($n=117$) had moderate expression, and 36% ($n=73$) weakly expressed MET (**Appendix Table A9B**). All controls also expressed MET and there was no significant difference between tumor and controls ($p=0.40$). The average number of ULMS cells per sample expressing MET was 85% ($\pm 9\%$). Expression of AXL was strong in 20% ($n=40$), weak in 58% ($n=113$) and absent in 22% ($n=43$) of ULMS; no controls had high expression. AXL expression increased significantly in ULMS versus controls ($p=0.0168$) and the average number of cells per ULMS sample positively expressing AXL was 40% ($\pm 33\%$).

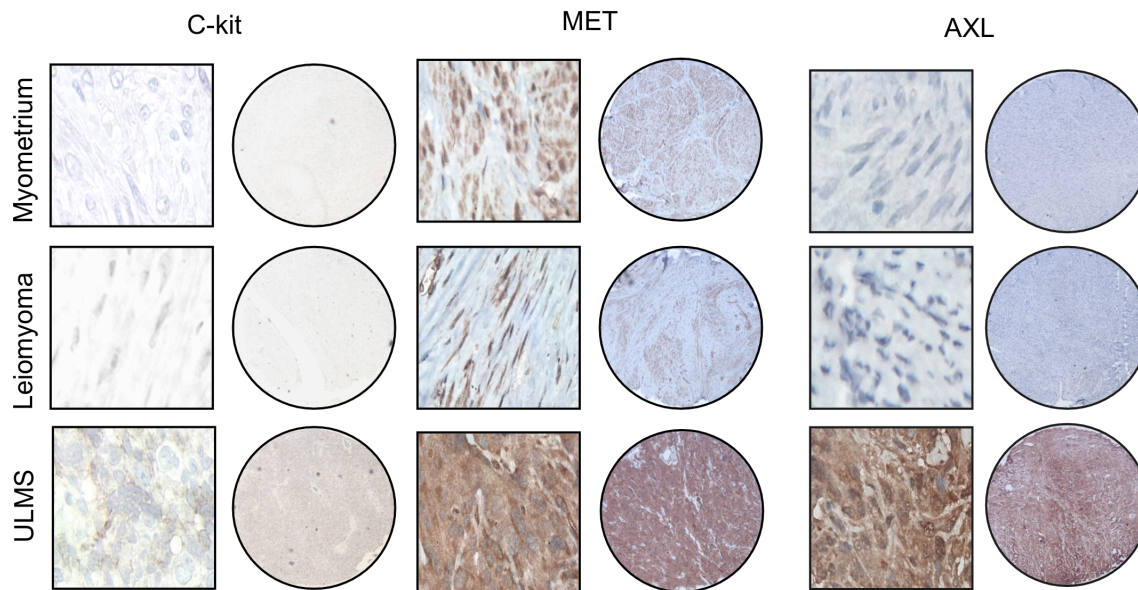


Figure 17. c-KIT, MET, and AXL expression in ULMS compared to controls. Photographic representation of receptor tyrosine kinases c-KIT, MET, and AXL in ULMS indicate that c-KIT is not highly expressed in ULMS or controls. MET and AXL expressions are elevated in ULMS compared to controls.³³⁰

Biomarker correlation to tumor status

Several tyrosine kinase receptors and one ligand were differentially expressed depending on tumor status (**Table 15B**) Expression of EGFR and IGF-1R was significantly increased in advanced versus primary lesions ($p=0.0009$ and $p=0.0349$) while PDGF- α expression decreased in metastatic versus local disease ($p=0.0238$) and AXL expression was significantly elevated in advanced compared with primary ULMS ($p=0.048$).

Outcome analysis

RTK receptor and ligand intensities were correlated with patient outcome to identify molecular prognosticators and potential therapeutic targets. Only intensity expression of PDGF- β correlated to recurrence-free survival ($p=0.0193$ [HR=2.12, 95% CI: 1.13-3.99]; **Table 16A**). PDGF- β intensity and distribution expressions also correlated to

metastasis-free survival ($p=0.0439$ [HR=0.30, 95% CI: 0.09-0.97] and $p=0.0215$ [HR=0.98, 95% CI: 0.97-1.00], respectively). c-KIT intensity expression was the only mTOR pathway component identified to correlated to enhanced disease specific survival ($p=0.0284$ [HR=5.28, 95% CI: 1.19-23.35]), however, few c-KIT positive ULMS were identified and this correlation likely does not reflect true correlation. Of note, PDGFR- β and PDGF- β distribution expressions correlated with DSS ($p=0.0394$ [HR=1.02, 95% CI: 1.00-1.04] and $p=0.0284$ [HR=5.28, 95% CI: 1.19-23.35], respectively; **Appendix Table A10**).

Although enhanced activation of several RTKs we observed (including IGF-1R, PDGFR- β , and AXL) might contribute to ULMS mTOR pathway activation, they do not appear to be promising prognosticators. No single receptor is likely to be the solo or even primary contributor; however, hyperactivation of several receptors in concert may underline the observed elevated activity of the mTOR pathway. It is logical to focus therapeutic efforts on downstream loci where RTK signals converge in the mTOR pathway, given that no single receptor is the likely cause of pathway activation.

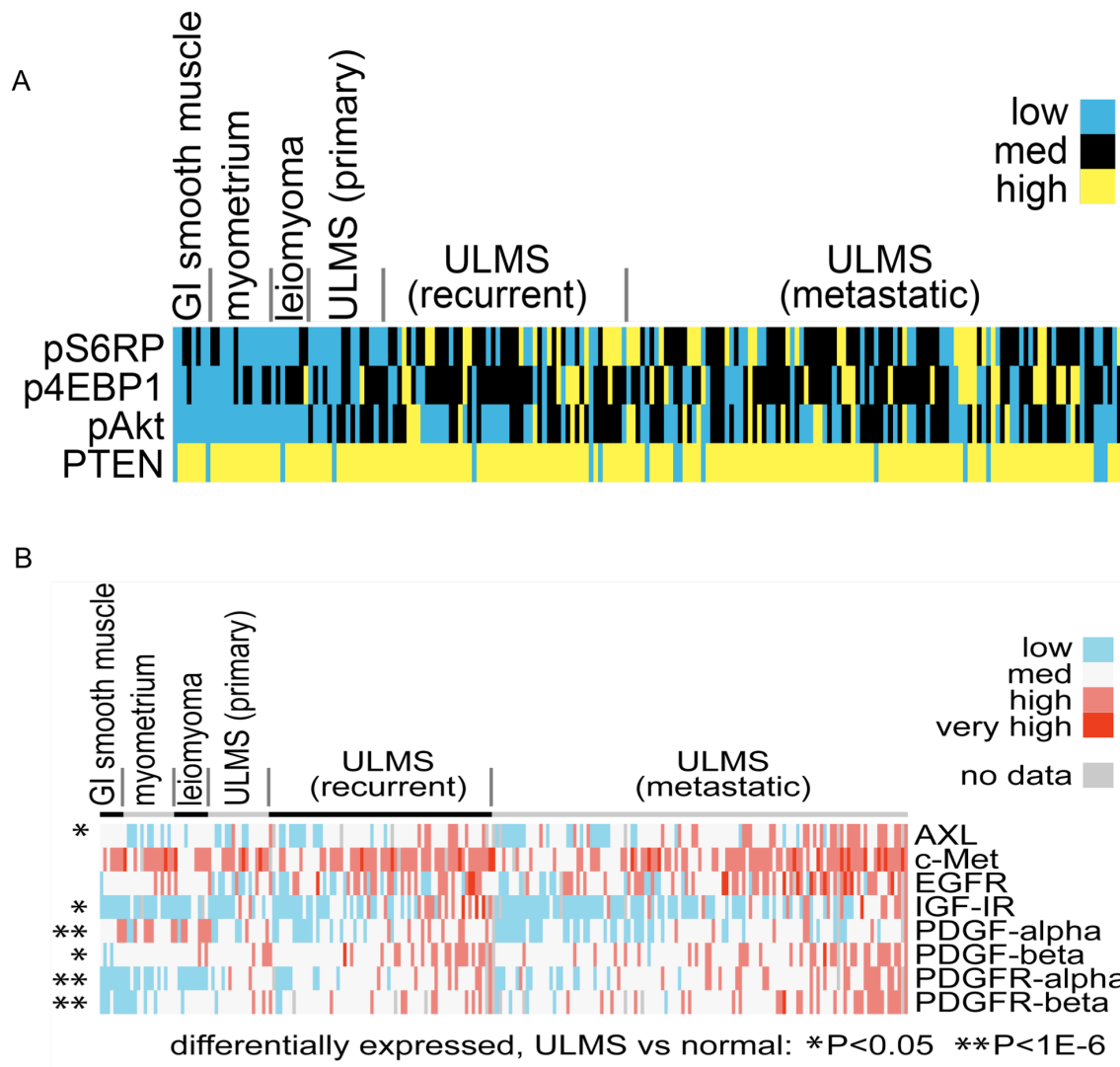


Figure 18. Heat map representation of mTOR activation in biomarkers. Heat map representation of mTOR activation in associated biomarker expression levels in each of the TMA spots for which all markers were available for A) mTOR pathway componentry. The mTOR pathway is highly active in ULMS compared to controls. Of note, PTEN levels were entered as negative (“lost”) in blue and as positive (“expressed”) in yellow; PTEN loss was not commonly observed in ULMS. B) differential expression of tyrosine kinase receptors/ligands in ULMS compared with controls.^{249,330}

Table 14. Biomarker correlation to disease status for mTOR pathway componentry and tyrosine kinase receptors/ligands.

Marker	Control				Tumor		p
	Smooth Muscle		Leiomyoma		ULMS		
	Total n=	Moderate- Strong Intensity (%)	Total n=	Moderate - Strong Intensity (%)	Total n=	Moderate - Strong Intensity (%)	
A. mTor pathway componentry							
pS6RP	25	7 (28%)	8	2 (25%)	200	145 (73%)	<0.0001
cyt. p4EBP1	23	10 (43%)	7	2 (29%)	193	150 (78%)	<0.0001
nuc. p4EBP1	24	5 (22%)	7	3 (43%)	192	117 (61%)	0.0001
pAKT	25	0 (0%)	7	0 (0%)	187	110 (59%)	<0.0001
PTEN Loss [@]	25	2 (8%)	8	1 (13%)	203	15 (7%)	0.3670
B. Tyrosine kinase receptors/ligands							
EGFR	25	3 (12%)	8	2 (25%)	199	72 (36%)	0.2239
IGF-1R	25	0 (0%)	6	0 (0%)	199	31 (16%)	0.0003
PDGFR- α	25	0 (0%)	8	0 (0%)	201	83 (41%)	<0.0001
PDGFR- β	25	0 (0%)	8	0 (0%)	196	43 (22%)	<0.0001
PDGF- α	25	10 (40%)	8	0 (0%)	203	17 (8%)	<0.0001
PDGF- β	25	2 (8%)	8	0 (0%)	203	56 (28%)	0.0009
c-KIT	25	0 (0%)	8	0 (0%)	207	5 (2%)	0.46*
MET	25	14 (56%)	8	6 (79%)	204	131 (64%)	0.4013
AXL	25	0 (0%)	8	0 (0%)	196	40 (20%)	0.0168

Total n= indicates the number of evaluable samples in each dataset

[@] is defined as having a score of 0 on a scale of 0-3 (defined in *) and indicates complete loss of protein expression

*p values were calculated by Fisher's exact test

All p-values compared control (myometrium, GI smooth muscle, leiomyoma) versus ULMS; bolded values indicate statistical significance ($p < 0.05$)

Table 15. Biomarker correlation to tumor status for mTOR pathway componentry and tyrosine kinase receptors/ligands.

	Primary ULMS		Recurrent ULMS		Metastatic ULMS		
		Moderate- Strong		Moderate - Strong		Moderate - Strong	
Marker	Total n=	Intensity (%)	Total n=	Intensity (%)	Total n=	Intensity (%)	<i>P</i>
A. mTor pathway componentry							
pS6RP cyt.	18	6 (33%)	61	47 (76%)	120	92 (77%)	0.0259^b
p4EBP1 nuc.	16	9 (56%)	59	50 (85%)	118	91 (77%)	0.1762 ^b
p4EBP1	16	4 (25%)	59	39 (66%)	118	74 (63%)	0.3032 ^b
pAKT	16	9 (56%)	58	35 (60%)	113	66 (58%)	0.3878 ^b
PTEN Loss [@]	18	1 (6%)	64	4 (6%)	121	10 (8%)	0.91 ^{a*}
B. Tyrosine kinase receptors/ligands							
EGFR	17	2 (12%)	65	23 (36%)	117	47 (40%)	0.0009^b
IGF-1R	18	2 (11%)	64	14 (22%)	117	15 (13%)	0.0349^b
PDGFR- α	18	2 (11%)	63	57 (90%)	120	24 (20%)	0.1165 ^b
PDGFR- β	18	2 (11%)	65	8 (12%)	120	7 (6%)	0.0926 ^a
PDGF- α	18	3 (17%)	61	10 (16%)	117	30 (26%)	0.0238^a
PDGF- β	18	4 (22%)	64	19 (30%)	121	33 (27%)	0.4659 ^b
c-KIT	18	0 (0%)	65	3 (5%)	124	2 (2%)	0.46 ^{b*}
MET	17	11 (65%)	64	46 (72%)	123	74 (60%)	0.1238 ^a
AXL	18	2 (11%)	61	8 (13%)	117	30 (26%)	0.048^a

Total n= indicates the number of evaluable samples in each dataset

@ is defined as having a score of 0 on a scale of 0-3 and indicates complete loss of protein expression

^a compares local (primary and recurrent) to metastatic lesions

^b compares primary to advanced (recurrent and metastatic) lesions

*p values were calculated by chi-squared test, except where 50% of the cells had expected counts less than 5%, in which case Fisher's exact test was applied

All p-values compared control (myometrium, GI smooth muscle, leiomyoma) versus ULMS; bolded values indicate statistical significance ($p < 0.05$)

Table 16. Biomarker correlation to outcome.

A. Biomarker intensity expression univariate correlation to recurrence-free (RFS) and metastasis-free (MFS) survival				
RFS (univariable)			MFS (univariable)	
Marker	<i>P</i>	Hazard Ratio (95% CI)	<i>P</i>	Hazard Ratio (95% CI)
pS6RP	0.1871	-	0.1381	-
cyt. p4EBP1	0.0733	-	0.1122	-
nuc. p4EBP1	0.0567	-	0.8343	-
pAKT	0.5227	-	0.5592	-
PTEN	0.9087	-	0.9308	-
EGFR	0.5005	-	0.8481	-
IGF-1R	0.0791	-	0.7211	-
PDGFR- α	0.1153	-	0.4692	-
PDGF- α	0.7117	-	0.1136	-
PDGFR- β	0.1124	-	0.5837	-
PDGF- β	0.0193	2.12 (1.13-3.99)	0.0439	0.30 (0.09-0.97)
c-KIT	0.9570	-	0.1340	-
MET	0.6183	-	0.1913	-
AXL	0.1220	-	0.8066	-
B. Biomarker intensity expression univariate and multivariate correlation to disease specific survival				

DDS (univariable)		
Marker	<i>P</i>	Hazard Ratio (95% CI)
pS6RP	0.1759	-
cyt. p4EBP1	0.0856	-
nuc. p4EBP1	0.0750	-
pAKT	0.9899	-
PTEN	0.7488	-
EGFR	0.6093	-
IGF-1R	0.6697	-
PDGFR- α	0.0786	-
PDGF- α	0.1671	-
PDGFR- β	0.1889	-
PDGF- β	0.4958	-
c-KIT	0.0284	5.28 (1.19-23.35)
MET	0.1237	-
AXL	0.8277	-

Chapter 5. Creation and Development of Critical Bioresources

One of the most significant obstacles hindering sarcoma research is the remarkable lack of commercially available bioresources. Currently, only one uterine sarcoma and two leiomyosarcoma cell lines are commercially available through the American Type Culture Collection (ATCC); only one is thought to actually represent human uterine leiomyosarcoma. Due to this profound lack of bioresources, we deemed it necessary to create our own. Towards this goal, we worked in close collaboration with the MD Anderson Cancer Center Tissue Bank and Surgery Department to consent patients pre-operatively. Through these mechanisms, we have secured multiple human specimens of leiomyosarcoma for scientific research. Tissues were transported to the laboratory from surgery, cells were disaggregated, and primary cell strains were created and grown as previously described.³³² To date, we have created >15 Uterine LMS cell strains and several additional extra-uterine LMS strains, expanding on available bioresources and furthering our scientific studies. Table 14 shows the novel ULMS and select LMS cell strains that we have created; bolded strains identify strains that were used in this body of this work.

Prior to experimental use, all cell strains/cell lines were characterized and confirmed as LMS or ULMS. First, cell growth and proliferation via MTS assay were examined (**Figure 19**). Photographs of Leio285 cells at 24, 48, 72, and 96hr demonstrate high proliferation rates for this cell line with a doubling rate of about 24hr (Figure 18).

Table 17. Novel LMS and ULMS cell strains created by our lab at the UTMDACC Sarcoma Research Center from primary patient tumors.

Name	Age	Race	Diagnosis	Tumor Site
Leio016	71	Caucasian	Uterine LMS	Retroperitoneal Recurrence
Leio096	56	Caucasian	Uterine LMS	Lung Metastases
Leio196	61	Caucasian	Uterine LMS	Bladder Recurrence
Leio278	58	Caucasian	Uterine LMS	Ureter Recurrence
Leio285	59	Caucasian	Uterine LMS	Lung Metastases
Leio2987	53	Caucasian	Uterine LMS	Pelvic Recurrence
Leio303	46	Caucasian	Uterine LMS	Mesentery Recurrence
Leio360	46	Caucasian	Uterine LMS	Pelvic Recurrence
Leio485	54	Caucasian	Uterine LMS	Pelvic Recurrence
Leio505	60	Caucasian	Uterine LMS	Lung Metastases
Leio594	61	African American	Uterine LMS	Retroperitoneal Recurrence
Leio661	71	Caucasian	Uterine LMS	Pelvic Recurrence
Leio780	48	Caucasian	Uterine LMS	Lung Metastases
Leio938	77	Caucasian	Uterine LMS	Pelvic Recurrence
Leio012	42	African American	Retroperitoneal LMS	Retroperitoneal Recurrence
Leio987B	50	Hispanic	Abdominal LMS	Lung Metastases

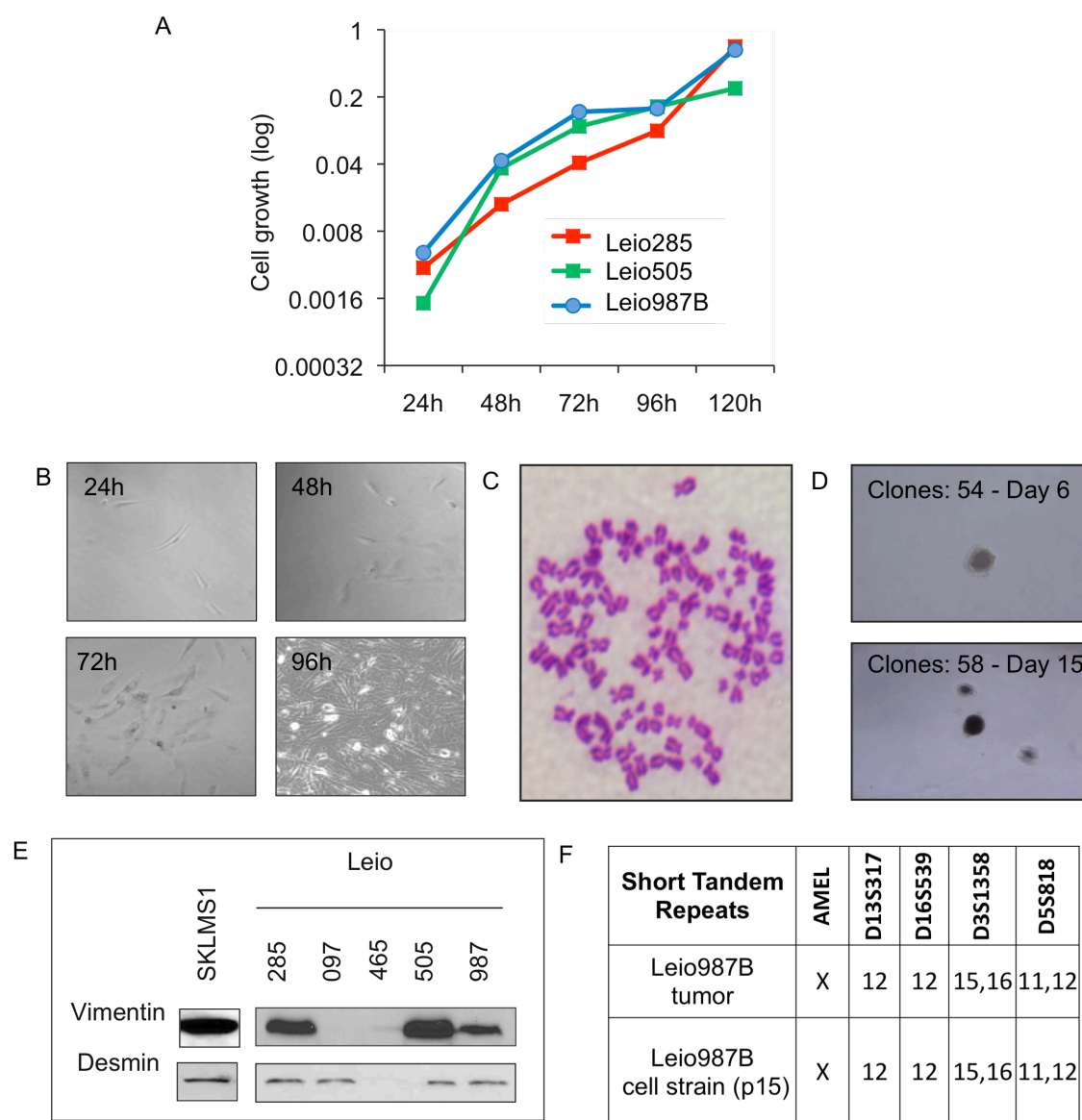


Figure 19. Primary cell cultures were characterized for *in vitro* growth and proliferation ability. MTS proliferation assays showed doubling times of 24 to 48 h for LMS cell strains (Leio285, Leio505, and Leio987B) and B) microscope images of Leio285 cells at 24, 48, 72, and 96 h confirmed doubling times of 24-48 h. Newly created cell strains are comprehensively characterized for C) karyotype assessment via GIEMSA (the complex karyotype of Leio177 is shown), D) clonogenic ability (Leio285 shown), and E) WB expression of vimentin and desmin to validate strains as LMS/ULMS. F) Short tandem repeats (STR) are acquired for all ULMS cell strains and cells are tested against the original tumor every 5-10 passages to ensure quality.

Next, the karyotypes of each cell strain/line were evaluated using GEMSA and clone-forming ability was evaluated via clonogenic assays (Figure 18, Leio177). As an example, GEMSA results using Leio177 are presented and show a complex karyotype; the figure also confirms that Leio285 has clone-forming ability. Additionally, immunoblotting was performed to ensure that all cells express vimentin, a marker of all mesenchymal cells, and desmin, a marker of muscle differentiation. Cells that did not express both markers could not be confirmed as LMS/ULMS and were not used in further studies. For example, Figure 7 showed vimentin and desmin WB in several cell strains. According to our standards, Leio285, Leio505, and Leio987B were considered viable LMS/ULMS cell strains while Leio097 and Leio465 were not confirmed and were not used further. Sequencing for p53 was also performed on select cell strains/cell lines (data not shown) to examine gene mutations in exons 5-9 of the gene. No mutations were found within this region for Leio285 or Mes-Sa, so these cells were deemed “*wild type*”. Notably, two mutations were found at codons 245 and 247 of exon 7 in SKLMS1. These alterations were in accordance with known SKLMS1 mutations.³³³ Short tandem repeat (STR) fingerprinting was used to validate cell strain integrity. Cell strain profiles were matched to the DNA profile of the solid tumor from which they were created for validation purposes (Figure 18, Leio987B). Fingerprints for commercially available cell lines were verified against known fingerprints. Of note, many primary ULMS cell strains start to show reduced proliferation and lose several of their morphological features, including spindle shape, at passages of 30 and higher. For this reason, all experiments were performed with cells in passages 5-15 to ensure that results are accurate and consistent.

To date, we have not successfully developed novel xenograft tumor mouse models for many of our LMS/ULMS cell strains. While we have experimented with several ULMS xenografts using our primary cell strains, none yielded reproducible *in vivo* growth. However, we have developed successful xenograft models of commercially available cells SKLMS1 and Mes-Sa for therapeutic experiments. The SKLMS1 xenograft model employed in this dissertation is further discussed in the materials and methods section. In total, we have created several xenograft-based mouse models for *in vivo* studies in ULMS. Cell strain/line and xenograft studies remain ongoing and development of additional resources is forthcoming.

Chapter 6: Targeting ULMS mTOR deregulation *in vitro*

Utilizing our ULMS focused TMA, we demonstrated that the mTOR pathway was hyperactivated in these malignancies, correlating with disease status, tumor status, and patient outcome. From the following studies, we hypothesized that the mTOR pathway might be a viable therapeutic target in ULMS and designed a series of *in vitro* and murine-based experiments.

The mTOR pathway is overexpressed in ULMS cells

First, we evaluated if cultured human ULMS cells recapitulated clinical findings by overexpressing activated mTOR downstream effectors (pS6K and p4EBP1). Cells tested included SKLMS1, Mes-Sa, and four cell strains recently isolated by our laboratory from primary patient cultures. SKLMS1 is well-characterized LMS cell line of gynecological origin (ATCC), the ULMS cell strains were isolated and characterized as described above in materials and methods. Mes-Sa, a commercially available (ATCC), poorly differentiated uterine sarcoma cell line was also included; is unclear whether this cell line represents a ULMS or an alternative uterine sarcoma subtype, but it was included as a relevant positive control (we previously demonstrated that Mes-Sa had loss of PTEN expression.¹⁸⁷ Lastly, normal smooth muscle cell primary cultures (NSMC) were used as controls (**Figure 20**). Western blots (WBs) showed increased pS6K and p4EBP1 expression in tumor cells versus control (**Figure 20**); increased levels of pAKT were observed in tumor cells (**Figure 20**). Excluding Mes-Sa, none of the ULMS cell strains exhibited loss of PTEN expression (**Figure 20**). Together, these data are in concordance with data obtained

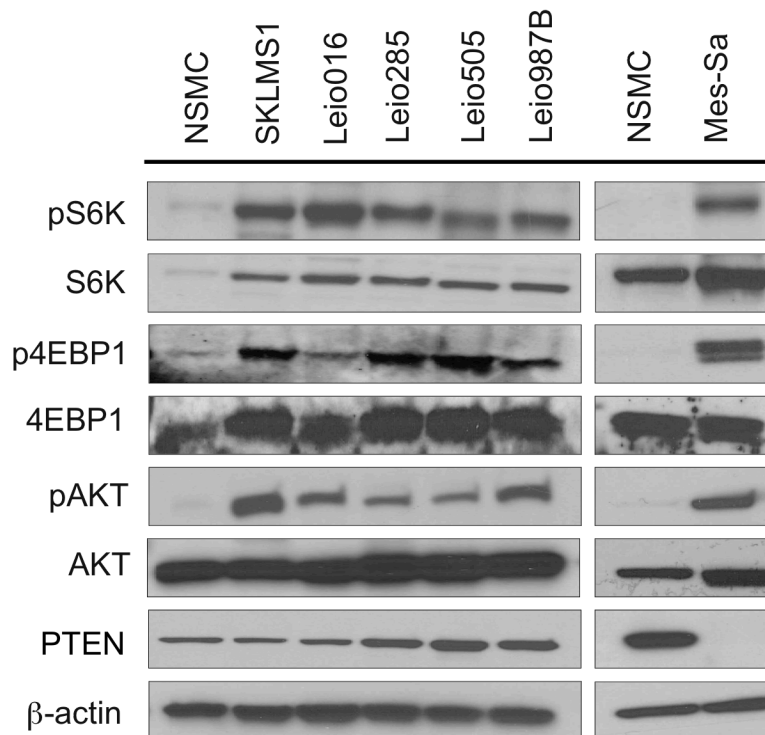


Figure 20. mTOR pathway is commonly activated in ULMS cells. Western blot analyses demonstrate increased phosphorylation of the mTOR downstream effectors S6K and 4EBP1, and the mTOR upstream regulator, AKT, in protein extracts of human ULMS cell strains/lines as compared to expression observed in normal smooth muscle cells (NSMC). Only Mes-Sa cells exhibited loss of PTEN expression.²⁴⁹

from our TMA and confirm that (similar to the observations in human specimens) activation of the mTOR axis is observed in ULMS cells growing in culture, rendering this a relevant model to test the effects of mTOR blockade.

mTOR blockade inhibits ULMS growth in vitro and in vivo

Rapamycin was utilized to examine effects mTORC1 inhibition on ULMS cell growth. Tumor cells were treated with incremental drug doses for 4h; decreased phosphorylation of S6K and 4EBP1 were detected in all ULMS cells; even at doses as low as 0.1nM (**Figure 21**). Functionally, a dose-dependent reduction in tumor cell

growth in response to rapamycin (0.01-50nM/96h) was observed in all cell lines/strains examined with GI₅₀ levels of ~ 1nM (**Figure 22A**). Notably, NSMC were not significantly affected by rapamycin treatment.

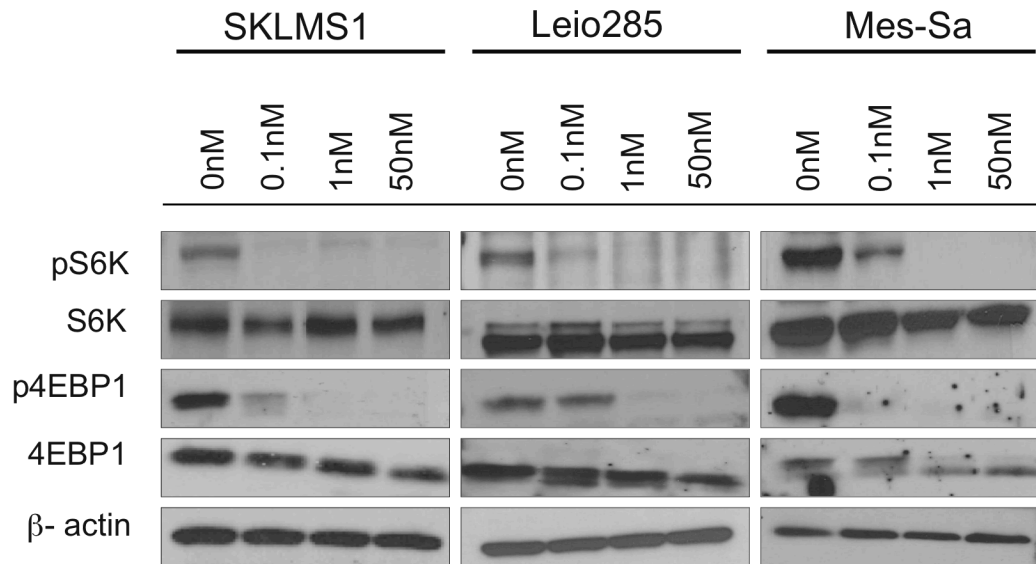


Figure 21. The mTOR pathway is inhibited with rapamycin. Rapamycin (0.1-50nM/4h) blocks the activation of the mTOR downstream targets S6K and 4EBP1 (WB analyses).²⁴⁹

Additionally, mTORC1 blockade inhibited colony formation capacities of ULMS cells; rapamycin pre-treatment (24h) significantly reduced the number of large colonies by 40-50% ($p=0.0007$, 0.0005 , and 0.03 for SKLMS1, Leio285, and Mes-Sa, respectively); when administered continuously, rapamycin almost abrogated colony formation ($p<0.0001$, <0.0001 and $=0.002$ for SKLMS1, Leio285, and Mes-Sa, respectively; **Figure 22B**). Rapamycin has been suggested to promote G1 arrest and play a cytostatic rather than cytotoxic role in tumor growth inhibition

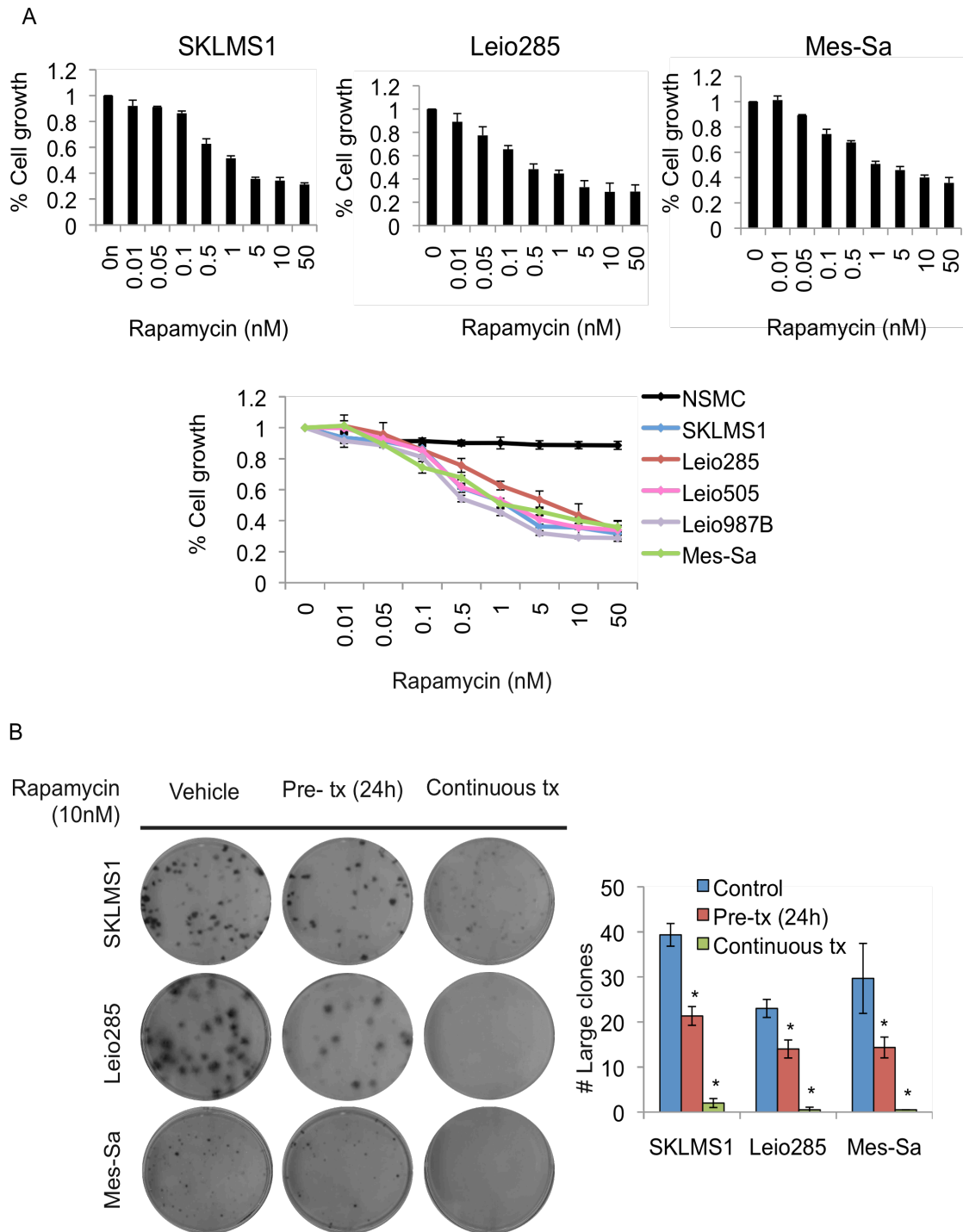
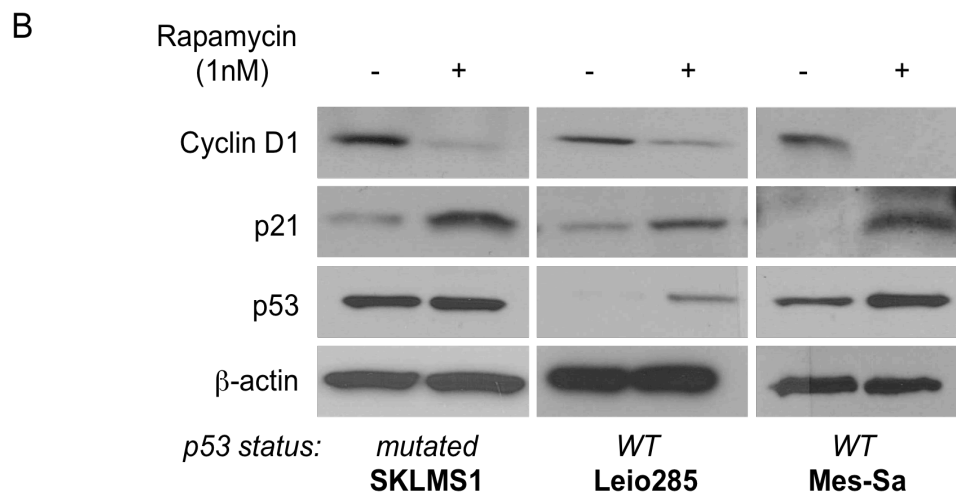
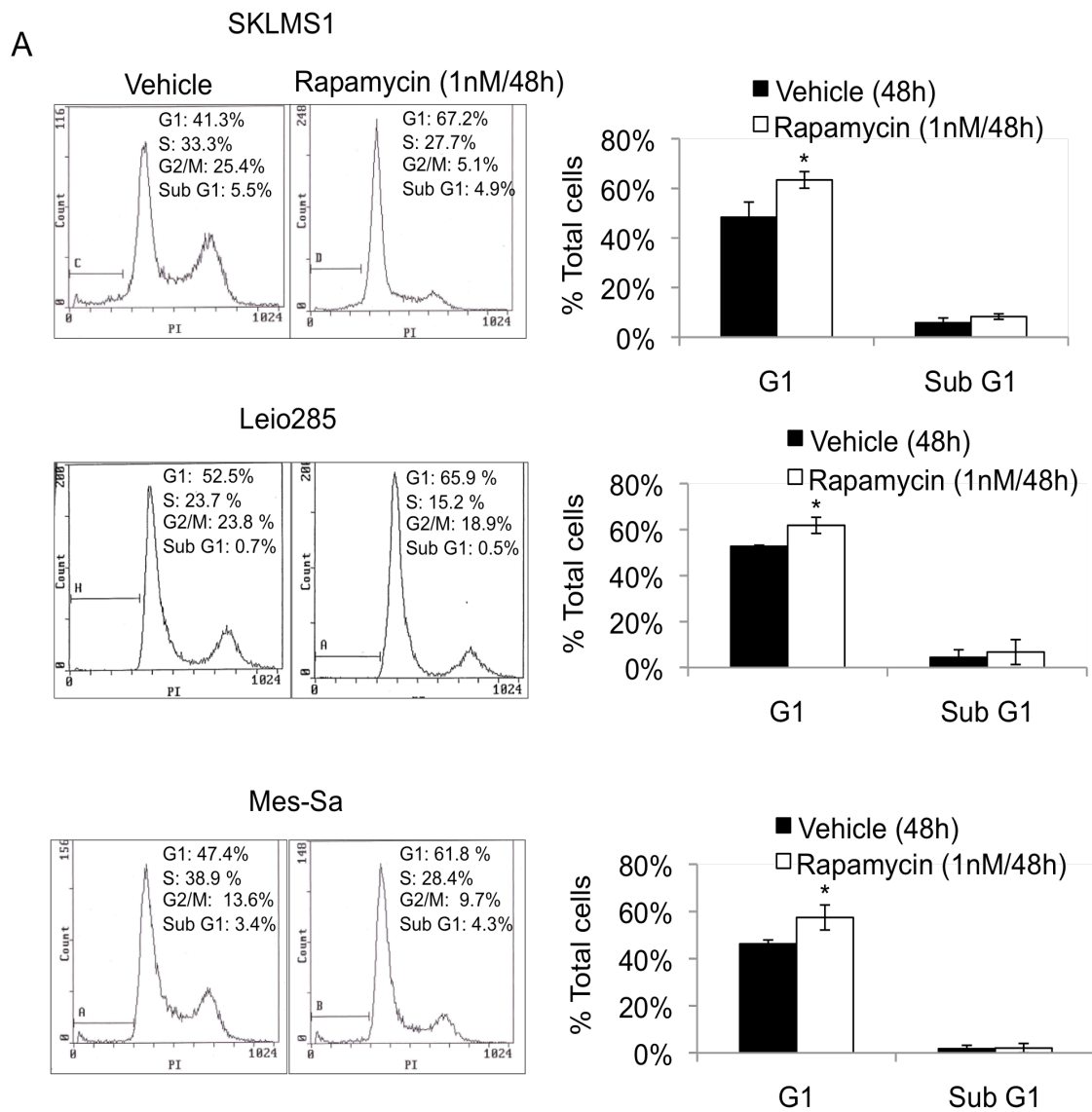


Figure 22. mTOR blockade using rapamycin inhibits cell growth. A) MTS assays demonstrating a rapamycin-induced (96h) dose-dependent decrease in ULMS cell growth. In addition, B) rapamycin (both as pre- and continuous treatments) inhibits the colony formation capacity of ULMS cells. [Graphs represent the average of at least 2 repeated experiments \pm SD; * denotes statistically significant effects($p < 0.05$)].²⁴⁹



C

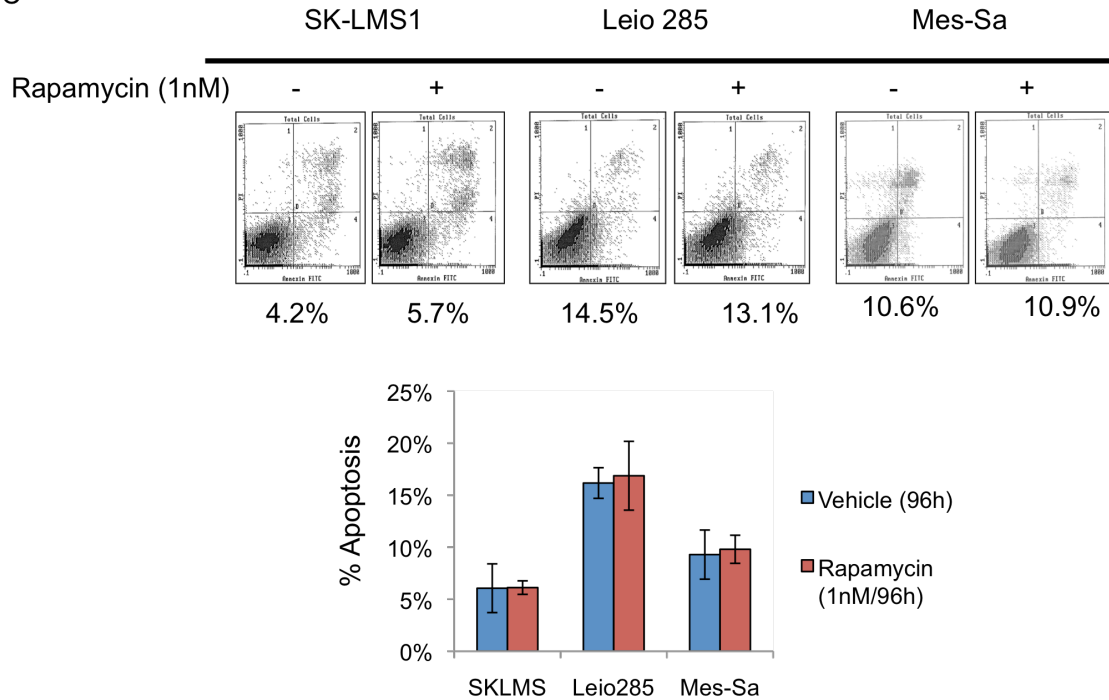


Figure 23. mTOR blockade using rapamycin induces G1 cell cycle arrest. A) Rapamycin treatment (1nM/48h) results in a G1 cell cycle arrest in ULMS cells. B) WB analyses demonstrate decrease in cyclin D1 and increased p21 expression in treated cells, independent of p53 mutational status. Increased p21 protein expression was found in cells harboring wild-type p53. C) No significant increase in apoptosis was noted by PI/Annexin V FACS analysis following rapamycin treatment (1nM/96h). [Graphs represent the average of at least two repeated experiments \pm SD; * denotes statistically significant effects ($p < 0.05$)].²⁴⁹

Therefore, the effects of rapamycin on cell cycle progression and apoptosis were evaluated. Rapamycin treatment (1nM/48h) induced substantial G1 cell cycle arrest ($p=0.02$, 0.01 , and 0.03 in SKLMS1, Leio285, and Mes-Sa, respectively; **Figure 23A**). This effect could partly be secondary to the observed decrease in cyclin D1 expression and increased p21 expression in treated cells (**Figure 23B**). Interestingly, we found that G1 cell cycle arrest occurred independently of p53 mutational status. In both wild-type p53 cell strains/lines (Leio285 and Mes-Sa), an increase in p53 protein expression was observed; increase was also found in p53 mutated cells (SKLMS1). In all cells, increased levels of p21 were observed independent of enhanced p53

expression or mutational status (**Figure 23B**). Notably, increased sub-G1 fractions were not noted in response to rapamycin as per PI staining FACS analysis (**Figure 23A**). Furthermore, Annexin-V/PI staining FACS analysis, conducted after 96h rapamycin treatment, failed to demonstrate significant apoptosis (**Figure 23C**).

Based on the above findings, we next sought to evaluate whether rapamycin effects could also be observed *in vivo*. Although ULMS primary cultures can be utilized for experimental studies *in vitro*, none of the cell strains available to us consistently grow *in vivo*. In contrast, SKLMS1 cells reproducibly grow as xenografts when injected into immunocompromised mice; therefore, this experimental model was selected for therapeutic experiments. Rapamycin (or vehicle control) treatment was initiated after tumor establishment (~4-5mm in largest dimension). Mice in both groups were followed for tumor size and toxicity; treatment was terminated when tumors in the control group reached an average of 1.5cm in largest dimension. Treatment with rapamycin resulted in tumor growth delay compared to vehicle-treated tumors (**Figure 24A**). Average tumor volumes recorded at termination of the study were control group: $1361\text{mm}^3 \pm 354$ vs. rapamycin group: $895\text{mm}^3 \pm 449$ ($p=0.0519$; **Figure 24A**). While trending towards statistical significance, these data revealed that rapamycin exerted only marginal, cytostatic effects on ULMS growth. Similarly, a decrease in tumor weight was noted (control = $1.97\text{g} \pm 1.11$ vs. $1.1\text{g} \pm 0.72$) although it did not reach statistical significance ($p=0.11$; **Figure 24B**). To confirm that rapamycin inhibits mTORC1 activity *in vivo*, formalin-fixed, paraffin-embedded tumor sections from mice

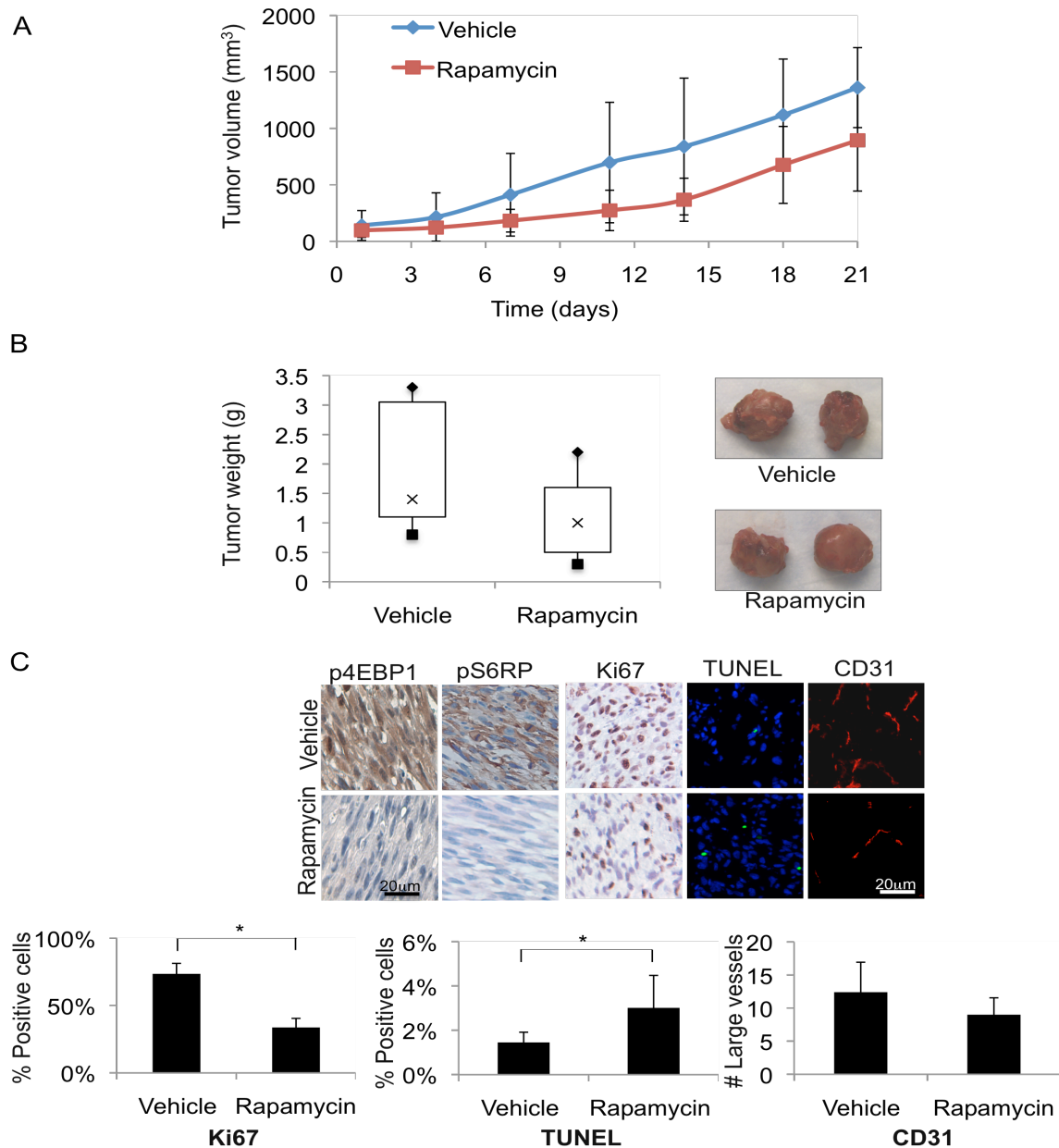


Figure 24. Rapamycin treatment delays the growth of ULMS xenografts. A) Treatment with rapamycin resulted in SKLMS1 xenograft tumor growth delay compared to control vehicle-treated tumors. Rapamycin treatment induced a decrease trending towards statistical significance ($p=0.0519$) in tumor volume at termination of the study. B) A decrease in tumor weight was also noted, although it did not reach statistical significance ($p=0.11$): C) Immunohistochemical (IHC) staining confirmed decreased p4EBP1 and pS6RP expression in rapamycin treated tumors. Rapamycin treated tumors exhibited decreased proliferation (measured by Ki67), increased apoptosis (measured via TUNEL), and decreased (although not statistically significant) CD31 positivity (original images were captured at 400x magnification). [* denotes statistically significant effects ($p<0.05$)].²⁴⁹

of both study arms were immunohistochemically evaluated. Decreased p4EBP1 and pS6RP expression was observed in rapamycin-treated tumor samples (**Figure 24C**). Of note, a significant ($p=0.001$) decrease in the number of Ki67 expressing tumor cells was observed in rapamycin related samples (**Figure 24C**). A relatively small but significant increase in TUNEL expression was noted with rapamycin treatment ($p=0.005$; **Figure 24C**). Finally, a reduction in CD31 positive blood vessels was found in rapamycin treated tumors (9.5 ± 0.99) compared to controls (12.4 ± 4.5), although this reduction did not reach statistical significance ($p=0.2$). Taken together, observations made in this preclinical model recapitulate effects noted in human clinical studies, supporting identification of additional ULMS molecular aberrations therapeutically targetable in combination with mTOR.

Rapamycin in combination with doxorubicin offers little therapeutic advantage.

Patients with recurrent or metastatic ULMS often have tumors that cannot be wholly resected surgically, necessitating chemotherapy as first-line treatment. We examined if the addition of a commonly used ULMS chemotherapeutic agent (doxorubicin) to rapamycin treatment was therapeutically beneficial. Uterine sarcoma cells (Mes-Sa) and a doxorubicin-resistant Mes-Sa strain (Mes- Sa D5X) were employed for these studies. Mes-Sa cells responded moderately to low dose doxorubicin with a GI_{50} of about 0.5uM whereas growth of doxorubicin resistant Mes-Sa D5X cells was not significantly affected by chemotherapy (**Figure 25A**). Rapamycin (0.5nM) and doxorubicin (150nM) in low-dose combination (48h) significantly reduced cell growth (**Figure 25B**). Additionally, an MTS assay (48h) was performed to examine anti-proliferative effects; in combination, rapamycin and doxorubicin were not synergistic;

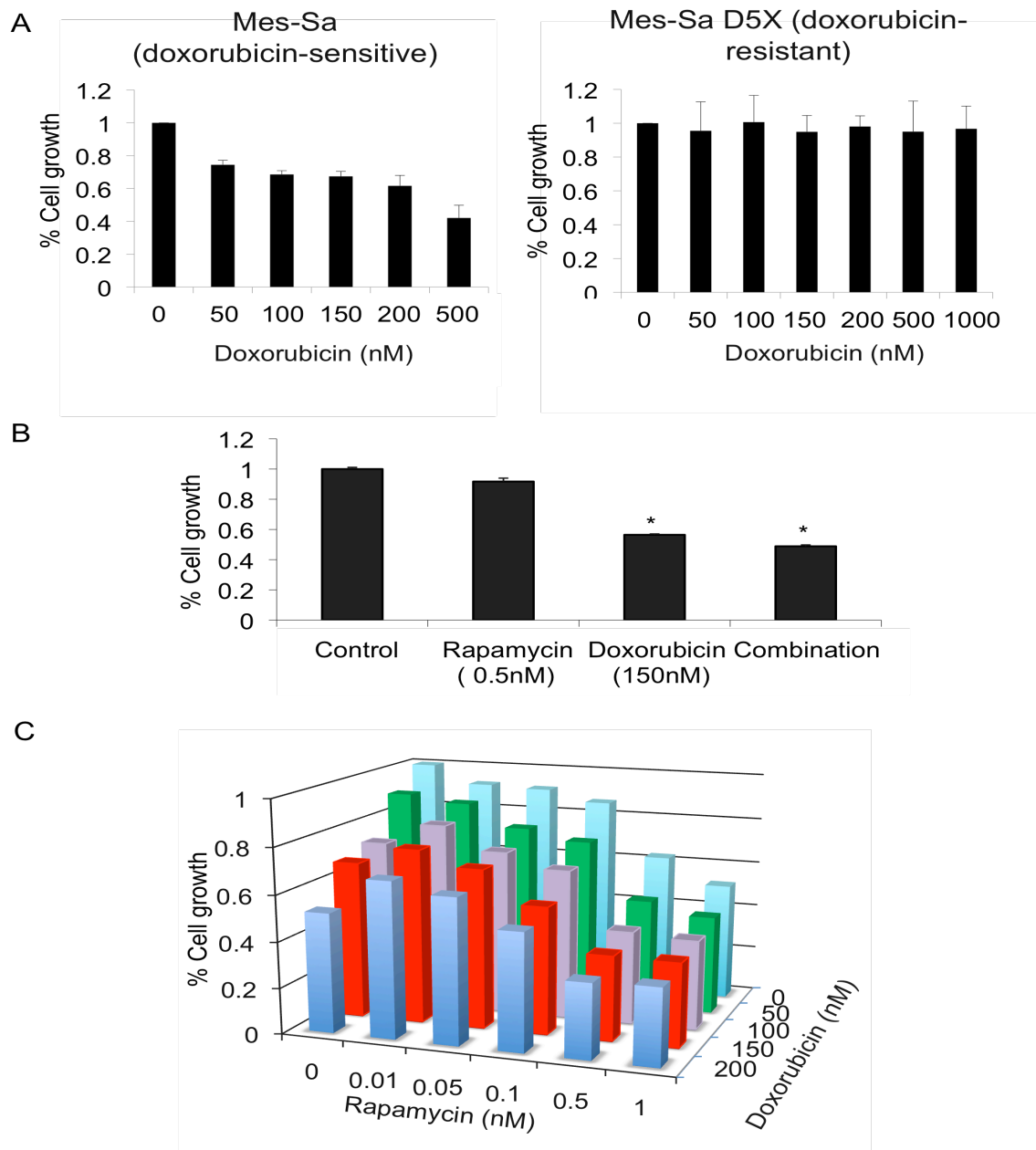


Figure 25. Rapamycin and doxorubicin combined treatment yielded additive anti-ULMS effects. A) MTS proliferation assay was employed to determine growth inhibition effects of rapamycin in sensitive (Mes-Sa) and resistant (Mes-Sa D5X). B) Combination therapy with low dose rapamycin and doxorubicin does not yield significantly beneficial results over single agent doxorubicin and C) rapamycin and doxorubicin do work synergistically in ULMS. [* denotes statistical significance ($p < 0.05$, compared to control)].

effects were likely additive (**Figure 25C**). Taken together, these data do not support the use of rapamycin in combination with conventional chemotherapies in the treatment of ULMS; molecular-based therapies may present with improved response when combined with rapamycin as anti-ULMS therapies and such therapies should therefore be evaluated.

Chapter 7: Aurora A kinase deregulation in ULMS and inhibition via MLN8237

In the current era of molecularly targeted therapies, personalized agents hold the promise of increased efficacy and decreased toxicity compared to conventional chemotherapy. To identify novel, molecularly based targets for ULMS therapy, we entered into collaboration with Dr. Matthew L. Anderson, an investigator from Baylor College of Medicine. Together, we validated Aurora A kinase as a viable therapeutic target and carried out several studies that examined the effects of Aurk A inhibition in ULMS.

Aurk A is upregulated/overexpressed in human ULMS tissues and cells

In collaboration, an Illumina Gene Chip Array of 12 ULMS specimens (FIGO stage 1) and 10 healthy myometrium samples (**Figure 26A**) was used to compile genome-wide transcriptional profiles of more than 75 ULMS patients to compare gene expression of nearly 50,000 genes.²⁵⁰ From this dataset, we identified several differentially expressed genes in human ULMS versus healthy myometrium and leiomyoma. Of the overexpressed genes, more than half were known to be integrally involved with centrosome and spindle assembly, and functioned during mitosis. Notably, Aurora A and B kinases were identified as upregulated by 7.9-fold ($p < 10^{-11}$) and 3.5-fold ($p < 10^{-6}$), respectively. Using semi-quantitative PCR (**Figure 26B**) and western blotting (**Figure 26C**), we validated that Aurk A is overexpressed in human ULMS tissues.²⁵⁰ Next, we confirmed via western blotting that Aurk A kinase is overexpressed in a large panel of ULMS

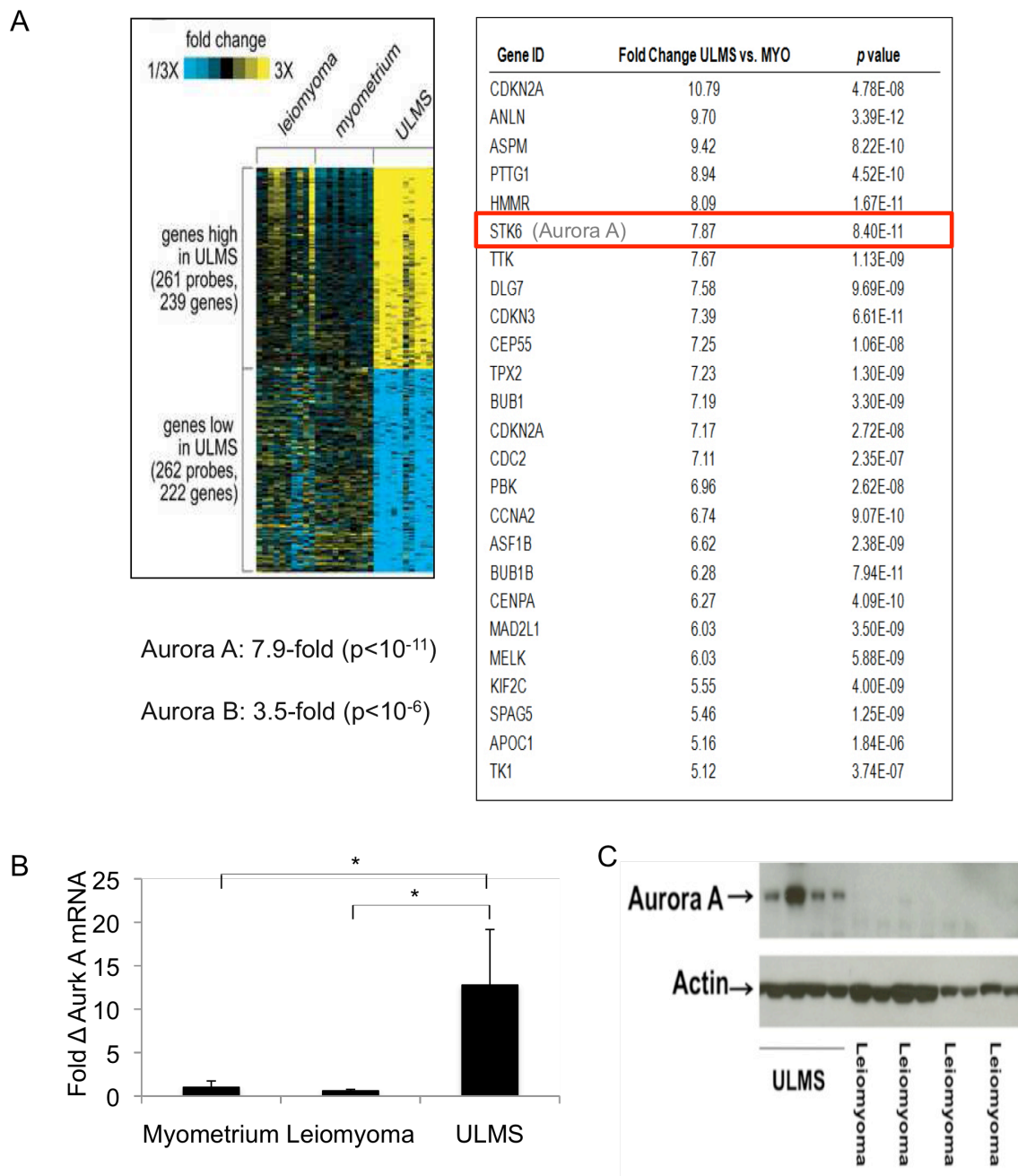


Figure 26. Aurk A kinase is upregulated and overexpressed in human ULMS. A) A gene array conducted with Dr. Matthew Anderson (Baylor College of Medicine) reveals several genes differentially expressed in healthy myometrium, leiomyoma, and FIGO stage I ULMS. Genes identified included Aurk A and Aurk B kinases. B) Aurk A mRNA was upregulated in human ULMS by about 15-fold and C) Aurk A was overexpressed in human ULMS compared with leiomyoma controls. Taken together, these data advocate a role for Aurk A kinase in ULMS.²⁵⁰

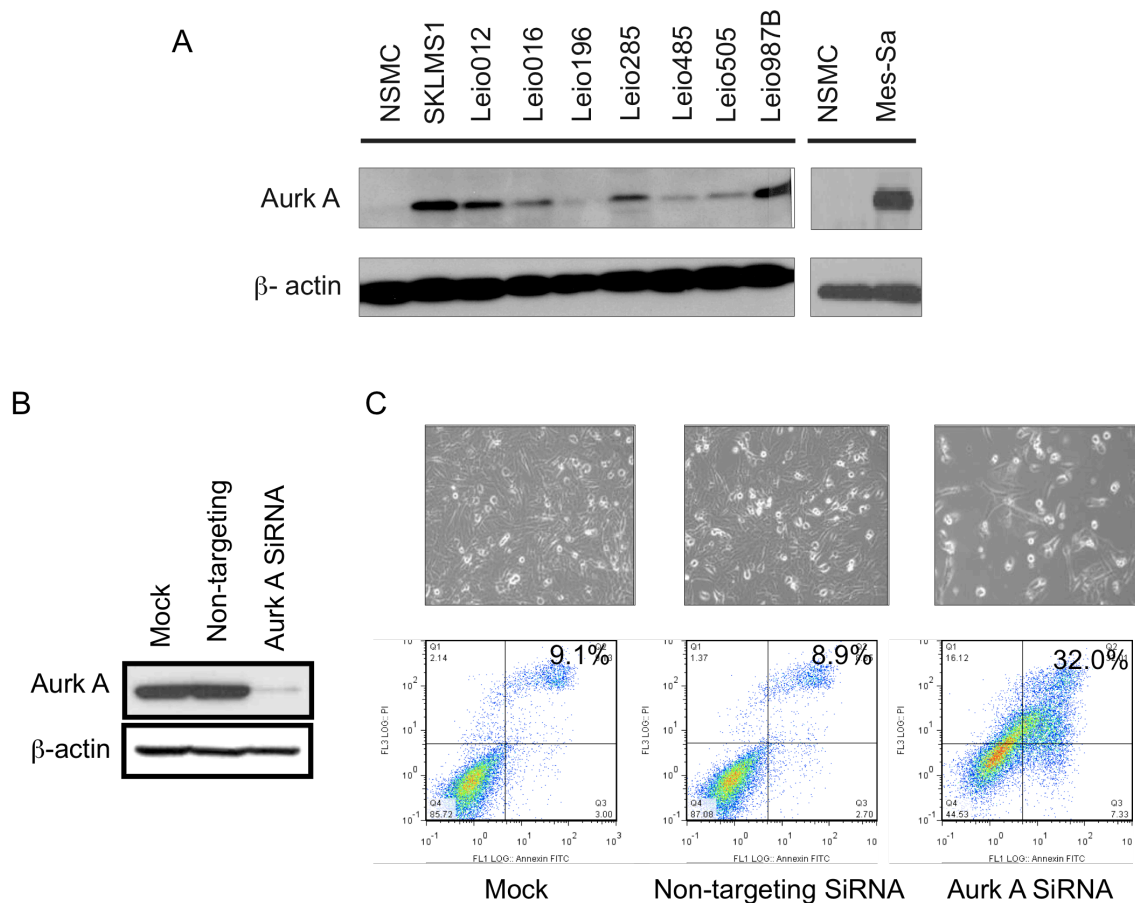


Figure 27. Aurk A kinase is highly expressed in human ULMS and knock down induces apoptosis. A) WB analysis demonstrated increased Aurk A protein expression in a panel of ULMS cell strains/lines compared to normal smooth muscle cells (NSMC). B) Aurk A was readily knocked down using an SiRNA approach and C) knockdown induced significant apoptosis compared with mock and non-targeting controls.²⁵⁰

cell strains/lines (**Figure 27A**) as compared to a smooth muscle control (NSMC). To examine if Aurk A may be a viable target, an SiRNA approach was used (knock down confirmed in **Figure 27B**). Knock down induced significant apoptosis (32% compared with 9.1% and 8.9% for mock- and non-targeting SiRNA-treated cells, respectively (**Figure 27C**). Taken together with preliminary conclusions from the gene array, our results suggest that Aurk A may be a relevant target to pair with mTOR inhibition for

improved ULMS therapy. and suggest that it may be a viable target for anti-ULMS therapies.

The Aurora A kinase inhibitor, MLN8237, inhibits ULMS cell growth and induces G2/M cell cycle arrest and apoptosis

Next, we aimed to evaluate the effects of Aurk A inhibition in ULMS to validate it as a promising co-target for combination therapy with rapamycin. Towards this aim, we acquired a novel, investigational, orally bioavailable, and selective Aurk-A inhibitor, MLN8237 (Millennium Pharmaceuticals), and first examined its anti-tumor effects in our *in vitro* ULMS model. MTS assays demonstrated marked MLN8237 dose-dependent (0-100nM/96h) ULMS cell growth inhibition; estimated GI₅₀ levels of ~ 75nM were observed in several cell lines evaluated (**Figure 28A**). Notably, a large number of spherical, floating cells were observed with MLN8237 treatment, suggesting that the drug may induce apoptosis. Furthermore, MLN8237 abrogated ULMS colony formation capacity: 24h pre-treatment with MLN8237 resulted in markedly reduced numbers of colonies (p=0.003, 0.01, and 0.0004 for SKLMS1, Leio285, and Mes-Sa, respectively); under continuous treatment almost no colonies were observed (p=0.0002, =0.0002, and <0.0001 for SKLM1, Leio285, and Mes-Sa, respectively; **Figure 28B**). Next, the effects of MLN8237 on cell cycle progression were evaluated. MLN8237 treatment (75nM/48h) resulted in a marked G2/M cell cycle arrest (p=0.009, =0.03, and <0.0001 for SKLMS1, Leio285, and Mes-Sa, respectively; **Figure 29A**); significant increase in sub-G1 fraction was noted, indicating probable apoptosis (p=0.03, 0.04, and 0.04 for SKLMS1, Leio285, and Mes-Sa, respectively).

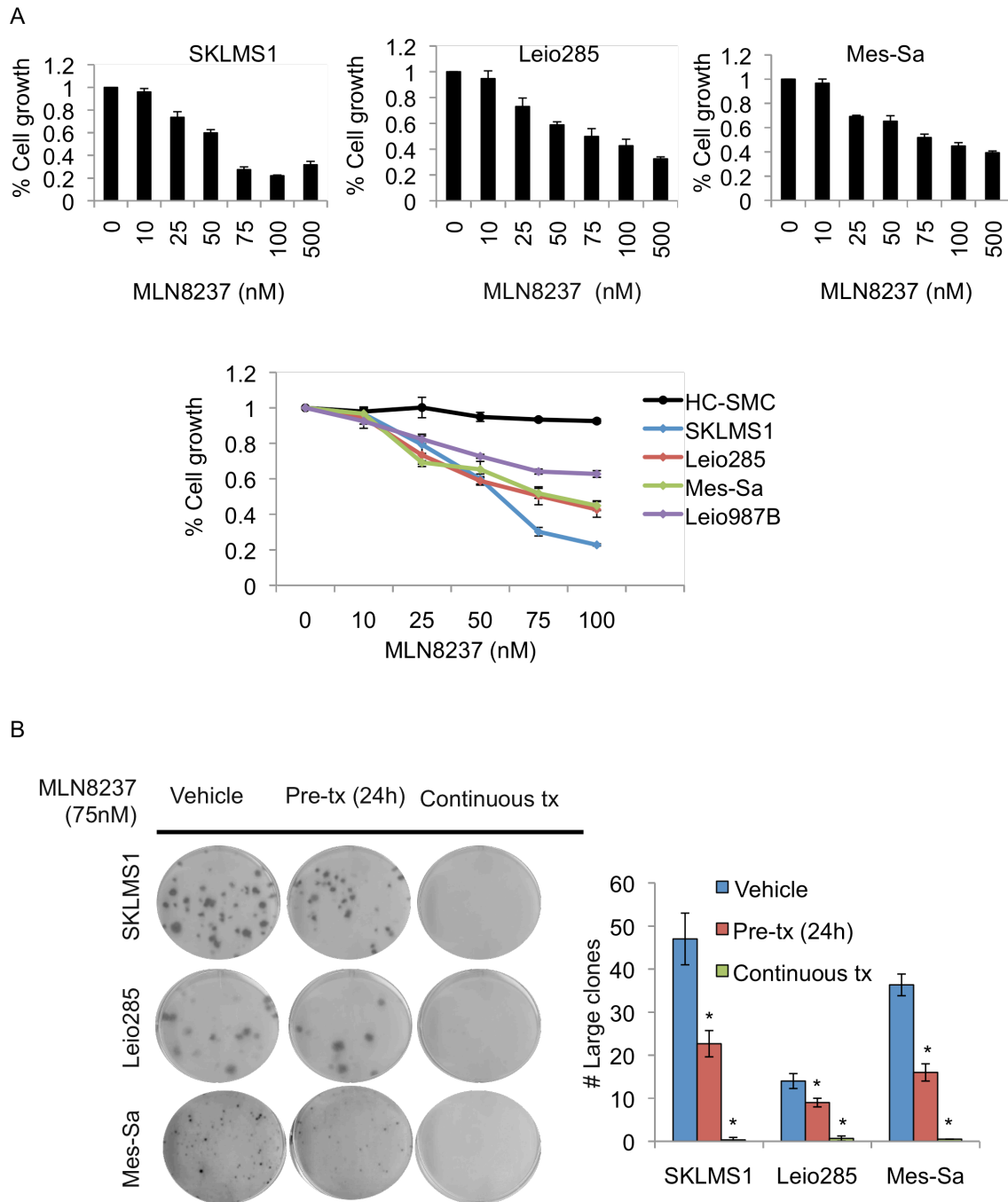


Figure 28. The Aurk A inhibitor, MLN8237, inhibits cell growth in ULMS. A) MTS assays demonstrated marked MLN8237 dose-dependent (0-500nM/96h) ULMS cell growth inhibition. B) MLN8237 (both as pre- and continuous-treatment) abrogated the colony formation capacity of ULMS cells). [Graphs are the average of at least two independent studies; * denotes statistically significant effects ($p < 0.05$)].²⁴⁹

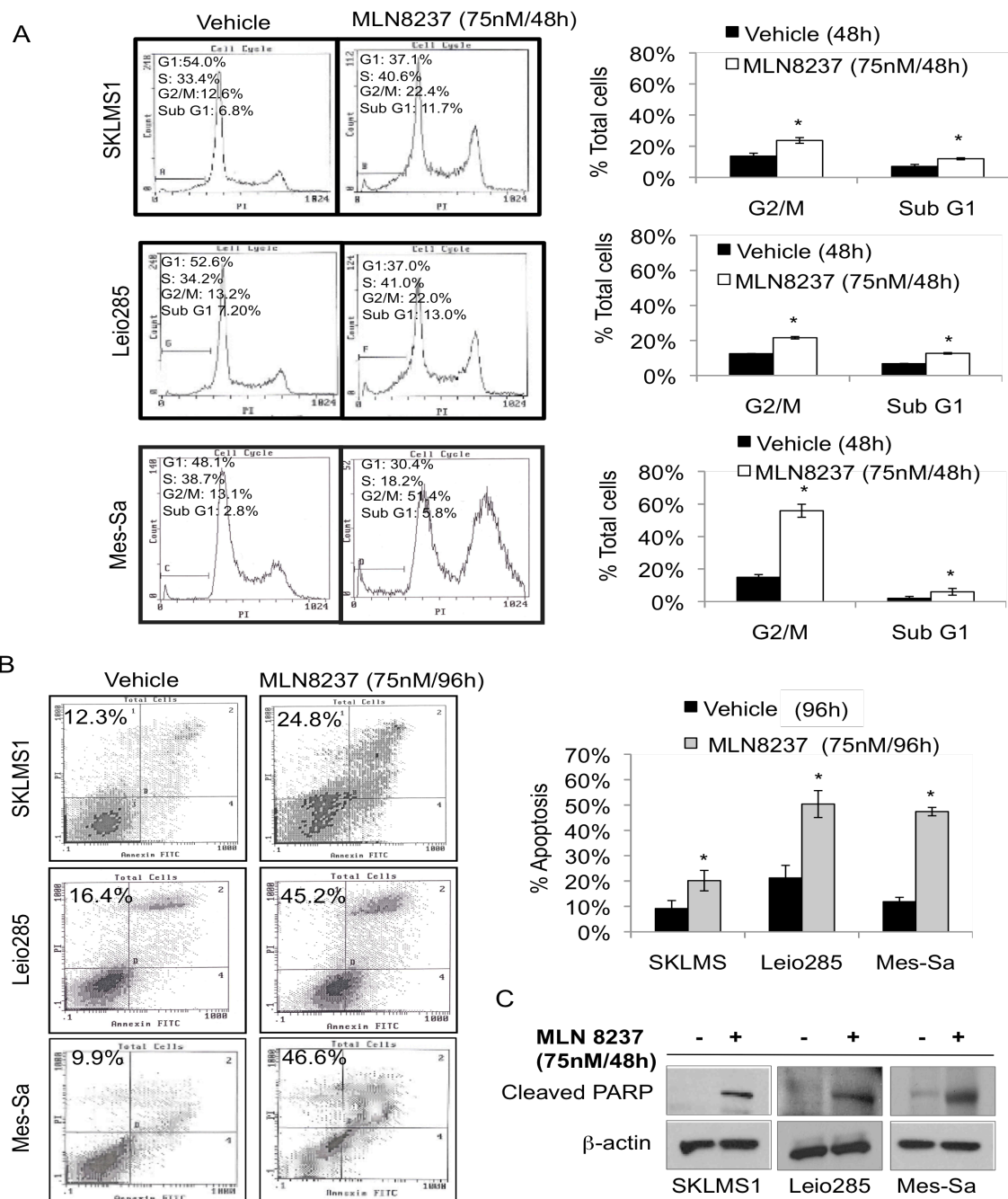


Figure 29. The Aurk A inhibitor, MLN8237, induced G2/M cell cycle arrest and apoptosis. A) MLN8237 treatment (75nM/48h) resulted in a G2/M cell cycle arrest in ULMS cells. Furthermore, increased sub-G1 fraction was observed; B) an increase (~2-4 fold) in apoptosis was observed in MLN8237 treated cells compared to vehicle treated controls (Annexin-V/PI staining FACS analysis). C) WB analyses further demonstrated increased cleaved PARP in response to treatment. [Graphs represent the average of at least two repeated experiments \pm SD; * denotes statistically significant effects ($p < 0.05$)].²⁴⁹

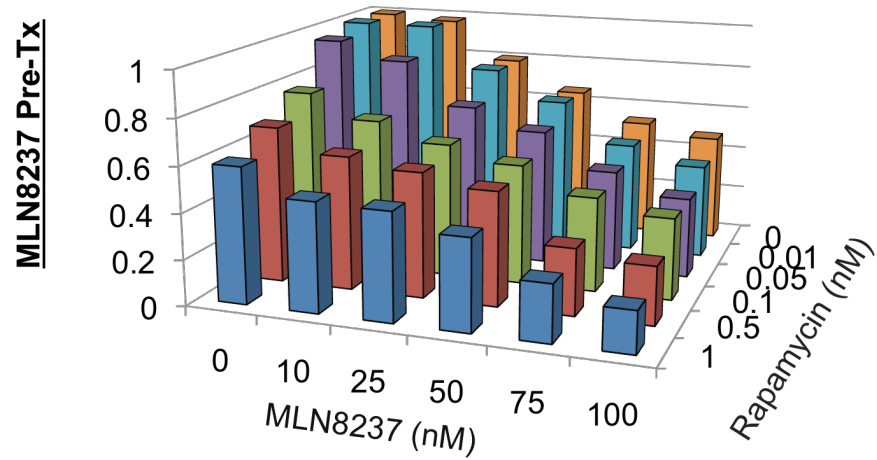
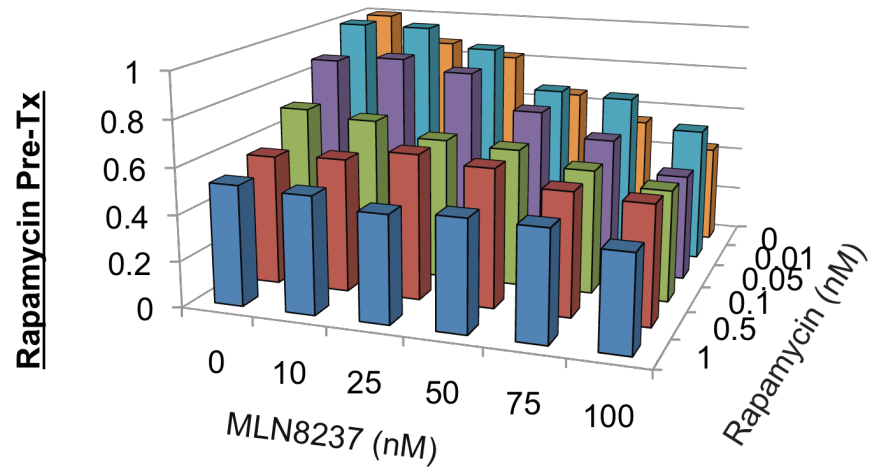
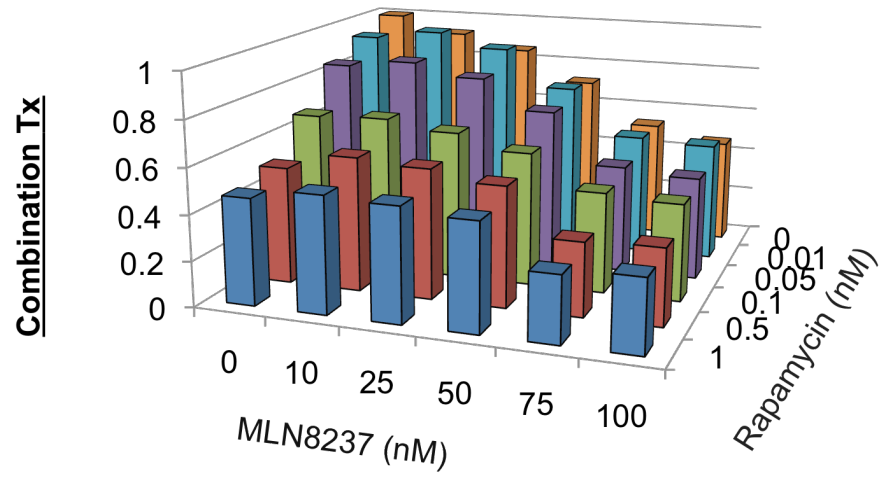
To determine the impact of MLN8237 on ULMS apoptotic cell death, Annexin-V/PI staining FACS analyses were conducted (**Figure 29B**) following 96h of treatment. A significant increase (~2-4 fold) in apoptosis was observed in MLN8237 treated cells compared to vehicle treated controls ($p=0.02$, $=0.002$, and <0.0001 , respectively for SKLMS1, Leio285, and Mes-Sa). A shorter treatment of 48h also showed substantial apoptotic activity (data not shown). Increased cleaved PARP was noticed after 48h of treatment, providing further evidence of MLN8237-induced apoptosis (**Figure 29C**). Together, these data confirm Aurk-A as a candidate anti-ULMS therapeutic target and demonstrate potential efficacy of MLN8237, suggesting that Aurk A and mTOR dual blockade in ULMS may yield improved therapeutic response over mTOR inhibition alone.

Chapter 8. Dual targeting of mTOR and Aurk A in ULMS

Combining rapamycin and MLN8237 results in superior (synergistic) anti-ULMS effects

Based on previous results, we sought to evaluate the usefulness of mTOR and Aurk A dual blockade in the treatment of ULMS. First, we determined ULMS growth effects of combined rapamycin and MLN8237. MTS assays of treated SKLMS1 cells were conducted to determine the combination index (CI), enabling assessment of potential interactions between these drugs (**Figure 30A**). Several scheduling regimens were tested: 1) simultaneous co-administration of rapamycin (increasing doses; 0-1nM) and MLN8237 (increasing doses; 0-100nM) for 96h; 2) 24h pre-treatment with rapamycin followed by co-treatment with MLN8237 for 72 hours; 3) 24h MLN8237 pre-treatment followed by 72hr co-treatment with rapamycin. Interestingly, while the first two regimens failed to demonstrate synergism between the drugs (for some doses antagonistic responses were even observed; $CI > 1.1$), isobologram analyses revealed that growth-inhibitory effects of the drug combination were strongly synergistic when administered as per the third schedule ($CI < 0.9$; **Figure 30A**). Similarly to our multi-dose synergy experiment in SKLMS1, we confirmed that MLN8237 and rapamycin worked in synergy using two other ULMS cell lines/strains with single, low-dose treatments assessed via MTS assay (as per the above schedule). In doses below the GI_{50} for both rapamycin and MLN8237, reductions in cell proliferation by $>50\%$ in Leio285 and Mes-Sa were observed in the combination group compared to control ($p < 0.0001$ for Leio285; $p = 0.0006$ for Mes-Sa) and in either agent alone ($p < 0.02$, **Figure 30A**). Similarly, combination therapy induced a superior inhibitory effect on colony formation compared to either agent alone (**Figure 30B**) and combination

A



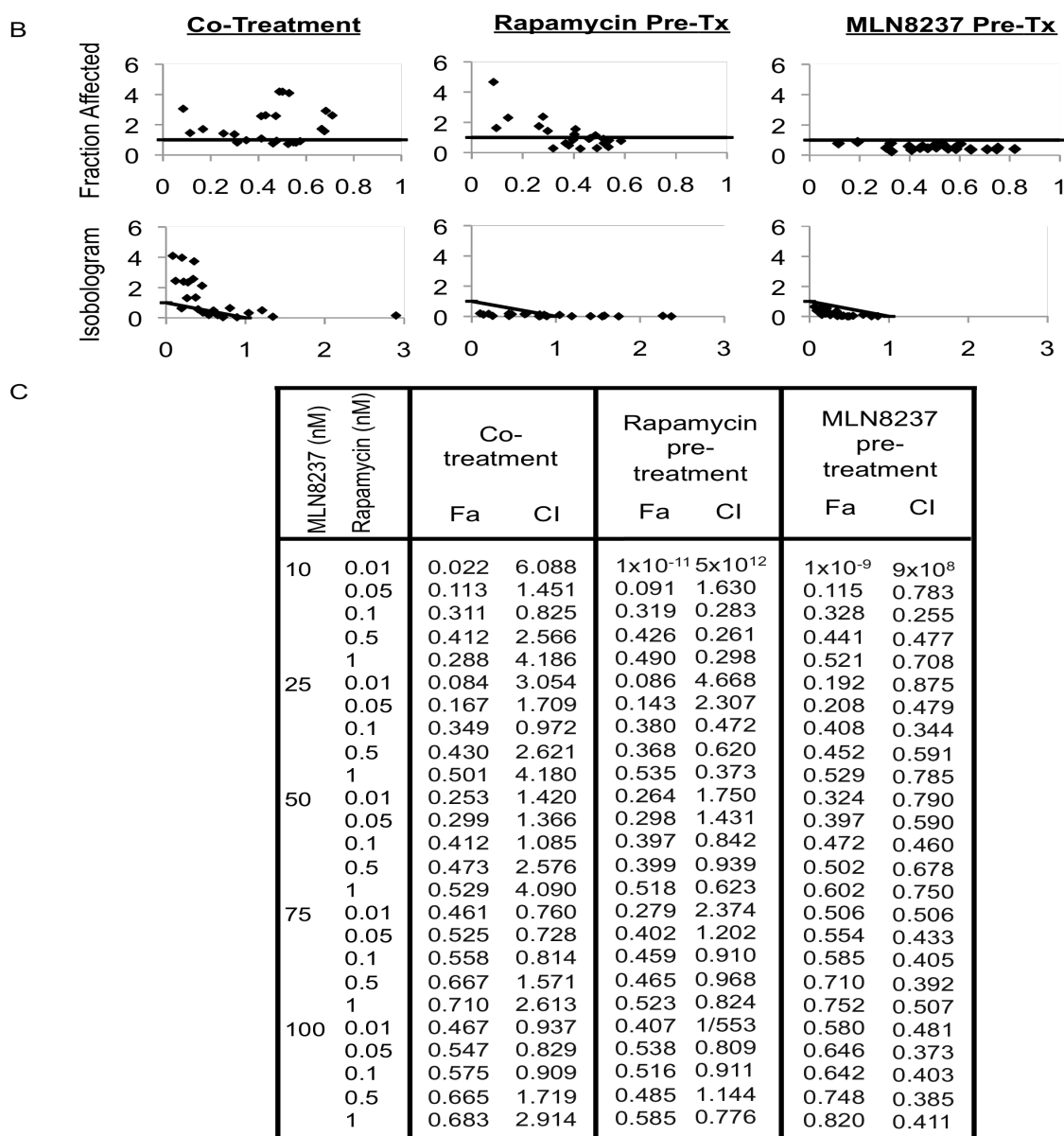


Figure 30. Combined mTOR and Aurk A targeting results in superior and synergistic ULMS effects *in vitro*. A) MTS assays were conducted using increasing doses of both rapamycin and MLN8237, three different scheduling regimens were used: 1. simultaneous co-administration of rapamycin (increasing doses; 0-1nM) and MLN8237 (increasing doses; 0-100nM) for 96h (upper graph). 2. 24h pre-treatment with rapamycin followed by co-treatment with MLN8237 for 72h (middle graph) and 3. 24h MLN8237 pre-treatment followed by 72h co-treatment with rapamycin (bottom graph). B) Fraction affected and isobologram analyses revealed that growth-inhibitor effects of the drug combination were strongly synergistic when administered per the third schedule (CI<0.9). C) Fraction affected and combination indices are given for all dosing schedules and confirm that the pre-treatment with MLN8237 schedule is synergistic; alternative schedules examined are not.²⁴⁹

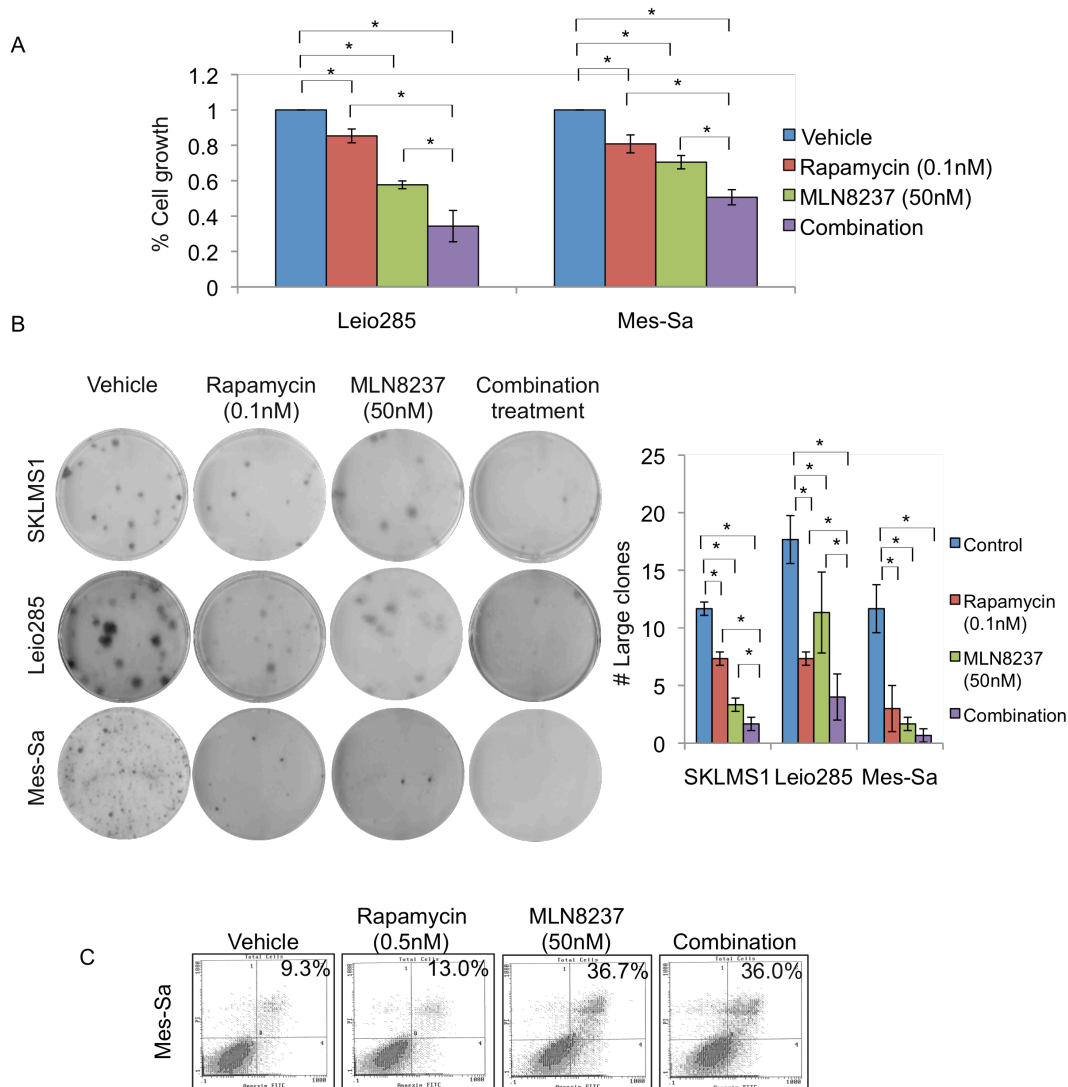


Figure 31. Dual inhibition of mTOR and Aurk A yields superior and synergistic anti-ULMS effects *in vitro*. A) Superior anti-growth effects are observed in Leio285 and Mes-Sa cells in response to dual mTOR/Aurk A inhibition (administered in low doses as per the aforementioned schedule) compared to either agent alone. B) Furthermore, combination therapy induces a superior inhibitory effect on colony formation compared to either agent alone and C) the superior effects of combination therapy are not due to an increase in apoptosis compared to levels induced by MLN8237 alone. [Graphs represent an average of at least three repeated experiments \pm SD; * denotes statistically significant effects ($p < 0.05$)].²⁴⁹

therapy did not increase levels of apoptosis compared to MLN8237 treatment alone (36.7% and 36.0%, respectively; **Figure 31C**).

Dual blockade of mTOR and Aurk A kinase in ULMS is effective *in vivo*.

Lastly, to determine whether the effects noted *in vitro* could be recapitulated *in vivo*, a four-armed therapeutic study was conducted comparing the effect of combination treatment to each drug alone or vehicle control. Of note, in accord with *in vitro* findings, mice were treated with MLN8237 and rapamycin sequentially. No major side effects or discomforts were noted; average mouse weights at the termination of the study were: control – 24.9g±0.7, rapamycin – 24.3g±2, MLN8237 – 23.6g±2.2, and combination – 22.9g±2.3. MLN8237 as a single agent inhibited SKLMS1 xenograft growth compared to control (average tumor volumes at study termination were MLN8237 - 765mm³±429 and control - 1361mm³±354, $p=0.012$; **Figure 32A**). Most importantly, combination therapy resulted in significant growth abrogation compared to rapamycin, MLN8237, or control alone; average of combination treatment tumor volumes at study termination was 227mm³±185 ($p=0.0016$, $=0.0047$, and <0.0001 , respectively; **Figure 32B**). Average tumor weights recorded at termination of the study were control group: 2.0g±1.1; rapamycin group: 1.1g±0.72; MLN8237 group: 1.2g±0.99; and combination group: 0.15g±0.13 (**Figure 32C**). In summary, dual inhibition of mTOR and Aurk A kinase in ULMS using rapamycin and MLN8237 resulted in superior and synergist effects compared to either therapy alone. H&E staining confirmed spindle shaped leiomyosarcoma cells with a large number of mitosis present (**Figure 33**). Interestingly, large circular cells with high mitotic counts were observed in the MLN8237 and co-treated tumors. Tumors treated with MLN8237

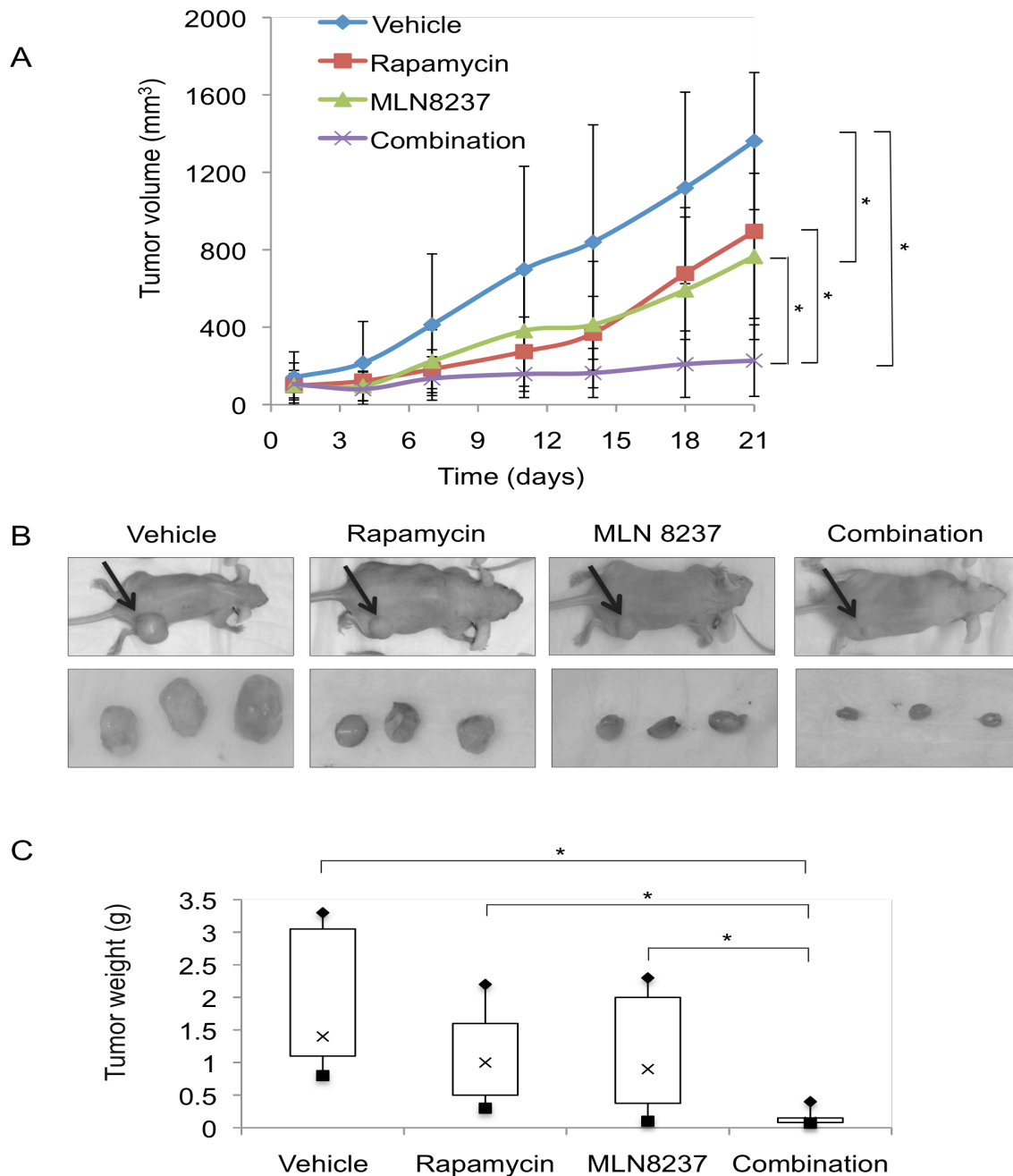


Figure 32. Combined mTOR and Aurk A targeting results in superior anti-ULMS effects *in vivo*. A) The impact of combined therapy was assessed *in vivo* using SKLMS1 xenografts growing in hairless SCID mice. MLN8237 as a single agent significantly inhibited tumor growth as compared to control. Most importantly, combination therapy resulted in significant growth abrogation as compared to rapamycin, MLN8237, or vehicle. B) Tumors are visibly smaller in mice treated with combination therapy than in any other group. C) Combination treated mice exhibited the most significant decrease in tumor weight as compared to all other therapeutic arms.²⁴⁹

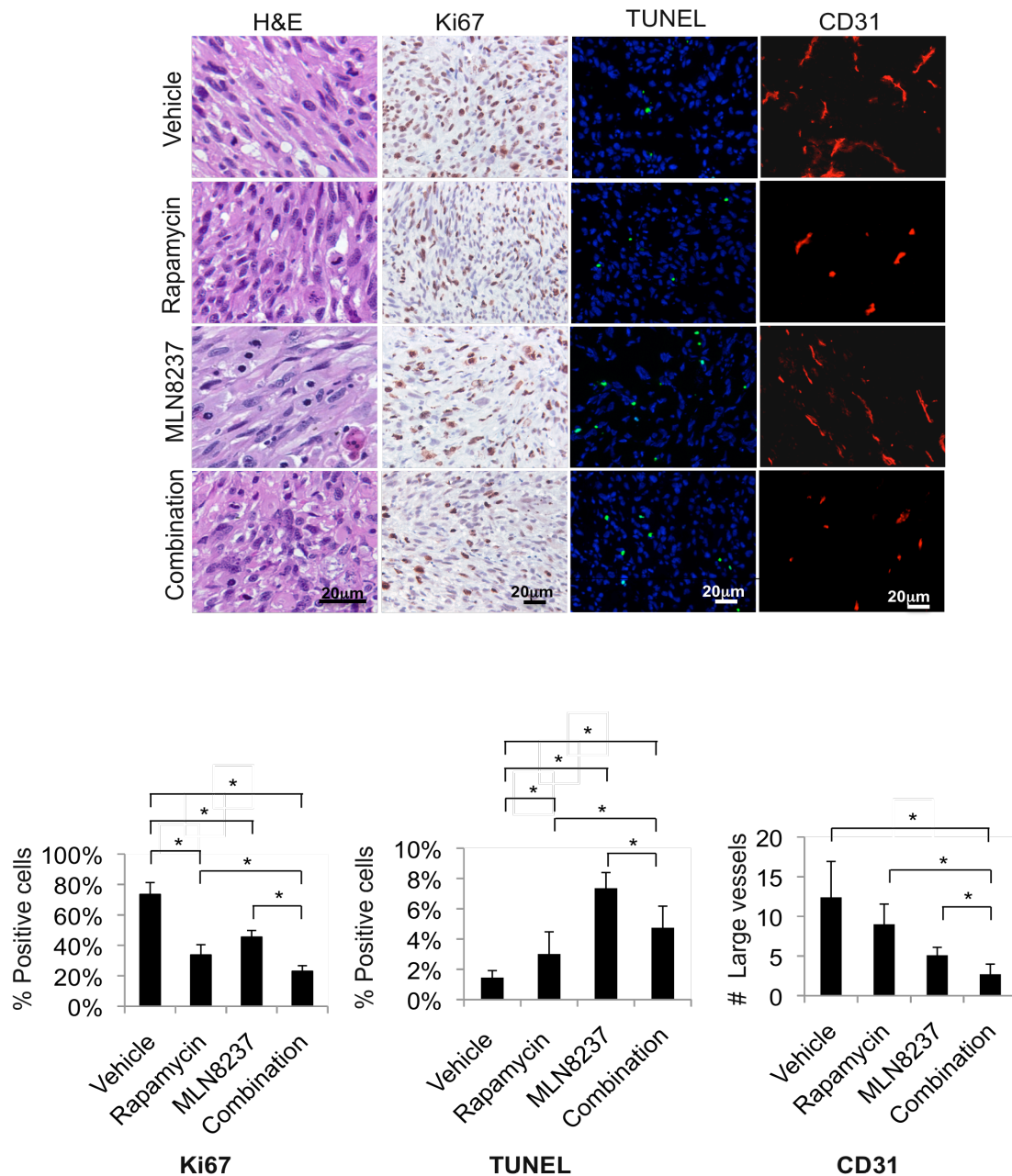


Figure 33. Dual mTOR and Aurk A inhibition in ULMS *in vivo* yielded markedly improved anti-ULMS effects. A) IHC analyses demonstrated decreased Ki67 positive staining cells in all treatment groups, the most pronounced was in combination treatment tumors. An increase in TUNEL positive cells was noted in all treated tumors. Moreover, combination treated tumors exhibited the greatest decrease in CD31 positivity. [All graphs represent an average of at least two repeated experiments; * denotes statistically significant effects ($p < 0.05$)].²⁴⁹

were more solid with a rubbery texture upon resection whereas tumors treated with rapamycin or vehicle had a very soft, mushy texture. IHC analysis demonstrated significantly decreased Ki67 positive staining cells in all treatment groups compared to control ($p < 0.002$), most pronounced were combination treated tumors. An increase in TUNEL positive cells was noted in all treated tumors. Moreover, combination treated tumors exhibited the greatest decrease in CD31 positivity (**Figure 33**). Based on these results, we hypothesized that MLN8237 may be exerting an anti-angiogenic effect on our tumors. To examine if MLN8237 induced apoptosis of blood vessel cells, co-immunofluorescence studies were conducted on resected tumors staining for TUNEL and CD-31. No co-localization was observed (data not shown). Taken together, these data suggest that mTOR blockade in combination with Aurora A kinase inhibition results in significant anti-ULMS effects *in vitro* and *in vivo*, a finding of potential clinical utility.

Chapter 10: Discussion

Clinical and translational relevance

ULMS, though relatively uncommon, is a devastating disease accounting for more than 25% of uterine cancer-related deaths. More than 50-80% of ULMS patients will face recurrent disease and only 8-28% of women with advanced disease will survive five years. Caregivers, patients, and their families are confronted with a comparative lack of robust prognostic markers or efficacious therapeutic strategies. Significant improvement in ULMS treatment mandates enhanced knowledge of the molecular forces driving ULMS, an objective hampered by the global lack of vital bioresources such as clinical databases, human tissue repositories, cell lines, and animal models. This body of work seeks to bridge this large research gap by utilizing the remarkable bioresources available at The University of Texas MD Anderson Cancer Center.

Our work represents one of the largest cohort (>100 patients) and arguably, the most comprehensive molecular expression studies in ULMS to date. Many markers that we examined were differentially express in ULMS compared to controls and could be used as potential biomarkers and most importantly, therapeutic targets. We observed pronounced loss of both smooth muscle and gynecological differentiation markers as well as deregulation of many molecules important in cancer such as Bcl-2, VEGF, and several tumor suppressors/oncoproteins. Further, our observations on expression profiles of several less recognized molecules such as survivin, AXL, and nuclear and membranous β -catenin, suggest potentially novel roles for proteins in ULMS and cancer. Unfortunately, few prognostic markers were identified.

In addition to the clinical and translational relevance of this project, there are also basic and pre-clinical science applications with the potential to facilitate increased clinical/translationally relevant work in the future. As mentioned, ULMS research is significantly hampered by the lack of clinical databases, human tissue studies, cell lines, and animal models essential to contemporary molecular oncology research. Ongoing efforts to consent patients for the development of vital ULMS cell lines will help to generate critical bioresources for continued ULMS research. Securing such requisite ULMS investigative tools is a significant part of this proposal; achieving this goal will have positive impacts extending considerably beyond the laboratory.

Towards identifying deregulated molecules for use in developing novel therapies for ULMS, we detected significant upregulation in both mTOR and Aurk A kinase. Using rapamycin (mTOR inhibitor) and MLN8237 (Aurk A inhibitor), we targeted both molecules in a dual blockade therapeutic approach. Our studies demonstrate that dual inhibition of mTOR and Aurk A kinase via rapamycin and MLN8237 yields remarkable synergistic anti-ULMS effects *in vitro* and in murine studies. In our work we provide pre-clinical evidence to support clinical investigation into dual inhibition of mTOR and Aurk A. We believe that combining these therapies in the clinic could prove to be of great benefit in the treatment of ULMS. Furthermore, the results of these studies can easily and directly translate into clinical investigations with the hope of positively impacting our ability to preserve the lives of patients burdened by this disease.

In summary, enhanced knowledge of the clinical presentation, patient and tumor variables, therapeutic response, and outcome of a large cohorts of patients, coupled with the establishment of a large, clinically annotated microarray enabled the assessment of molecular marker expression, will help scientists and physicians secure new insights into drug design and targeting as well as clinical care. Furthermore, the development of *in vitro* and *in vivo* ULMS models markedly enhances the ability to investigate the efficacy of novel therapies. More importantly, this research may provide physicians with new clinically relevant insights that can translate directly to improved patient care via evidence-based treatment regimens. By identifying and validating molecularly based, translationally relevant therapies and providing a rationale to translate these into clinical trials, this study hopes to facilitate progress in ULMS treatment and outcomes.

Tissue microarray

A significant limitation to therapeutic advancement in ULMS is the lack of comprehensive research. Few published studies report data derived from cohorts of >100 patients; most are small reports of 5-30 patient cohorts and virtually all of these examine five or fewer molecular markers. To our knowledge, the tissue microarray study represented here comprises one of the largest and most comprehensively examined ULMS series to date utilizing 109 patients and nearly 250 tumor samples. Notably, only 21 primary ULMS samples are included in this TMA compared with more than 100 metastatic cases. One explanation for this discrepancy is that MD Anderson Cancer Center is a tertiary cancer care facility and as such, is more likely to

treat advanced cancers. Additionally, many women with ULMS undergo surgery at primary care centers for conditions presumed benign, such as a symptomatic uterine leiomyoma (uterine fibroids).³⁰ These lesions are benign and can be identified in approximately two-thirds of women; as such, snap frozen tissues are not necessarily preserved or retrievable for study at a tertiary center.^{25,30} Regardless, the composition of tumors in this TMA slightly favors advanced stage ULMS; given that stage at diagnosis correlates directly with survival, our TMA can be a good tool to examine molecular deregulations contributing to tumorigenesis and identify molecular prognosticators, especially in aggressive and lethal late stage tumors.

Smooth muscle differentiation markers

Pathologic diagnosis of ULMS, especially abdominal/pelvic recurrences and distant metastases, is sometimes confounded by variability in tumor cell morphology and growth patterns. Loss of smooth muscle and gynecological-specific markers in advanced or dedifferentiated tumors can also be deceiving, rendering definitive diagnosis difficult. Potential loss of differentiation markers in ULMS could contribute significantly to difficulty with diagnosis, and awareness of the frequency of loss of differentiation markers in ULMS could aid pathologists in the diagnosis of more complicated ULMS cases. We examined the ULMS samples in our TMA for evidence of smooth muscle marker loss and found loss of at least one smooth muscle marker in more than one-third of our ULMS; only 2% of ULMS tissues lost all of the four smooth muscle markers examined. Loss of each of the smooth muscle markers was significant in tumor versus controls and correlated to decrease recurrence-free survival. In general, tumors with aggressive histologies were more likely to lose

muscle markers, stressing the importance of additional diagnostic tools for accurate diagnosis.

Gynecological differentiation markers

In addition to loss of smooth muscle differentiation markers, ULMS exhibited marked loss of gynecological markers WT-1, estrogen receptor, and progesterone receptor. ULMS arise from the uterine smooth muscle layer and are thus a gynecological malignancy. Coosemans et al. reported that many uterine sarcomas express WT-1 and found in a 38 tissue series that 76% of ULMS had WT-1 expression. Our results in a much larger cohort of ULMS suggest that WT-1 is not as frequently expressed and is only found in about 55% of ULMS. Uterine tissues highly express many hormone receptors, underscoring the possibility that ULMS could be hormonally regulated and therefore, targeted via hormone-based therapies.^{334,335} Indeed, several groups have studied hormone receptor expression in ULMS and leiomyoma. Leitao et al. reported marked decrease in hormone receptors in ULMS compared to leiomyoma; estrogen receptor expression was reduced from 78% in leiomyoma to only 40% in leiomyosarcoma. Similarly, progesterone receptor levels were reduced from 88% in leiomyoma to 38% in leiomyosarcoma; both findings were statistically significant. Progesterone receptor expression also predicted lower risk of recurrence in ULMS.³³⁵ Several studies, including ours, confirm these results in LMS and ULMS.^{60,334,336,337} In our studies, estrogen receptor was significantly decreased from 100% in healthy myometrium to 75% in leiomyoma. A further decrease was seen in ULMS, where estrogen receptor was reduced more than two-fold from 100% myometrium to only 45% in ULMS. Similarly, expression of progesterone receptor

decreased by about 30% in leiomyoma and by about 70% in ULMS versus myometrium.

The loss of hormone receptors such as PR and ER may have significant biological effects and likely contributes to tumorigenesis. For example, a breast cancer study found that loss of PR correlated to overexpression of HER2, a kinase receptor that activates cell growth signaling and can function in tumorigenesis.³³⁸ Similarly, loss of ER can have profound biological effects, such as epigenetic silencing, on downstream targets, including PR, cathepsin D, cyclin D1, and c-myc and in breast cancer, loss of ER is associated with malignant progression.³³⁹⁻³⁴¹ Notably, a study in breast cancer concluded that tumors originally ER negative will recur as ER negative tumors, but that tumors originally ER positive can lose ER expression; loss of ER significantly predicted poor response to further endocrine-based therapy, making the tumors harder to treat and therefore, more deadly.³⁴²

In general, few correlations have been identified between estrogen/progesterone receptor expression and survival in ULMS. In one study, neither receptor was associated with risk of recurrence³³⁴ and in another, the expression of progesterone, but not estrogen, suggested a risk of recurrence.³³⁵ A third study suggested that elevated estrogen and progesterone receptor expressions positively impacted survival.³⁴³ Our findings indicate that estrogen receptor, but not progesterone correlated with longer recurrence-free survival, but that neither receptor was predictive of better disease-specific survival. The implications of these findings may have implications in the management of ULMS patients.

Most women with ULMS undergo dual hysterectomy/oophorectomy and may therefore be candidates for hormone-based therapy (e.g. estrogen replacement). Several studies have shown that hormone replacement therapy, especially estrogen-based therapies, can fuel tumor development and re-growth in breast cancer.³⁴⁴ Our examination of estrogen and progesterone receptor expression in ULMS concludes that expression of either receptor is markedly reduced in ULMS compared with leiomyoma or myometrium, suggesting that hormone therapy may not be a viable option in ULMS. These results are in agreement with several independent studies.^{334,335} Even if the clinical benefit of hormone replacement therapy were substantial in ULMS patients, the risk: benefit ratio must be carefully considered. Taken together, these findings suggest that ULMS is not likely driven appreciably driven by estrogen or progesterone. Interestingly, drugs targeting hormone receptors (e.g. tamoxifen and medroxyprogesterone acetate) slightly increase the likelihood of developing uterine sarcoma and ULMS.^{344,345} The use of hormone replacement therapy following hysterectomy/oophorectomy in women with ULMS remains controversial; until further evidence provides definitive conclusions, it is prudent to err on the side of caution when using hormone replacement therapies.

Cancer-related biomarkers

In order to understand the traditional cancer biology governing tumorigenesis in ULMS, expression of several proteins identified as deregulated in cancer were examined. Therapeutic exploitation of molecular factors underlying tumorigenesis (e.g., invasion-facilitating matrix metalloproteinases in highly metastatic or VEGF in

highly vascular tumors) has had mixed therapeutic results.³⁴⁶⁻³⁴⁹ A better understanding of the basic cancer biology in ULMS will further development of therapeutic interventions.

Proliferation

Ki67 is a nuclear protein found throughout the cell cycle except in G0 that likely functions in ribosomal RNA transcription and is a marker of cellular proliferation. The precise function of Ki67 in the cell cycle remains unclear.³⁵⁰ Several studies in a variety of cancers have shown high Ki67 expression correlated with aggressive tumor phenotypes and less favorable outcomes.³⁵¹⁻³⁵³ In rapidly proliferating ULMS cells compared to slower growing, regulated normal tissues, and logic dictates that Ki67 would be highly expressed. Several studies support this logic, including our own.^{337,354-356}

Cyclin D1, a cyclin family protein that regulates cell cycle transition from G1 to S phases, has previously been examined as a marker of proliferation.^{339,357,358} Cyclin D1 serves as a co-factor for many transcriptional factors; it binds CDK4/6 to activate the complex that phosphorylates the retinoblastoma (Rb) tumor suppressor protein. Rb hyperphosphorylation triggers the release of Rb-sequestered E2F and leads to transcription of genes required for transition into S phase; cyclin D1 also functions as a transcriptional cofactor.³⁵⁹ Overexpression of cyclin D1 has been identified in several human tumors and Sherr et al. hypothesize that cyclin D1 overexpression promotes cell proliferation and differentiation via by facilitating a quicker G1/S transition.³⁶⁰ Our studies and the studies of others agree that although cyclin D1 can

serve as a marker of proliferation, it is not a strong prognostic marker in many human cancers, including ULMS.³⁶⁰⁻³⁶²

Survival

Tumor cells often have dysregulations that promote cell survival and allow bypass of biological cell-death signals when signaling, cell division, or DNA integrity surveillance mechanisms go awry. Apoptosis, a programmed cell death mechanism, is triggered by a number of intrinsic and extrinsic initiators such as TNF- α or mitochondrial dysregulation-mediated cytochrome *c* release.³⁶³ In healthy cells, a tightly regulated balance between pro-apoptotic and anti-apoptotic proteins directs cell fate; however, overexpression of anti-apoptotic molecules in many types of cancers shifts the equilibrium to favor cell survival.³⁶³

Bcl-2 is a prominent member of an anti-apoptotic family of proteins that play critical homeostatic roles in apoptosis. Bcl-2 prevents cytochrome *c* release induced by pro-apoptotic proteins (i.e. Bad, Bax, and Bok) from the mitochondria, thereby inhibiting apoptosis and counteracting pro-death signaling.³⁶⁴ To thwart pro-death signals and promote cell survival despite dysfunction, many tumors upregulate Bcl-2 expression. In fact, Bcl-2 is upregulated in several tumor types including cancers of the prostate, bladder, lung, and breast and also in leukemia.³⁶⁵⁻³⁶⁹ High expression of Bcl-2 is observed in some sarcomas (e.g. synovial sarcoma) but not in others (e.g. MPNST).³⁷⁰ Our study found that myometrium and leiomyoma controls had higher Bcl-2 high intensity and distribution expressions than ULMS; less than half of ULMS expressed the pro-survival molecule. These findings in a large (>200 sample) cohort

confirm Zhai et al. reports of Bcl-2 expression in only 43% ULMS and substantially higher expression in leiomyoma within a small cohort study.³³⁷ Although it is a pro-survival molecule and therefore might be expected to aid tumors, we found that high intensity and distribution Bcl-2 expression correlated with improved disease-specific survival. While this is in contrast to Brambilla et al.³⁷¹ and Kim et al.³⁷², our findings are supported by several independent groups who also found that Bcl-2 expression correlates with a more favorable outcome.^{337,365,373-378} The potential mechanisms to shed light on why Bcl-2 expression is associated with improved survival are unclear,³⁷⁷ however, Bcl-2 has several functions other than anti-apoptotic activity by binding with and neutralizing pro-apoptotic family members such as Bax. Bcl-2 has been found to play a protective role in oxidative stress, binds beclin-1 to block beclin-1 induced autophagy and cell death, and can modulate calcium levels.³⁷⁹⁻³⁸¹ In colorectal cancer, loss of Bcl-2 was linked to metastatic progression.³⁷⁹ It is possible that Bcl-2, through mechanisms yet to be elucidated, can also prevent tumors from metastatic progression, thereby improving survival.

Survivin is a pro-life molecule that plays a major role in the pro- versus anti-apoptotic molecular balance that regulates apoptosis. As a member of the IAP (inhibitor of apoptosis) family, survivin binds to effector caspases to prevent propagation of pro-death signals that would mechanistically commit the cell to apoptosis; it can be regulated by β -catenin activation and WNT signaling. Most importantly, survivin is differentially expressed in tumor, but not terminally differentiated, healthy cells, making it an attractive potential biomarker and therapeutic target. While role(s) for overexpressed survivin in malignancies that are unrelated to anti-apoptotic regulation

have not yet been elucidated, localization to mitotic spindle poles during G2/M suggests an additional function in mitotic regulation. Overexpression of survivin was previously documented in sarcoma,³⁸² but to our knowledge, has not yet been reported in ULMS. Our findings conclude survivin expression in both nuclear and cytoplasmic compartments is elevated in ULMS compared with controls; increase in nuclear, but not cytoplasmic, expression was significant. Intensity expression of both nuclear and cytoplasmic survivin were predictive of shorter recurrence free survival and nuclear distribution expression also correlated to shorter RFS. In non-small cell lung cancer, nuclear expression predicted longer recurrence-free and overall survival.³⁸³ It also correlated to poorer prognosis in glioblastoma;³⁸⁴ cytoplasmic expression did not correlate with survival in the same study.³⁸⁴ However, survivin was expressed significantly in uterine cervical squamous cell carcinoma compared with controls, but was not predictive of outcome.³⁸⁵ In breast cancer, the expression of surviving predicted early recurrence.³⁸⁶ Our study found neither cytoplasmic nor nuclear survivin expression to correlate with disease-specific survival. These findings indicate that nuclear survivin expression may be utilized as a biomarker and prognostic factor, and may be targeted for therapy to reduce the risk of recurrence in ULMS.

Invasion

Many factors are responsible for a tumor's ability to invade and ultimately, metastasize. Fidler et al. describes the steps necessary to facilitate tumor invasion and metastasis.³⁸⁷ One critical step of this process is tumor extravasation, which requires degradation of extracellular matrix adjacent to the tumor. Matrix

metalloproteinases are a part of a family of zinc-dependent endopeptidases that facilitate breakdown of the tumor matrix and surrounding stroma. Several matrix metalloproteinases are responsible for breakdown of extracellular matrix (including MMP2 and MMP9). In this study, no significant increase in MMP2 expression was identified and any observed MMP2 expression in ULMS was rare. These findings contrast with most published reports in ULMS which indicate that around 50% of ULMS express MMP2.¹⁰⁴ Inhibition of MMP9 in a rat sarcoma model inhibited tumor metastasis, indicating that MMP9 may be critical for tumor progression.³⁸⁸ In our ULMS TMA, we did not observe significantly increased MMP9 expression and therefore, cannot support the previous study. Since there are many proteins that can function similarly in the MMP family, it is also feasible that other MMP family members play more predominant roles to facilitate invasion and metastasis in ULMS. Nevertheless, the effects of MMP2 and MMP9 are likely minor in ULMS.

Angiogenesis

Angiogenesis is an essential part of tumor proliferation and progression. Blood vessels deliver molecular oxygen and critical nutrients via the bloodstream to rapidly dividing tumor cells and remove waste to keep the tumor microenvironment conducive for tumor growth. Vascular endothelial growth factor (VEGF) is a critical molecule responsible for endothelial cell migration, a critical step in vasculogenesis and angiogenesis.³⁸⁹ VEGF binds to and activates VEGF receptors, thereby initiating formation and remodeling of blood vessels within the tumor microenvironment.²⁶⁵ Our studies suggested that VEGF is overexpressed in human ULMS; significantly elevated levels were also identified in tumor progression groups. Together, these data

demonstrate that VEGF is important in ULMS and may represent a therapeutic target to halt disease progression. Elevated VEGF expression has been previously noted and inhibitors of VEGF and its receptor examined in many tumor models including Ewing's sarcoma, angiosarcoma, and leiomyosarcoma.^{134,390,391} While largely ineffective in most cancers, VEGF receptor inhibitors in leiomyosarcoma have shown therapeutic benefit and prolonged disease stabilization.¹³⁴ The role of VEGF, VEGFR, and endothelial cells in ULMS is unique because in this mesenchymal cell model, endothelial cells are critical and the two must work together to facilitate tumor growth.

Tumor suppressors and oncoproteins

Expression and function of several tumor suppressors and oncoproteins can directly contribute to tumorigenesis. The p53 tumor suppressor is perhaps one of the most widely studied and frequently mutated proteins in cancer.³⁹² The protein has many cellular functions including DNA damage repair, regulation of cell proliferation via p21, and transcription of many pro-survival molecules.³⁹³ It is regarded as the "Guardian of the Genome" for its widely diverse functions and effects. When stable, p53 forms a tetramer, translocates into the nucleus, and acts as a transcription factor to induce transcription of many pro-apoptotic genes such as Bax, Apaf-1, and PUMA as well as growth arrest-inducing p21 and Gadd45. It also transcribes MDM-2 as part of a self-regulating negative feedback mechanism.³⁹³ A loss of function mutation in p53 results in accumulation of the protein within in the nucleus. We report a statistically significant accumulation of p53 in more than 30% of ULMS, suggesting that p53 mutation in ULMS is common. Further, p53 mutation is more prevalent in advanced ULMS

compared with primary disease and may partially contribute to accelerated tumorigenesis and aggressiveness in advanced lesions.

As a critical molecule for cellular equilibrium and homeostasis, p53 is closely regulated by MDM-2, an oncoprotein that functions as an E3 ubiquitin ligase and ubiquitinates p53, resulting in degradation of the protein. High levels of MDM2 correlate with low levels of stable and transcriptionally functional p53, resulting in a shift in equilibrium favoring cell survival.³⁹³ Several studies in de-differentiated liposarcoma have established overexpressed MDM2 and in cases of retroperitoneal sarcomas, MDM2 overexpression may sometimes be utilized to help distinguish between ULMS and LPS.³³² We and others report significant MDM2 overexpression in ULMS.^{394,395} Interestingly, overexpression in our series is found predominantly in local lesions and is significantly lower in metastatic ULMS.

p16 is a cyclin-dependent kinase inhibitor and tumor suppressor that negatively regulates cell cycle progression. Functional p16 sequesters CDK4, preventing CDK4-mediated phosphorylation of Rb and subsequent pro-growth downstream effects. We and several others observed diffuse and strong p16 expression in ULMS compared with leiomyoma or myometrium.³⁹⁶⁻⁴⁰¹ While it is unclear why a tumor suppressor would be consistently overexpressed in ULMS compared to benign leiomyoma, Atkins et al. suggest that overexpression could be the result of gene mutation or loss of protein functionality.³⁹⁷ Although at least one small cohort study found that elevated and diffuse p16 expression correlated statistically with poor clinical course, we did not find any significant association between p16 expression and outcome. Notably,

several studies, including ours, agree that healthy myometrium minimally express p16 while some leiomyomas express p16 weakly and focally suggesting that gain of p16 expression may be an important molecular event in tumor progression.^{396,397,399} Taken together, these data suggest that p16 could be used as a molecular biomarker to aid in the discrimination of ULMS from leiomyoma in cases of complex or atypical cases.

The retinoblastoma protein (Rb) is a widely recognized tumor suppressor frequently mutated or lost in cancers. Phosphorylated Rb protein is a gatekeeper of the cell cycle and regulates G1 progression to S phase, halting the cycle in G1 if damaged DNA is present. When Rb is lost or has a nonfunctional mutation, tumor cells with frequently altered and damaged DNA is able to progress through G1 into S phase and continue cellular replication. Notably, women with hereditary Rb syndromes that are characterized by loss of Rb have a higher risk for developing ULMS.⁴⁰² We examined Rb expression in a large ULMS cohort and concluded that expression is significantly reduced in tumor versus controls and therefore, it can serve as a biomarker for ULMS. Taken together, we found deregulations in p53, MDM2, p16, and Rb are relatively common in ULMS and likely contribute to tumorigenesis. We found that all tumor oncoproteins examined were overexpressed compared with controls and the single tumor suppressor we examined, Rb, was significantly reduced compared with controls. Notably, the tumor suppressors and oncoproteins we examined interact with one another and in concert, can control the cell's fate via regulatory mechanisms; their expressions likely rely on regulation by each other.

Signaling: β -catenin

β -catenin is a central signaling molecule that initiates the WNT pathway and is critical in wound healing and cell proliferation.⁴⁰³ Further, dysregulation of WNT pathway through β -catenin has been shown to promote mTOR activation and propagate pro-life signals. Accumulation of β -catenin in the cytoplasm can be a result of mutation in the CTNNB1 gene (this gene encodes for β -catenin protein) and results in nuclear translocation and transcriptional activation of many pro-growth and pro-survival genes.⁴⁰⁴ Additionally, a cre-recombinase driven mouse model featuring constitutively activated β -catenin within the uterine mesenchyme resulted in the development of uterine sarcomas and activation of the mTOR pathway in mice.⁴⁰⁵ Given these implications and that cancer has been described as both a hyper-proliferative disorder and a “wound that over heals,”⁴⁰⁶ we examined β -catenin expression in human ULMS. Several investigators have found cytoplasmic β -catenin overexpression in more than half of LMS and ULMS.^{407,408} Our results identified significant β -catenin overexpression in 64% of ULMS and agree well with existing reports.⁴⁰⁸ Further, we found high cytoplasmic β -catenin to be statistically correlated to recurrence-free survival in ULMS.

Nuclear β -catenin has been observed in many cancers,^{409,410} however, several small studies have failed to observe nuclear expression in LMS/ULMS.^{411,412} Our data disagree with these previously published reports in ULMS and suggest that nuclear β -catenin is in fact overexpressed in a small number (~7%) of ULMS cases. Although the percentage of ULMS expressing nuclear β -catenin was small, the difference

between ULMS and control expression was significant. Recently, Kildal et al. reported that 23% of ULMS (n=231) expressed nuclear β -catenin and that high expression correlated with lower 5-year survival.⁴⁰⁸ Although expression is much more prevalent than in our study, we did find that nuclear β -catenin correlated with RFS and together, our findings agree that nuclear β -catenin is expressed in ULMS and could potentially serve as a molecular biomarker or therapeutic target.⁴⁰⁸ Notably, our study also found nuclear β -catenin correlated with tumor status; higher levels of nuclear expression were found in recurrent and metastatic lesion than in primary ULMS, suggesting a role for nuclear β -catenin in advanced ULMS.

Membranous β -catenin expression has been observed in many epithelial-derived cancers;^{413,414} however, studies in sarcoma have largely failed to demonstrate appreciable membranous β -catenin expression.^{411,412,415-417} In epithelial cells, membranous β -catenin functions in the cadherin-cadherin cellular adhesion process; loss of membranous β -catenin associated with greater tumor invasiveness and worse outcome.⁴¹⁸⁻⁴²² Kildal et al. were the first to demonstrate membranous β -catenin in uterine sarcoma and concluded that expression was limited only to ULMS (25% ULMS expressed membranous β -catenin); endometrial stromal sarcoma, carcinosarcoma, and healthy myometrium controls did not express membranous β -catenin.⁴⁰⁸ Further, they reported that elevated membranous β -catenin correlated to poor survival.⁴⁰⁸ Using our TMA, we found 20% of ULMS stained positively for membranous β -catenin compared with none of the controls. Our data agree well with findings from Kildal et al.; however, we did not observe a significant association of

membranous β -catenin to tumor status or outcome. Together, these data conclude that membranous β -catenin is differentially expressed in ULMS over other uterine sarcomas and suggest a potential role in ULMS development and progression. The underlying cause of membranous β -catenin and its functional significance remains unclear, however, a ULMS-specific role is likely.

mTOR associated proteins

The mTOR pathway is a major cell growth-signaling pathway in sarcoma. mTOR is center in the signaling cascades and contributes to cell survival, growth, migration, and cell cycle progression via regulation of mRNA translation.¹¹⁴ Enhanced mTOR signaling, serving as a convergence point for multiple upstream molecular deregulations, is commonly observed in many malignancies, making this axis an attractive anti-cancer therapeutic target.¹⁰⁹ Recent data suggest a role for this pathway in LMS.^{107,196,423,424} In 2007, Hernandez et al. proposed that elevated activation of the mTOR pathway was critical in the development of leiomyosarcoma.⁹⁸ Using GEM mouse models, researchers conditionally knocked out PTEN, causing rapid development of leiomyosarcoma.⁹⁸ Recently, Setsu et al. suggested that the AKT/mTOR pathway is highly activated in soft tissue leiomyosarcomas and upregulation of this pathway was significantly associated with shorter survival.¹⁰⁷ Studies here extend these observations to ULMS, a particularly devastating LMS subset, demonstrating increased ULMS mTOR activation.

To provide rationale for further studies, we queried a ULMS gene array that compared ULMS to normal myometrium and leiomyoma.²⁵⁰ We compared gene signatures in

ULMS to three known signatures associated with PI3K/AKT/mTOR pathway hyperactivation. The CMap signature was based on “The Connectivity Map” dataset and mapped expression profiles of several different cell lines treated with PI3K inhibitors to identify genes with reduced expression following treatment.³²⁷ The second gene set used for comparison was the Saal profile, which mapped gene expression in breast cancers with characterized PTEN loss (and therefore, highly activated mTOR pathways).³²⁸ The third and final gene expression model that we used was the Majumder gene set examining gene regulation in mice with overexpressed AKT1.³²⁶ Together, the three gene expression profiles were used to characterize a set of genes associated with PI3K/AKT/mTOR pathway activation. The PI3K/AKT/mTOR pathway was highly active in ULMS compared with myometrium. These results confirm that on the level of RNA expression, there is marked enrichment of PI3K/AKT/mTOR pathway gene activations, suggesting that the mTOR pathway is overexpressed and may be a viable therapeutic target. Towards that end, we have evaluated expression of several proteins in this pathway to identify possible biomarkers, prognosticators, and therapeutic targets.

The functions and significance of p4EBP1, pS6RP, and pAKT are reviewed in the introduction to this work. In our study, enhanced mTOR signaling occurred with disease progression; activation of mTOR downstream effector 4EBP1 correlated with poor prognosis in localized ULMS patients, making it a potential ULMS molecular prognosticator. While this is the first report to examine mTOR pathway levels in ULMS, findings agree well with elevated levels of activated mTOR pathway proteins and correlation to poor prognosis found in a recent LMS study.¹⁰⁷ Activated 4EBP1,

S6RP (used as a surrogate for S6K), and AKT statistically correlated with one another and in tumors versus controls, suggesting they are viable biomarkers in ULMS. However, only nuclear distribution expression of p4EBP1 correlated with outcome.

PTEN

Loss of PTEN was previously implicated in driving tumorigenesis through elevating mTOR pathway activity in a transgenic LMS mouse model.⁹⁸ Furthermore, the gene encoding PTEN is located on chromosome 10q23 in a region that is frequently lost in LMS and in other STS.^{184,425-429} Although PTEN loss can mTOR pathway activation, Rivera et al. show that loss may not be predictive of response to mTOR inhibitors temsirolimus or ridaforolimus²⁴⁰ and studies suggest that PTEN loss may not be associated significantly with clinical outcome in some cancers, including LMS.^{107,236} We found that loss of PTEN expression occurred in only 7% of the ULMS samples in our study. This is in accord with previous reports demonstrating a low PTEN mutation rate (~5%) in ULMS.⁴³⁰ Notably, two smooth muscle controls and one leiomyoma exhibited loss of PTEN. All control tissues on our TMA were acquired from patients upon tumor resection and PTEN loss in control tissues is likely contributed to the fact that they were taken from areas near a malignant lesion and therefore, may show some signs of tumorigenicity. In contrast to epithelioid sarcomas, where 88% of samples had identified PTEN reduction or loss and PTEN loss was a major contributing factor to AKT/mTOR pathway activation, our study and those of others have shown that PTEN loss is not frequent in ULMS and therefore, is not a prominent mechanism underlying mTOR activation.^{99,184,238,431,432} However, there may be a small

subset of patients with highly active mTOR signaling resulting, at least in part, from PTEN loss.

Receptor tyrosine kinases

mTOR activation can also be due to multiple upstream molecular derangements acquired throughout the tumorigenic process.¹⁵⁰ Among possible derangements, overexpression and activation of tyrosine kinase receptors (RTKs) can contribute to mTOR activation. As part of a complex signaling network, the mTOR pathway is activated by a number of receptor tyrosine kinases at the cell surface that internalize mitogenic signals. Overexpression or activation of RTKs has been identified in many sarcomas.^{332,433-435} While knowledge is limited, several studies suggest that RTKs such as PDGF- α and IGF-1R are over-expressed in ULMS.^{436,437} In this study, we sought to identify RTKs that were overexpressed and potentially contributing to mTOR activation by evaluating their expression levels in human ULMS.

The epidermal growth factor (EGFR, Her-1, Erb-B1) is a class I RTK that regulates mitosis and can be over-expressed in cancer.¹⁸⁴ Relatively few studies have examined EGFR expression in uterine sarcoma or uterine leiomyosarcoma, but our studies supports existing reports that EGFR is not differentially expressed in ULMS and leiomyoma; it is not likely a contributing force to mTOR activation.⁴³⁸ No significant genetic amplifications or alterations have been noted.⁴³⁹ Notably, overexpression of nuclear EGFR in breast cancer cells resulted in EGF accumulation in the nucleus; nuclear accumulation led to EGF-mediated induction of Aurk A expression via cofactor STAT5.⁴⁴⁰ While an interesting observation, we did not find

overexpression of EGFR in ULMS and therefore, it is not likely responsible for the marked upregulation of Aurk A that we observed.

Next, we evaluated the insulin growth factor receptor 1 (IGF-1R), an RTK that has previously been reported to be overexpressed in ULMS.⁴³⁶ The IGF-1R plays roles in enhancement of cellular proliferation, and differentiation.³⁸⁹ Cell growth inhibition resulted when IGF-1R was not present within the cell.⁴⁴¹ Enhanced expression of IGF-1R has been observed in many cell lines and human cancers including breast, colonic, cervical, and prostatic cancers.⁴⁴²⁻⁴⁴⁸ One group documented the overexpression of IGF-1R in ULMS⁴³⁶ and several publications indicate that it may be a viable therapeutic target for sarcomas.^{109,134,449} Our study concluded that IGF-1R is overexpressed significantly in ULMS versus leiomyoma and myometrium controls and suggests a potential role for IGF-1R as a therapeutic target; it likely contributes to mTOR activation in ULMS. Notably, IGF-1R expression did not correlate to outcome.

The PDGF family of RTKs represent a diverse family of receptors that hetero- or homodimerize to activate and transmit pro-growth signals intracellularly.⁴⁵⁰⁻⁴⁵³ The effects of PDGF on the uterus and leiomyomas was reviewed by Ciarmela et al.⁴⁵⁴ When fully activated, PDGFR- α and - β are known to trigger downstream activation of the PI3K/AKT/mTOR pathway. As such, we evaluated the expressions of PDGFR- α , PDGFR- β , and the PDGF- α and - β ligands that bind to and activate the receptors. Our studies concluded that PDGFR- α expression is significantly higher in smooth muscle controls than in ULMS; less than half of ULMS had moderate-high level expression. Further, PDGFR- β expression was found to be moderate- high in almost

one-quarter of ULMS compared with no controls. While existing literature on PDGF receptor and ligand expression in ULMS is sparse, our results agree well with existing reports.^{406,437,455} Notably, we found PDGFR- β distribution expression to correlate significantly to DSS. Adams et al., examined a small subset of human ULMS tissues for PDGF- α mutation; no mutations were detected.⁴³⁷ Given the effectiveness of imatinib mesylate, a dual c-KIT and PDGFR inhibitor, several groups have suggested its use in ULMS.^{220,456} Clinical trials with imatinib mesylate in ULMS have been largely disappointing and failed to yield significantly improved results.²²⁰ Examining expression of RTKs is crucial to understanding PI3K/AKT/mTOR activation in ULMS; however, evaluation of ligand expression may also provide useful insight. We investigated expressions of PDGF- α and PDGF- β ligands and found PDGF- β was significantly overexpressed compared with controls. Conversely, PDGF- α expression was significantly reduced in ULMS. PDGF- β intensity expression correlated to both recurrence-free and metastasis-free survival while PDGF- α expression predicted only shorter MFS. PDGF-mediated activation of PDGFRs may contribute to marked hyperactivation of the mTOR pathway in ULMS, however, it is unlikely the predominant or even a major contributor.

c-KIT (CD117) is a receptor tyrosine kinase activated by the stem cell factor (SCF) ligand; it propagates pro-survival and pro-growth signals via PI3K/AKT/mTOR signaling.⁴⁵⁷ Deregulation of c-KIT in at least one other STS subtype tumors is well established and has been molecularly targeted with c-KIT inhibitor imatinib with excellent therapeutic benefit. Some reports suggest that c-KIT is overexpressed in ULMS.^{458,459} However, we and several others did not observe c-KIT expression in

ULMS, suggesting that c-KIT would not be a viable therapeutic target.⁴⁶⁰⁻⁴⁶² This hypothesis is supported by *in vitro* work by Merimsky et al. where the use of imatinib for c-KIT inhibition in ULMS cells was examined; inhibition of c-KIT had no effect on cell growth.²²⁰

MET is a unique RTK activated by hepatocyte growth factor (HGF). The receptor can activate the PI3K/AKT/mTOR pathway in two ways: 1) MET can directly activate PI3K, and 2) MET can activate the Ras oncoprotein, stimulating activation of the AKT pathway through an intersecting cell growth regulation pathway. Deregulation of MET in sarcoma and in LMS, has been identified;^{332,463,464} amplification of MET gene copy number is also observed in LMS.⁸⁹ We found no statistical difference in MET expression between tumor and controls. Although Gao et al. showed that a MET inhibitor effectively inhibit the growth of SKLMS1 and SKLMS1 xenografts.⁴⁶⁵ MET is not differentially expressed in tumor versus normal tissue controls and therefore, MET inhibition likely does not represent a viable therapeutic option in human ULMS.

The final tyrosine kinase receptor that we examined is a TAMM family receptor named AXL.⁴⁶⁶ Activated by Gas6 ligand, AXL signals through the mTOR pathway via PI3K, is shown to be overexpressed in cancer, and is thought to contribute to tumorigenesis.⁴⁶⁷ However, relatively fewer studies have focused on AXL. Interestingly, AXL knock out mice are viable and do not have show significant phenotypic consequences, indicating that if AXL function is critical in tumorigenesis, its functions may be redundant with other receptor(s). Further, if any two of the three known family receptors (AXL, Tyro 3, and Mer) are knocked out, mice are still viable

and fertile. Triple knockdown results in viable but infertile males who produce no viable sperm.⁴⁶⁸ Our studies suggested that AXL expression was significantly elevated in ULMS compared with normal controls and further, that AXL expression rises significantly with tumor progression. To the best of our knowledge, this study is the first to date to evaluate AXL expression in ULMS or LMS. Interestingly, Yang et al. provided evidence of a c-KIT to AXL “switch” in GIST where resistant tumors show downregulated c-KIT accompanied by upregulation of AXL.⁴⁶⁹ A c-KIT to AXL switch would be an interesting explanation of observed ineffectiveness of c-KIT inhibitors in ULMS. Notably, our studies shown overexpression of AXL and very minimal expression of c-KIT in ULMS. Perhaps there is a correlation between low c-KIT and high AXL expression due to a “c-KIT to AXL switch.” If the switch is reversible, inhibition of AXL may lead to enhanced c-KIT expression, which could be potentially targeted with imatinib or other c-KIT inhibitors.

A marked deregulation in tyrosine kinase receptors has been shown in other sarcomas.^{332,434,435} We found deregulation in several RTKs in human ULMS that quite likely have functional significance and might explain AKT/mTOR pathway activation in ULMS. However, we did not examine the activated (phosphorylated) levels of each receptor, but rather the receptor expression as a whole. In several studies, it is apparent that overexpression does not necessarily lead to high activation and hyperactivation is not necessarily a result of enhanced expression.⁴⁷⁰ Regardless of the cause of mTOR upregulation in ULMS, mTOR downstream targets pS6RP and p4EBP1 are highly activated and overexpressed in ULMS, providing a rationale for

further studies *in vitro* and *in vivo* to examine the potential therapeutic benefits of targeting mTOR in ULMS.

mTOR pathway and inhibition via rapamycin in ULMS cell strains/lines

Few, if any, studies have examined mTOR pathway activation in ULMS *in vitro*. Elevated mTOR activity was found in one LMS cell line (SKLMS1) in two independent studies.^{268,471} Here, we demonstrated high AKT/mTOR pathway activity in a large panel of ULMS cell lines and strains. We also showed that mTOR activation contributes to ULMS cell growth and cell cycle progression; rapamycin arresting cells in G1 without inducing apoptosis.¹³⁵ These results agree with nearly all other published reports on the effects of rapamycin *in vitro* against cancer.^{209,472-475} While Huang et al. report that G1 arrest is dependent on p53 status, we and others observe G1 arrest and accompanying effects in ULMS cells independent of p53 mutational status.⁴⁷⁶ The latter finding is of clinical relevance given that p53 mutations occur in human ULMS⁹² and that p53 mutation can contribute to conventional chemotherapeutic resistance.⁴⁷⁷

Several reports show that rapamycin and its analogs have only modest effects in human STS, despite marked upregulation of the mTOR pathway in several STS, LMS, and ULMS.^{107,209,210,473,478-481} Our results from murine xenograft models agree well with human clinical studies and demonstrate that despite excellent *in vitro* sensitivity, rapamycin is not a sufficient monotherapy *in vivo* for the treatment of ULMS.

While encouraging, these data also demonstrate the limitation of single agent mTOR inhibitors as treatment for ULMS, resulting in cytostatic effects that might not be sufficient to improve patient outcomes, reminiscent of mTOR blockade-based trials in other human solid tumors and in LMS.^{238,482} Identifying additional targets for inhibition in combination with mTOR is thus urgently needed, a strategy currently being evaluated in conjunction with radiotherapy- conventional chemotherapy-, and molecular-based therapies in the context of several malignancies.⁴⁸³ Murphy et al. report that inhibition of mTOR sensitizes STS and blood vessels to radiotherapy.⁴⁸⁴ Since the mTOR pathway is upregulated in ULMS, low-dose rapamycin treatment may be a therapeutic benefit in combination with radiotherapy. Each modality alone has shown minimal effects on cell growth, but together, they are proposed to act synergistically and may be of patient benefit.⁴⁸⁴

Following promising results of a case study of gemcitabine and rapamycin in LMS, Merimsky et al. carried out several *in vitro* experiments to evaluate the efficacy of the combined therapy in LMS and ULMS.²²⁰ In this study, combination therapy did not show significant therapeutic benefit compared with gemcitabine alone.²²⁰ Imatinib in combination with rapamycin was also examined and found to have no effects on LMS or ULMS alone or in combination with rapamycin, presumably because we and others have found that its c-KIT target is not expressed in ULMS.²¹⁹ Towards evaluating mTOR blockade in combination with conventional chemotherapies, we utilized doxorubicin and rapamycin in combination therapy *in vitro* against ULMS. Our results did not yield synergistic anti-growth effects and were similar to previously noted outcomes achieved with combination gemcitabine and rapamycin,²²⁰ suggesting that

standard conventional first-line chemotherapies (gemcitabine or doxorubicin) do not yield superior results when combined with mTOR blockade. In this new era of personalized medicine, mTOR inhibition combined with molecularly based therapies should be further examined.

Recently we have identified that ULMS overexpress gene products regulating centrosome structure and function, highlighting a role for Aurk A as a novel ULMS therapeutic target. In our recent study a Gene Chip Array was used to gather genome-wide transcriptional profiles for more than 75 ULMS patients, comparing mRNA expression of nearly 50,000 genes to profiles acquired from leiomyoma and health myometrium.²⁵⁰ Several genes were identified as up- or down-regulated in ULMS; many of the genes up-regulated were centrosome-related proteins including Aurk A (7.9-fold up-regulation).²⁵⁰ Few studies have examined Aurk A expression or function in sarcoma^{317,485,486} and to the best of our knowledge, this and our previous study were the only to validate Aurk A overexpression in LMS or ULMS. Additionally, SiRNA knockdown induced marked apoptosis in our studies, indicating functional importance for Aurk A in cell survival.²⁵⁰ Next, we validated that Aurk A is upregulated and overexpressed in ULMS. *In vitro*, we found that ULMS cells are sensitive to MLN8237 at doses similar to those previously reported,³²⁴ observing significant treatment-induced growth inhibition, G2/M arrest, and a 2- to 4- fold increase in apoptosis compared with vehicle treated cells.³¹⁵ Interestingly, several potential non-kinase functions for Aurk A have recently emerged.²⁵⁵ MLN8237 is thought to inhibit only functions related to kinase activity and therefore, may not completely inhibit Aurk

A functions. Altogether, our studies, backed by literature, suggest that Aurk A kinase is a viable therapeutic target.

We hypothesized that since Aurk A inhibition via MLN8237 only killed about 25-50% of ULMS cells *in vitro*, a combined therapy approach with mTOR may yield superior effects. Additionally, dual therapy approaches often require lower doses of drug, resulting in lower clinical toxicity and side effects and enhanced quality of life for the patient while on therapeutic interventions. Aware that a critical next step in utilizing mTOR inhibition for ULMS treatment mandates developing effective drug combinations and taking into account our finding that Aurk-A might be a novel ULMS therapeutic target, we evaluated dual mTOR and Aurk-A blockade in our preclinical models. To the best of our knowledge this therapeutic combination has not been previously reported.

Timing of drug administration in combination therapies is an important consideration. Several reports conclude that drug administration timing significantly affects the outcome of the drug combination.¹⁰ For example, doxorubicin and TRAIL combination therapy yields superior results in STS, but only when doxorubicin is administered first, suggesting that it may sensitize cells to the secondary treatment.¹⁰ Notably, Wang et al. found that dual inhibition induced an elevated level of apoptosis compared to either compound alone or the sum of the two compounds.¹⁰ Merimsky et al. touch on the subject in 2004, stating:

“The use of such combinations requires smart planning and choice of drugs to be combined, their proper dosing as well as correct sequence and schedule of application. This is of crucial importance to enable synergism rather than antagonism between combined drugs.”²¹⁹

In the study Merimsky referred to, he combined rapamycin and gemcitabine therapies for the treatment of metastatic LMS. By combining a traditional chemotherapeutic agent, gemcitabine, and a cell signaling inhibitor, rapamycin, cancer cells were selectively targeted at 2 different phases (G1 phase – rapamycin, S phase – gemcitabine).²¹⁹

We evaluated three timing combinations in our synergy study: co-treatment, rapamycin pre-treatment, and MLN8237 pre-treatment. Our data demonstrate that MLN8237 synergizes with rapamycin to induce superior *in vitro*. Notably, synergism was found only when MLN8237 was administered prior to rapamycin treatment, highlighting the importance of scheduling sequences in development of therapeutic combination regimens. While it is unclear if pre-treatment with MLN8237 sensitized ULMS cells to rapamycin therapy, it was logical to first administer a cytotoxic agent to arrest cells in G2/M and kill as many tumor cells as possible. Interestingly, enhanced apoptosis was not observed in combination therapy groups compared to MLN8237 groups. The cells able to evade apoptosis via MLN8237 were then trapped in G1 by rapamycin and experienced cytostatic growth inhibition effects. These results likely reflect the differential impact of these compounds on cell cycle progression, although

the exact mechanisms underlying the observed anti-tumor effects remain to be determined.

A pronounced synergistic effect is observed when ULMS are pre-treated with MLN8237 then co-treated with MLN8237 and rapamycin, suggesting a potential link between Aurk A and mTOR. Interestingly, Saeki et. al published a potential model for Aurk A-mediated malignant transformation.⁴⁸⁷ In this model, they predicted that Aurk A overexpression alone was not sufficient for tumorigenesis, but coupled with cellular effectors or mTOR pathway activation, could contribute greatly to full transformation. This predicted model is the presents a potential link between mTOR and Aurk A.⁴⁸⁷ Wang et. al show induction of mTOR activation in a transgenic mouse model featuring an *Aurk A* transgene leading to overexpression of Aurk A kinase.⁴⁸⁸ Although the group could not recapitulate these effects in cultured cells, they concluded that marked activation of the mTOR pathway was induced by Aurk A kinase overexpression.⁴⁸⁸ They concluded that the activation may be a long-term effect and therefore, not observed in shorter cell-based experiments.⁴⁸⁸ Aurk A kinase has demonstrated nuclear localization and functions within the nucleus during mitosis, however, cytoplasmic localization of Aurk A has also been observed irrespective of cell cycle in many cancers. The cytoplasmic function of Aurk A remains unclear, but as a Ser/Thr kinase, it is possible that Aurk A may phosphorylate AKT, TSC2, or other mTOR-associated proteins, thereby activating the pathway and promoting cell growth. In this hypothesis, targeting Aurk A first would not only result in apoptosis, but also in a reduction of mTOR activity. This decline in mTOR activity, coupled with suppression from combination MLN8237 and rapamycin treatment could explain the synergistic

effects that are observed with this unique dosing schedule. Studies examining the recognition and activation sites of Aurk A and AKT/mTor pathway components as well as binding and functional studies would be required to confirm this hypothesis.

In vivo, combination therapy produced stabilization of disease in 100% of mice in that treatment group (n=8) for the duration of the 3-week treatment. Additional studies are necessary to see if complete or partial response can be achieved with an extended therapeutic treatment window. Also, further studies are necessary to define the duration of disease stabilization resulting from dual inhibition of mTOR and Aurk A kinase in ULMS. We found that MLN8237 treatment resulted in polyploidy in many cells, which agree well with published reports using Aurk A inhibitors in cancer.³¹⁵ Also, we found a marked reduction in CD31 expression indicative of angiogenic inhibition with MLN8237 and in combination treatment. To evaluate whether MLN8237 induced apoptosis in blood vessels, a co-immunofluorescence study for CD31 and TUNEL in xenograft tissues from all treatment groups was performed (data not shown). We did not observe any co-localization, indicating that MLN8237 likely does not have direct anti-angiogenic properties. Further, we believe that the significant size reduction in MLN8237-treated and co-treated tumors may be the culprit for fewer blood vessels, as smaller tumors do not require extensive vasculature networks compared with larger tumors. Combination therapy *in vivo* resulted in synergistic, anti-ULMS effects compared to either agent alone (although MLN8237 was given at a dose lower than the maximal tolerated dose (MTD)).⁴⁸⁹

Our studies demonstrate that dual inhibition of mTOR and Aurk A kinase via rapamycin and MLN8237 yields remarkable synergistic anti-ULMS effects *in vitro* and in murine studies. Additionally, several recent clinical trials with rapalogs and case studies with Aurk A inhibitors suggest that neither compound is therapeutically sufficient in humans. However, both drugs are fairly well tolerated at clinically relevant doses. In our work we provide pre-clinical evidence to support clinical investigation into dual inhibition of mTOR and Aurk A. We believe that combining these therapies in the clinic could prove to be of great benefit in the treatment of ULMS.

In summary, ULMS is a dismal disease and few women will survive more than 5 years after diagnosis. The aim of this work was to provide a better understanding of the deregulations promoting tumorigenesis in ULMS so that more efficacious therapies can be designed and tested clinically. Our findings suggest that deregulations leading to enhanced mTOR signaling and Aurk A overexpression play important roles in ULMS tumorigenicity. Dual blockade of mTOR and Aurk A in ULMS worked synergistically to abrogate cell proliferation *in vivo* and *in vitro*. Our results provide supporting justification for clinical trials to evaluate combined mTOR and Aurk-A inhibitors to enhance the anti-ULMS effects observed with either inhibitor alone.

Chapter 11: Future Directions

While we examined several possible causes of deregulation in the mTOR pathway, it is a complex system and several other proteins may contribute to mTOR pathway activation. More than 20 families of receptor tyrosine kinases have been discovered. In our study, we examined seven major tyrosine kinase receptors that have previously been indicated in cancer. However, we did not investigate all receptors and it is possible that deregulation of these receptors may play into the significant hyperactivation of the mTOR pathway. For example, kinase receptor ROR2 was recently found to be overexpressed in LMS and functioned to enhance tumor invasiveness; knockdown of ROR2 in LMS resulted in tumor mass decline. A number of additional molecules could be responsible for driving tumorigenesis in ULMS including RTKs such as ROR2, signaling kinases such as MAPK, tumor suppressors such as BRCA1, and tumor oncoproteins such as Ras. A further evaluation of such proteins may yield additional biomarkers, prognosticators, and therapeutic targets.

Mutations in or silencing of upstream effectors may also contribute to mTOR activation. For example, activating mutations in AKT, PI3K, and Ras have been identified in cancer.^{107,490} Recently, Setsu et al. examined PI3KCA and AKT1 gene mutations in LMS to determine if mutation, at least in part, could contribute to high activity of mTOR pathway activity they observed. No mutations were observed at hotspots of either gene. This study suggests that gene mutations in PI3KCA and AKT1 are not common in LMS and are not responsible for increased activity of the mTOR pathway.¹⁰⁷ Several other mutations could contribute to mTOR activity

including those in PTEN, TSC1/2, Rheb, mTOR, or RTKs and should be investigated further.

MicroRNAs (miRNAs) have recently emerged as regulatory mechanisms for several critical cancer-related proteins.⁴⁹¹ Very few studies have been conducted to identify functional and critical miRNAs in uterine leiomyosarcoma. In a recent study by Nuovo et al., MiR-221 was identified in 87% of ULMS (13/18), compared with no leiomyomas and was proposed as a means to differentiate between the two lesions.⁴⁹² Although few miRNA studies have been conducted in ULMS, additional studies may be warranted to identify a novel level of regulation in the disease. Further, miRNAs from the tumor microenvironment may also play roles in tumorigenesis and should be examined in ULMS.⁴⁹³⁻⁴⁹⁵

Our studies examined smooth muscle and gynecological differentiation markers. In addition to those reported in this dissertation, several additional diagnostic markers such as CD10, S-100 protein, and vimentin were evaluated. Tumors were also stratified into subtypes and evaluated for correlation to outcomes. An extra-uterine LMS tissue microarray was constructed and is currently being stained and scored for several markers for comparison to ULMS expression profiles. These data comprise a histology/diagnostic-based manuscript that is currently being drafted.

In our studies, we examined several cancer-related biomarkers and biomarkers relating to the mTOR pathway. These studies are presented and discussed in the body of this work. However, we also examined several other potential biomarkers with

evidence of importance in sarcoma and ULMS. For example, histone deacetylases (HDACs) are a class of proteins that deacetylate lysine residues on histones, thereby unraveling DNA and making it more accessible to transcriptional proteins. They are also thought to have non-histone targets and have distinct cellular locations, some being exclusively nuclear, some exclusively cytoplasmic, some membrane-associated, and a few HDACs are seen in both the nucleus and the cytoplasm. Many HDAC functions not related to their deacetylase activity have not yet been elucidated, but HDAC 8 was found to be overexpressed in leiomyosarcoma compared with normal controls. We have several ongoing studies in our lab involving HDAC inhibition in cancer and preliminary data shows that HDACs may be important in ULMS. We found that several HDACs were overexpressed significantly in ULMS compared to smooth muscle controls including HDAC2 (cytoplasmic and nuclear intensities), HDAC 3 (cytoplasmic and nuclear intensity), HDAC 6 (cytoplasmic intensity), HDAC 8 (cytoplasmic and nuclear intensities), and HDAC 10 (cytoplasmic intensity). Additionally, expression of several HDACs were found to correlate to survival. We are currently investigating the role of HDACs in sarcoma and will be further pursuing these findings.

Finally, we have completed construction and analysis on our large ULMS database, which includes more than three hundred identified ULMS patients from UTMDACC, regardless of tissue availability (this database includes patients on the ULMS TMA). In these patients, we have examined our database for several clinicopathologic parameters (i.e. stage, size, mitotic count, surgical margins, age, race, etc.) and correlated them to recurrence-free, metastasis-free, and disease specific survival.

Together with the cancer-related biomarkers reported in this work, we are finalizing a comprehensive manuscript for submission to report these data. This work will represent one of the largest cohort clinical database reports coupled with arguably the most comprehensive molecular study of ULMS to date.

Appendix

Appendix Table A1. Antibodies used for immunohistochemistry.

<i>Name of Marker</i>	<i>Company</i>	<i>City, State</i>	<i>Catalog #</i>	<i>Antibody Origin</i>	<i>Clone</i>
<i>Smooth muscle/gynecological differentiation markers</i>					
SMA	Sigma	St. Louis, MO	A2547	Mouse monoclonal	1A4
SMM	Dako	Carpinteria, CA	IR066	Mouse monoclonal	SMMS-1
Desmin	Sigma	St. Louis, MO	M0760	Mouse monoclonal	D33
Caldesmon	Dako	Carpinteria, CA	M3557	Mouse monoclonal	h-CD
WT-1	Dako	Carpinteria, CA	M3561	Mouse monoclonal	6F-H2
Estrogen Receptor	Novocastra	Buffalo Grove, IL	NCL-ER-6F11	Mouse monoclonal	6F11
Progesterone Receptor	Dako	Carpinteria, CA	M3568	Mouse monoclonal	PgR 1294
<i>Cancer-related markers</i>					
Ki-67	Dako	Carpinteria, CA	M7240	Mouse monoclonal	MIB-1
Cyclin D1	Labvision	Freemont, CA	RM-9104-S	Rabbit monoclonal	SP4
Bcl-2	Biogenex	San Ramon, CA	AM287	Mouse monoclonal	100
Survivin	Abcam	Cambridge, MA	Ab469	Rabbit polyclonal	-
MMP2	Chemicon	Billerica, MA	AB807	Rabbit polyclonal	-
MMP9	Chemicon	Billerica, MA	AB13458	Rabbit polyclonal	-
MDM2	Calbiochem	Rockland, MA	OP46	Mouse monoclonal	1F2
VEGF	Santa Cruz Biotechnology	Santa Cruz, CA	sc-152	Rabbit polyclonal	A20 / SC-152
p53	Dako	Carpinteria, CA	M7001	Mouse monoclonal	D0-7
p16	MTM Laboratories	Heidelberg, Germany	9517	Mouse monoclonal	E6H4
Rb	BD Biosciences	San Jose, CA	554136	Mouse monoclonal	G3-245
β -catenin	BD Biosciences	San Jose, CA	610154	Mouse monoclonal	14
<i>mTor pathway components</i>					
pS6RP (Ser235/236)	Cell Signaling	Danvers, MA	2211	Rabbit polyclonal	-
p4EBP1 (Thr70)	Cell Signaling	Danvers, MA	9455	Rabbit polyclonal	-
pAKT (Ser473)	Cell Signaling	Danvers, MA	9271	Rabbit polyclonal	-
PTEN	Dako	Carpinteria, CA	M3627	Mouse monoclonal	6H2.1
<i>Tyrosine kinase receptors/ligands</i>					
EGFR	Zymed	Carlsbad, CA	08-4205	Mouse monoclonal	31G7
IGF-1R	Ventana	Tucson, AZ	790-4346	Rabbit monoclonal	G11
PDGFR- α	Santa Cruz Biotechnology	Santa Cruz, CA	sc-338	Rabbit polyclonal	C-20
PDGFR- β	Santa Cruz Biotechnology	Santa Cruz, CA	sc-339	Rabbit polyclonal	P-20
PDGF- α	Santa Cruz Biotechnology	Santa Cruz, CA	sc-128	Rabbit polyclonal	N-30
PDGF- β	Santa Cruz Biotechnology	Santa Cruz, CA	sc-7878	Rabbit polyclonal	H-55
C-kit (CD117)	Dako	Carpinteria, CA	A4502	Rabbit polyclonal	CD117
MET	Santa Cruz Biotechnology	Santa Cruz, CA	SC-10	Rabbit polyclonal	C12
AXL	Cell Signaling	Danvers, MA	4566	Rabbit monoclonal	C44G1

Appendix Table A2. Protocols for immunohistochemistry experiments.

Name of Marker	Dilution	Blocking Agent(s)	HIER method	HIER buffer
<i>Smooth muscle/gynecological differentiation markers</i>				
SMA	1:80,000	Antibody diluent buffer (Ventana)	no antigen retrieval	no antigen retrieval
SMM	1:200	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 30min	Citrate buffer, pH6.0
Desmin	1:200	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 20min	Citrate buffer, pH6.0
Caldesmon	1:50	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 30min	Citrate buffer, pH6.0
WT-1	1:40	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 15min	Tris-EDTA buffer, pH8.0
Estrogen Receptor	1:35	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 30min	Citrate buffer, pH6.0
Progesterone Receptor	1:200	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 20min	Citrate buffer, pH6.0
<i>Cancer-related markers</i>				
Ki-67	1:100	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 25min	Citrate buffer, pH6.0
Cyclin D1	1:20	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 35min	TRIS-HCl, pH9.0
Bcl-2	1:20	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 25min	Citrate buffer, pH6.0
Survivin	1:500	5% horse serum & 1% goat serum	Steam Cooker for 40min	Citrate buffer, pH6.0
MMP2	1:500	5% horse serum & 1% goat serum	no antigen retrieval	No antigen
MMP9	1:1000	5% horse serum & 1% goat serum	no antigen retrieval	No antigen retrieval
VEGF	1:50	Antibody diluent buffer (Ventana)	Dako Auto Stainer, 45min	Citrate buffer, pH6.0
MDM2	1:20	Antibody diluent buffer (Diagnostic Biosystems)	Pressure cooker, 45 min	Citrate buffer, pH6.0
p53	1:100	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 5 min	Citrate buffer, pH6.0
p16	1:2	Antibody diluent buffer (Diagnostic Biosystems)	Pressure cooker, 45 min	Citrate buffer, pH6.0
Rb	1:100	Antibody diluent buffer (Diagnostic Biosystems)	Pressure cooker, 45 min	Citrate buffer, pH6.0
β-catenin	1:400	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 20min	TRIS-HCl, pH9.0
<i>mTor pathway components</i>				
pS6RP (Ser235/236)	1:200	5% horse serum & 1% goat serum	Microwave, 10min, 98°C	10mM Sodium citrate, pH6.0
p4EBP1 (Thr70)	1:400	5% horse serum & 1% goat serum	Microwave, 10min, 98°C	10mM Sodium citrate, pH6.0
pAKT (Ser473)	1:50	5% horse serum & 1% goat serum	Microwave, 10min, 98C	Borg decloaker, pH9.0
PTEN	1:100	Antibody diluent buffer (Ventana)	BOND MAX 20min, 100C	TRIS-HCl, pH9.0
<i>Tyrosine kinase receptors/ligands</i>				
EGFR	1:200	Antibody diluent buffer (Ventana)	no antigen retrieval	pre-tx P24 enzyme, 1 min
IGF-1R	1:2 pre-diluted	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 5min	Citrate buffer, pH6.0
PDGFR-α	1:50	Antibody diluent buffer (Ventana)	BOND MAX, 45min	Citrate buffer, pH6.0
PDGFR-β	1:50	Antibody diluent buffer (Ventana)	BOND MAX, 45min	Citrate buffer, pH6.0
PDGF-α	1:50	Antibody diluent buffer (Ventana)	BOND MAX, 45 min	Citrate buffer, pH6.0
PDGF-β	1:10	Antibody diluent buffer (Ventana)	Steam Cooker for 45 min	Tris/EDTA, pH8.0
C-kit (CD117)	1:100	Antibody diluent buffer (Ventana)	BOND MAX, 100°C, 15min	Citrate buffer, pH6.0
MET	1:100	5% horse serum & 1% goat serum	Steam Cooker for 45 min	DIVA decloaker, pH6.0
AXL	1:100	5% horse serum & 1% goat serum	Steam Cooker for 40min	Borg decloaker, pH8.0

Appendix Table A3. Antibodies used for western blotting (WB).

Protein	Antibody Origin	Clone	Dilution	Company	City, State	Catalog #	Blocking buffer	Incubation
pS6K (Thr389)	Mouse monoclonal	1A5	1:1000	Cell Signaling	Danvers, MA	9206	5% non-fat milk	overnight, 4°C
S6K	Rabbit monoclonal	49D7	1:1000	Cell Signaling	Danvers, MA	2708	5% non-fat milk	overnight, 4°C
p4EBP1 (Thr70)	Rabbit polyclonal	-	1:1000	Cell Signaling	Danvers, MA	9455	5% BSA	overnight, 4°C
4EBP1	Rabbit polyclonal	-	1:1000	Cell Signaling	Danvers, MA	9452	5% BSA	overnight, 4°C
pAKT (Ser473)	Rabbit monoclonal	193H12	1:1000	Cell Signaling	Danvers, MA	4058	5% BSA	overnight, 4°C
AKT	Rabbit polyclonal	-	1:1000	Cell Signaling	Danvers, MA	9272	5% BSA	overnight, 4°C
PTEN	Rabbit monoclonal	138G6	1:1000	Cell Signaling	Danvers, MA	9559	5% BSA	overnight, 4°C
Cyclin D1	Mouse monoclonal	DSC6	1:1000	Cell Signaling	Danvers, MA	2926	5% non-fat milk	overnight, 4°C
p21	Rabbit polyclonal	C-19	1:1000	Santa Cruz Biotechnology	Santa Cruz, CA	Sc-397	5% BSA	overnight, 4°C
p53	Mouse monoclonal	DO-1	1:1000	Santa Cruz Biotechnology	Santa Cruz, CA	sc-126	5% non-fat milk	overnight, 4°C
Aurora A	Rabbit polyclonal	-	1:1000	Cell Signaling	Danvers, MA	3902	5% non-fat milk	overnight, 4°C
Cleaved PARP	Rabbit polyclonal	-	1:1000	Abcam	Cambridge, MA	ab2322	5% BSA	overnight, 4°C
β-actin (HRP conjugate)	Mouse monoclonal	C4	1:3000	Santa Cruz Biotechnology	Santa Cruz, CA	sc-47778	Same as 1° Ab	45min, RT

Appendix Table A4. Differentiation marker expression in controls and ULMS.

A. Differentiation markers in controls												
Marker	GI Smooth Muscle				Myometrium				Leiomyoma			
	Total n=	negative	focal	diffuse	Total n=	negative	focal	diffuse	Total n=	negative	focal	diffuse
Smooth muscle differentiation markers												
SMA	10	0 (0%)	0 (0%)	10 (100%)	15	0 (0%)	0 (0%)	15 (100%)	7	0 (0%)	0 (0%)	7 (100%)
SMM	10	0 (0%)	0 (0%)	10 (100%)	15	0 (0%)	0 (0%)	15 (100%)	8	0 (0%)	1 (12%)	7 (88%)
Desmin	10	0 (0%)	0 (0%)	10 (100%)	15	0 (0%)	0 (0%)	15 (100%)	7	0 (0%)	0 (0%)	7 (100%)
Caldesmon	10	0 (0%)	0 (0%)	10 (100%)	15	0 (0%)	0 (0%)	15 (100%)	8	0 (0%)	0 (0%)	8 (100%)
Gynecological differentiation markers												
Nuclear WT-1	8	8 (100%)	0 (0%)	0 (0%)	14	1 (7%)	0 (0%)	13 (93%)	10	0 (0%)	0 (0%)	10 (100%)
Marker	Total n=	absent	low	high	Total n=	absent	low	high	Total n=	absent	low	high
ER	10	6 (60%)	0 (0%)	4 (40%)	15	0 (0%)	0 (0%)	15 (100%)	8	2 (25%)	0 (0%)	6 (75%)
PR	10	1 (10%)	3 (30%)	6 (60%)	14	0 (0%)	0 (0%)	14 (100%)	7	0 (0%)	2 (29%)	5 (71%)
B. Differentiation markers in ULMS												
Marker	Primary				Recurrent				Metastatic			
	Total n=	negative	focal	diffuse	Total n=	negative	focal	diffuse	Total n=	negative	focal	diffuse
Smooth muscle differentiation markers												
SMA	18	2 (11%)	0 (0%)	16 (89%)	66	0 (0%)	3 (5%)	63 (95%)	124	10 (8%)	11 (9%)	103 (83%)
SMM	18	1 (6%)	2 (11%)	15 (83%)	65	17 (26%)	4 (6%)	44 (68%)	121	25 (21%)	9 (7%)	87 (72%)
Desmin	18	0 (0%)	15 (89%)	2 (11%)	65	16 (25%)	5 (7%)	44 (68%)	123	12 (10%)	22 (18%)	89 (72%)
Caldesmon	18	2 (11%)	1 (6%)	15 (83%)	65	13 (20%)	9 (14%)	43 (66%)	122	23 (19%)	14 (11%)	85 (70%)
Gynecological differentiation markers												
Nuclear WT-1	18	5 (28%)	0 (0%)	13 (72%)	63	24 (38%)	0 (0%)	39 (62%)	121	62 (51%)	0 (0%)	59 (49%)
Marker	Total n=	absent	low	high	Total n=	absent	low	high	Total n=	absent	low	high
ER	18	10 (56%)	0 (0%)	8 (44%)	65	37 (57%)	8 (12%)	20 (31%)	124	67 (54%)	18 (15%)	39 (31%)
PR	18	7 (39%)	3 (17%)	8 (44%)	65	29 (45%)	5 (8%)	31 (48%)	117	42 (36%)	25 (21%)	50 (43%)

Appendix Table A5. Expression and distribution of cancer-related markers in controls.

GI Smooth Muscle						Myometrium					Leiomyoma				
A. Intensity															
Marker	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong
Survival															
Bcl-2	10	6 (60%)	1 (10%)	3 (30%)	0 (0%)	14	0 (0%)	3 (21%)	8 (57%)	3 (21%)	8	2 (25%)	3 (38%)	3 (38%)	0 (0%)
Cyt. survivin	10	0 (0%)	5 (50%)	4 (40%)	1 (10%)	15	0 (0%)	7 (47%)	8 (53%)	0 (0%)	8	0 (0%)	3 (38%)	4 (50%)	1 (13%)
Nuc. survivin	10	1 (10%)	8 (80%)	1 (10%)	0 (0%)	15	0 (0%)	15 (100%)	0 (0%)	0 (0%)	8	1 (13%)	7 (88%)	0 (0%)	0 (0%)
Invasion															
MMP-2	10	10 (100%)	0 (0%)	0 (0%)	0 (0%)	15	14 (93%)	1 (7%)	0 (0%)	0 (0%)	8	7 (88%)	1 (13%)	0 (0%)	0 (0%)
MMP-9	10	0 (0%)	4 (40%)	6 (60%)	0 (0%)	15	0 (0%)	3 (20%)	12 (80%)	0 (0%)	8	0 (0%)	4 (50%)	4 (50%)	0 (0%)
Angiogenesis															
VEGF	10	0 (0%)	4 (40%)	6 (60%)	0 (0%)	15	0 (0%)	6 (40%)	9 (60%)	0 (0%)	8	0 (0%)	4 (50%)	4 (50%)	0 (0%)
Oncoproteins and tumor suppressors															
MDM2	10	10 (100%)	0 (0%)	0 (0%)	0 (0%)	15	15 (100%)	0 (0%)	0 (0%)	0 (0%)	5	5 (100%)	0 (0%)	0 (0%)	0 (0%)
p53	10	9 (90%)	1 (10%)	0 (0%)	0 (0%)	15	7 (47%)	8 (53%)	0 (0%)	0 (0%)	7	3 (43%)	4 (57%)	0 (0%)	0 (0%)
p16	10	6 (60%)	1 (10%)	1 (10%)	2 (20%)	15	11 (73%)	0 (0%)	2 (13%)	2 (13%)	7	3 (43%)	1 (14%)	1 (14%)	2 (29%)
Rb	10	2 (20%)	8 (80%)	-	0 (0%)	14	3 (21%)	11 (79%)	-	0 (0%)	6	0 (0%)	6 (100%)	-	0 (0%)
Signaling															
Cyt. β -catenin	10	9 (40%)	6 (60%)	0 (0%)	0 (0%)	15	2 (13%)	13 (87%)	0 (0%)	0 (0%)	8	4 (50%)	4 (50%)	0 (0%)	0 (0%)

B. Distribution		GI Smooth Muscle				Myometrium					Leiomyoma				
Marker	Total n=	absent	low	high	Average (+/- SD)	Total n=	absent	low	high	Average (+/- SD)	Total n=	absent	low	high	Average (+/- SD)
Proliferation															
Ki67	10	10 (100%)	0 (0%)	0 (0%)	0% (+/- 0)	14	14 (100%)	0 (0%)	0 (0%)	0% (+/- 0)	6	6 (100%)	0 (0%)	0 (0%)	0% (+/- 0)
Cyclin D1	10	9 (90%)	1 (10%)	0 (0%)	2% (+/- 6)	15	14 (93%)	1 (7%)	0 (0%)	2% (+/-3)	8	6 (75%)	2 (25%)	0 (0%)	6% (+/- 12)
survival															
Bcl-2	10	6 (60%)	1 (10%)	3 (30%)	23% (+/-31)	14	0 (0%)	1 (7%)	13 (93%)	60% (+/-12)	8	2 (25%)	2 (25%)	4 (50%)	41% (+/-28)
Cyt. survivin	10	0 (0%)	2 (20%)	8 (80%)	59% (+/-23)	15	0 (0%)	1 (7%)	14 (93%)	67% (+/-13)	8	0 (0%)	3 (38%)	5 (63%)	55% (+/-24)
Nuc. survivin	10	2 (20%)	4 (40%)	4 (40%)	30% (+/-22)	15	0 (0%)	8 (53%)	7 (47%)	41% (+/-15)	8	2 (25%)	5 (63%)	1 (13%)	27% (+/-18)
Invasion															
MMP-2	10	10 (100%)	0 (0%)	0 (0%)	0% (+/-0)	15	14 (93%)	0 (0%)	1 (7%)	6% (+/-23)	8	7 (88%)	0 (0%)	1 (13%)	11% (+/-32)
MMP-9	10	0 (0%)	0 (0%)	10 (100%)	90% (+/-0)	15	0 (0%)	4 (27%)	11 (73%)	71% (+/-28)	8	0 (0%)	1 (13%)	7 (88%)	83% (+/-18)
Angiogenesis															
VEGF	10	0 (0%)	3 (30%)	7 (70%)	51% (+/-19)	15	0 (0%)	6 (40%)	9 (60%)	53% (+/-16)	8	0 (0%)	3 (38%)	5 (63%)	55% (+/-21)
Oncoproteins and tumor suppressors															
MDM2	10	10 (100%)	0 (0%)	0 (0%)	0% (+/-0)	15	15 (100%)	0 (0%)	0 (0%)	0% (+/-0)	5	5 (100%)	0 (0%)	0 (0%)	0% (+/-0)
p53	10	9 (90%)	1 (10%)	0 (0%)	2% (+/-6)	15	13 (87%)	2 (13%)	0 (0%)	3% (+/-4)	7	6 (86%)	1 (14%)	0 (0%)	4% (+/-4)
p16	10	9 (90%)	1 (10%)	0 (0%)	4% (+/-6)	15	12 (80%)	3 (20%)	0 (0%)	2% (+/-4)	7	5 (71%)	1 (14%)	1 (14%)	15% (+/-33)
Rb	10	3 (30%)	3 (30%)	4 (40%)	29% (+/-21)	15	6 (43%)	7 (50%)	1 (7%)	16% (+/-17)	6	0 (0%)	5 (83%)	1 (17%)	25% (+/-15)
Signaling															
Cyt. β-catenin	10	4 (40%)	6 (60%)	0 (0%)	15% (+/-18)	15	2 (13%)	12 (80%)	1 (7%)	16% (+/-12)	8	4 (50%)	4 (50%)	0 (0%)	11% (+/-16)

Nuc. β -catenin	10	10 (100%)	0 (0%)	0 (0%)	-	15	15 (100%)	0 (0%)	0 (0%)	-	8	8 (100%)	0 (0%)	0 (0%)	-
Membranous β -catenin	10	10 (100%)	0 (0%)	0 (0%)	-	15	15 (100%)	0 (0%)	0 (0%)	-	8	8 (100%)	0 (0%)	0 (0%)	-

Appendix Table A6. Expression and distribution of cancer-related markers in ULMS

A. Intensity		Primary ULMS				Recurrent ULMS					Metastatic ULMS				
Marker	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong
Survival															
Bcl-2	18	1 (6%)	7 (39%)	8 (44%)	2 (11%)	64	8 (13%)	30 (47%)	17 (27%)	9 (14%)	122	24 (20%)	49 (40%)	26 (21%)	23 (19%)
Cyt. survivin	18	0 (0%)	11 (61%)	7 (39%)	0 (0%)	63	0 (0%)	18 (29%)	45 (71%)	0 (0%)	120	0 (0%)	37 (31%)	83 (69%)	0 (0%)
Nuc. survivin	18	0 (0%)	16 (89%)	2 (11%)	0 (0%)	63	0 (0%)	42 (67%)	20 (32%)	1 (2%)	120	0 (0%)	82 (68%)	38 (32%)	0 (0%)
Invasion															
MMP-2	18	18 (100%)	0 (0%)	0 (0%)	0 (0%)	65	60 (92%)	3 (5%)	2 (3%)	0 (0%)	118	111 (94%)	2 (2%)	2 (2%)	3 (3%)
MMP-9	18	0 (0%)	7 (39%)	6 (33%)	5 (28%)	66	1 (2%)	23 (35%)	28 (42%)	14 (21%)	118	0 (0%)	53 (45%)	38 (32%)	27 (23%)
Angiogenesis															
VEGF	18	0 (0%)	9 (50%)	6 (33%)	3 (17%)	65	1 (2%)	28 (43%)	21 (32%)	15 (23%)	121	0 (0%)	32 (26%)	68 (56%)	21 (17%)
Oncoproteins and tumor suppressors															
MDM2	18	15 (83%)	1 (6%)	2 (11%)	0 (0%)	65	53 (82%)	1 (2%)	7 (11%)	4 (6%)	117	108 (92%)	4 (3%)	3 (3%)	2 (2%)
p53	17	6 (35%)	6 (35%)	5 (29%)	0 (0%)	65	19 (29%)	19 (29%)	27 (42%)	0 (0%)	122	62 (51%)	27 (22%)	32 (26%)	1 (1%)
p16	18	2 (11%)	6 (33%)	3 (17%)	7 (39%)	64	3 (5%)	6 (9%)	20 (31%)	35 (55%)	120	8 (7%)	17 (14%)	39 (33%)	56 (47%)
Rb	18	9 (50%)	3 (17%)	-	6 (33%)	62	38 (61%)	5 (8%)	-	19 (31%)	117	70 (60%)	19 (16%)	-	28 (24%)
Signaling															
Cyt. β -catenin	18	1 (6%)	9 (50%)	8 (44%)	0 (0%)	64	1 (2%)	20 (31%)	24 (38%)	19 (30%)	120	1 (1%)	40 (33%)	53 (44%)	26 (22%)
Nuc. β -catenin	18	18 (100%)	0 (0%)	-	0 (0%)	64	53 (83%)	0 (0%)	-	11 (17%)	121	118 (98%)	0 (0%)	-	3 (2%)
Membranous β -catenin	18	17 (94%)	0 (0%)	-	1 (6%)	64	51 (80%)	0 (0%)	-	13 (20%)	121	94 (78%)	0 (0%)	-	27 (22%)

B. Distribution		Primary ULMS				Recurrent ULMS				Metastatic ULMS					
Marker	Total n=	absent	low	high	Average (+/- SD)	Total n=	absent	low	high	Average (+/- SD)	Total n=	absent	low	high	Average (+/- SD)
Proliferation															
Ki67	18	9 (50%)	8 (44%)	1 (6%)	11% (+/-18)	64	9 (14%)	45 (70%)	10 (16%)	25% (+/-18)	123	32 (26%)	76 (62%)	15 (12%)	23% (+/-18)
Cyclin D1	18	12 (67%)	3 (17%)	3 (17%)	18% (+/-29)	65	47 (72%)	15 (23%)	3 (5%)	8% (+/-17)	124	96 (77%)	23 (19%)	5 (4%)	7% (+/-14)
Survival															
Bcl-2	18	1 (6%)	6 (33%)	11 (61%)	54% (+/-28)	64	8 (13%)	27 (42%)	29 (45%)	43% (+/-34)	122	26 (21%)	45 (37%)	51 (42%)	41% (+/-34)
Cyt. survivin	18	0 (0%)	5 (28%)	13 (72%)	59% (+/-21)	63	0 (0%)	2 (3%)	61 (97%)	73% (+/-11)	120	2 (2%)	15 (13%)	103 (86%)	68% (+/-20)
Nuc. survivin	18	4 (22%)	10 (56%)	4 (22%)	26% (+/-20)	63	0 (0%)	34 (54%)	29 (46%)	41% (+/-19)	120	5 (4%)	63 (53%)	52 (43%)	40% (+/-18)
Invasion															
MMP-2	18	18 (100%)	0 (0%)	0 (0%)	0% (+/-0)	66	61 (92%)	0 (0%)	5 (8%)	7% (+/-23)	118	111 (94%)	1 (1%)	6 (5%)	4% (+/-19)
MMP-9	18	0 (0%)	4 (22%)	14 (78%)	73% (+/-25)	65	0 (0%)	9 (14%)	56 (86%)	78% (+/-22)	118	0 (0%)	19 (16%)	99 (84%)	76% (+/-23)
Angiogenesis															
VEGF	18	1 (6%)	4 (22%)	13 (72%)	56% (+/-26)	65	3 (5%)	14 (22%)	48 (74%)	60% (+/-26)	121	0 (0%)	25 (21%)	96 (79%)	66% (+/-21)
Oncoproteins and tumor suppressors															
MDM2	18	16 (89%)	1 (6%)	1 (6%)	6% (+/-17)	65	59 (91%)	4 (6%)	2 (3%)	3% (+/-13)	117	115 (98%)	1 (1%)	1 (1%)	1% (+/-6)
p53	17	8 (47%)	3 (18%)	6 (35%)	32% (+/-35)	65	26 (40%)	14 (22%)	25 (38%)	34% (+/-34)	122	74 (61%)	15 (12%)	33 (27%)	22% (+/-31)
p16	18	4 (22%)	1 (6%)	13 (72%)	58% (+/-37)	64	7 (11%)	4 (6%)	53 (83%)	71% (+/-30)	120	18 (15%)	6 (5%)	96 (80%)	65% (+/-32)
Rb	18	11 (61%)	5 (28%)	2 (11%)	13% (+/-21)	62	46 (74%)	9 (15%)	7 (11%)	11% (+/-22)	117	91 (78%)	17 (15%)	9 (8%)	8% (+/-19)
Signaling															
Cyt. β -catenin	18	1 (6%)	8 (44%)	9 (50%)	52% (+/-31)	64	1 (2%)	13 (20%)	50 (78%)	65% (+/-26)	120	1 (1%)	30 (25%)	89 (74%)	61% (+/-26)

Appendix Table A7. Cancer-related biomarkers' correlation to outcome.

A. Biomarker distribution expression univariate correlation to recurrence-free (RFS) and metastasis-free (MFS) survival				
Marker	RFS (univariable)		MFS (univariable)	
	<i>P</i>	Hazard Ratio (95% CI)	<i>P</i>	Hazard Ratio (95% CI)
Ki67	0.1909	-	0.2044	-
Cyclin D1	0.5493	-	0.5559	-
Bcl-2	0.3655	-	0.7220	-
Cyt. survivin	0.1196	-	0.0680	-
Nuc. survivin	0.0077	1.03 (1.01-1.05)	0.2683	-
MMP2	0.9458	-	0.4031	-
MMP9	0.7799	-	0.9543	-
VEGF	0.4517	-	0.5928	-
MDM2	0.7489	-	0.7840	-
p53	0.0705	-	0.6729	-
p16	0.4584	-	0.7786	-
Rb	0.1351	-	0.6089	-
Cyt. β -catenin	0.0045	1.02 (1.01-1.04)	0.2190	-
Nuc. β -catenin	0.0001	5.81 (2.03-16.60)	0.6238	-
Memb. β -catenin	0.1519	-	0.5537	-
B. Biomarker distribution expression univariate and multivariate correlation to disease specific survival				
Marker	DDS (univariable)			
	<i>P</i>	Hazard Ratio (95% CI)		
Ki67	0.1240	-		
Cyclin D1	0.4388	-		
Bcl-2	0.0142	0.98 (0.97-1.00)		
Cyt. survivin	0.2564	-		
Nuc. survivin	0.6086	-		
MMP2	0.6272	-		
MMP9	0.9277	-		
VEGF	0.7665	-		
MDM2	0.8800	-		
p53	0.0680	-		
p16	0.8024	-		
Rb	0.3709	-		
Cyt. β -catenin	0.1403	-		
Nuc. β -catenin	0.3173	-		
Memb. β -catenin	0.5741	-		
All markers were calculated by Cox analysis.				

Appendix Table A8. Expression and distribution of mTor componentry and tyrosine kinase receptors/ligands in controls.

GI Smooth Muscle						Myometrium					Leiomyoma				
A. Intensity															
Marker	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong
<i>mTOR pathway componentry</i>															
pS6RP	10	0 (0%)	6 (60%)	4 (40%)	0 (0%)	15	0 (0%)	12 (80%)	3 (20%)	0 (0%)	8	0 (0%)	6 (75%)	2 (25%)	0 (0%)
Cyt. p4EBP1	10	0 (0%)	7 (70%)	2 (20%)	1 (10%)	13	0 (0%)	6 (46%)	7 (54%)	0 (0%)	7	0 (0%)	5 (71%)	2 (29%)	0 (0%)
Nuc. p4EBP1	10	0 (0%)	8 (80%)	2 (20%)	0 (0%)	13	0 (0%)	10 (77%)	2 (23%)	0 (0%)	7	0 (0%)	2 (57%)	3 (43%)	0 (0%)
pAKT	10	0 (0%)	10 (100%)	0 (0%)	0 (0%)	14	0 (0%)	14 (100%)	0 (0%)	0 (0%)	7	0 (0%)	7 (100%)	0 (0%)	0 (0%)
PTEN	10	2 (20%)	5 (50%)	3 (30%)	0 (0%)	15	0 (0%)	7 (47%)	8 (53%)	0 (0%)	8	1 (13%)	4 (50%)	3 (38%)	0 (0%)
<i>Tyrosine kinase receptors/ligands</i>															
EGFR	10	1 (10%)	8 (80%)	1 (10%)	0 (0%)	15	0 (0%)	13 (87%)	2 (13%)	0 (0%)	8	0 (0%)	6 (75%)	2 (25%)	0 (0%)
IGF-1R	10	9 (90%)	1 (10%)	0 (0%)	0 (0%)	15	11 (73%)	4 (27%)	0 (0%)	0 (0%)	6	6 (100%)	0 (0%)	0 (0)%	0 (0%)
PDGFR- α	10	9 (90%)	1 (10%)	0 (0%)	0 (0%)	15	11 (73%)	4 (27%)	-	0 (0%)	8	5 (63%)	3 (38%)	-	0 (0%)
PDGF- α	10	1 (10%)	5 (50%)	0 (0%)	4 (40%)	15	2 (13%)	7 (47%)	-	6 (40%)	8	0 (0%)	3 (38%)	-	0 (0%)
PDGFR- β	10	6 (60%)	4 (40%)	0 (0%)	0 (0%)	15	3 (20%)	12 (80%)	0 (0%)	0 (0%)	8	4 (50%)	4 (50%)	0 (0%)	0 (0%)
PDGF- β	10	2 (20%)	8 (80%)	0 (0%)	0 (0%)	15	0 (0%)	13 (87%)	2 (13%)	0 (0%)	8	0 (0%)	8 (100%)	0 (0%)	0 (0%)
c-KIT	10	10 (100%)	0 (0%)	0 (0%)	0 (0%)	15	15 (100%)	0 (0%)	-	0 (0%)	8	8 (100%)	0 (0%)	-	0 (0%)
MET	10	0 (0%)	4 (40%)	5 (50%)	1 (10%)	15	0 (0%)	5 (33%)	9 (60%)	1 (7%)	8	0 (0%)	2 (25%)	5 (63%)	1 (13%)
AXL	10	2 (20%)	8 (80%)	0 (0%)	0 (0%)	15	5 (33%)	10 (67%)	-	0 (0%)	8	2 (25%)	6 (75%)	-	0 (0%)

B. Distribution		GI Smooth Muscle				Myometrium					Leiomyoma				
Marker	Total n=	absent	low	high	Average (+/- SD)	Total n=	absent	low	high	Average (+/- SD)	Total n=	absent	low	high	Average (+/- SD)
<i>mTOR pathway componentry</i>															
pS6RP	10	0 (0%)	2 (20%)	8 (80%)	63% (+/-14)	15	0 (0%)	1 (7%)	14 (93%)	63% (+/-13)	8	0 (0%)	2 (25%)	6 (75%)	64% (+/-14)
Cyt. p4EBP1	10	0 (0%)	0 (0%)	10 (100%)	70% (+/-12)	13	0 (0%)	0 (0%)	13 (100%)	67% (+/-8)	7	0 (0%)	1 (14%)	6 (86%)	61% (+/-23)
Nuc. p4EBP1	10	0 (0%)	0 (0%)	10 (100%)	73% (+/-5)	13	0 (0%)	0 (0%)	13 (100%)	68% (+/-6)	7	0 (0%)	1 (14%)	6 (86%)	66% (+/-26)
pAKT	10	0 (0%)	2 (20%)	8 (80%)	68% (+/-23)	14	0 (0%)	7 (50%)	7 (50%)	44% (+/-22)	7	0 (0%)	0 (0%)	7 (100%)	77% (+/-5)
<i>Tyrosine kinase receptors/ligands</i>															
EGFR	10	0 (0%)	2 (20%)	8 (80%)	50% (+/-31)	15	0 (0%)	8 (53%)	7 (47%)	39% (+/-22)	8	0 (0%)	4 (50%)	4 (50%)	48% (+/-27)
IGF-1R	10	9 (90%)	1 (10%)	0 (0%)	4% (+/-13)	15	11 (73%)	0 (0%)	4 (27%)	19% (+/-33)	6	6 (100%)	0 (0%)	0 (0%)	0% (+/-0)
PDGFR- α	10	9 (90%)	1 (10%)	0 (0%)	1% (+/-3)	15	11 (73%)	3 (20%)	1 (7%)	7% (+/-18)	8	5 (63%)	2 (25%)	1 (12%)	9% (+/-17)
PDGF- α	10	2 (20%)	1 (10%)	7 (70%)	55% (+/-27)	15	2 (13%)	5 (33%)	8 (53%)	46% (+/-29)	8	0 (0%)	1 (13%)	7 (88%)	61% (+/-15)
PDGFR- β	10	6 (60%)	1 (10%)	3 (30%)	19% (+/-30)	15	3 (20%)	8 (53%)	4 (27%)	29% (+/-21)	8	4 (50%)	4 (50%)	0 (0%)	13% (+/-16)
PDGF- β	10	2 (20%)	5 (50%)	3 (30%)	30% (+/-28)	15	0 (0%)	7 (47%)	8 (53%)	45% (+/-25)	8	0 (0%)	6 (75%)	2 (25%)	34% (+/-24)
c-KIT	10	10 (100%)	0 (0%)	0 (0%)	-	15	15 (100%)	0 (0%)	0 (0%)	-	8	8 (100%)	0 (0%)	0 (0%)	-
MET	10	0 (0%)	0 (0%)	10 (100%)	79% (+/- 3)	15	0 (0%)	0 (0%)	15 (100%)	80% (+/-9)	8	0 (0%)	0 (0%)	8 (100%)	80% (+/-8)
AXL	10	2 (20%)	6 (60%)	2 (20%)	21% (+/-18)	15	7 (47%)	7 (47%)	1 (7%)	16% (+/-22)	8	4 (50%)	2 (25%)	2 (25%)	21% (+/-24)

Appendix Table A9. Expression and distribution of mTOR componentry and tyrosine kinase receptors/ligands in ULMS.

Primary ULMS						Recurrent ULMS					Metastatic ULMS				
A. Intensity															
Marker	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong	Total n=	absent	weak	moderate	strong
<i>mTOR pathway componentry</i>															
pS6RP	18	0 (0%)	12 (67%)	5 (28%)	1 (6%)	62	0 (0%)	15 (24%)	30 (48%)	17 (27%)	120	0 (0%)	28 (23%)	62 (52%)	30 (25%)
Cyt. p4EBP1	16	0 (0%)	7 (44%)	8 (50%)	1 (6%)	59	0 (0%)	9 (15%)	40 (68%)	10 (17%)	118	0 (0%)	27 (23%)	66 (56%)	25 (21%)
Nuc. p4EBP1	16	0 (0%)	12 (75%)	3 (19%)	1 (6%)	58	0 (0%)	19 (33%)	24 (41%)	15 (26%)	118	0 (0%)	44 (37%)	60 (51%)	14 (12%)
pAKT	16	0 (0%)	7 (44%)	9 (56%)	0 (0%)	57	0 (0%)	22 (39%)	27 (47%)	8 (14%)	113	0 (0%)	47 (43%)	51 (45%)	15 (13%)
PTEN	18	1 (6%)	3 (17%)	7 (39%)	7 (39%)	64	4 (6%)	10 (16%)	21 (33%)	29 (45%)	121	10 (8%)	12 (10%)	39 (32%)	60 (50%)
<i>Tyrosine kinase receptors/ligands</i>															
EGFR	17	7 (41%)	8 (47%)	2 (12%)	0 (0%)	65	15 (23%)	27 (42%)	20 (31%)	3 (5%)	117	17 (15%)	53 (45%)	38 (32%)	9 (8%)
IGF-1R	18	9 (50%)	7 (39%)	2 (11%)	0 (0%)	64	27 (42%)	23 (36%)	11 (17%)	3 (5%)	117	68 (58%)	34 (29%)	14 (12%)	1 (1%)
PDGFR- α	18	2 (11%)	14 (78%)	-	2 (11%)	63	2 (3%)	4 (6%)	-	57 (90%)	120	14 (12%)	82 (68%)	-	24 (20%)
PDGF- α	18	4 (22%)	12 (67%)	-	2 (11%)	65	14 (22%)	43 (66%)	-	8 (12%)	120	39 (33%)	74 (62%)	-	7 (6%)
PDGFR- β	18	1 (6%)	14 (78%)	3 (17%)	0 (0%)	61	2 (3%)	49 (80%)	10 (16%)	0 (0%)	117	5 (4%)	82 (70%)	29 (25%)	1 (1%)
PDGF- β	18	0 (0%)	10 (56%)	7 (39%)	1 (6%)	65	2 (3%)	24 (37%)	28 (43%)	11 (17%)	121	3 (2%)	50 (41%)	24 (20%)	44 (36%)
c-KIT	18	18 (100%)	0 (0%)	-	0 (0%)	65	62 (95%)	3 (5%)	-	0 (0%)	124	122 (98%)	2 (2%)	-	0 (0%)
MET	17	0 (0%)	6 (35%)	9 (53%)	2 (12%)	64	0 (0%)	18 (28%)	45 (70%)	1 (2%)	123	0 (0%)	49 (40%)	63 (51%)	11 (9%)
AXL	18	6 (33%)	10 (56%)	-	2 (11%)	61	12 (20%)	41 (67%)	-	8 (13%)	117	25 (21%)	62 (53%)	-	30 (26%)

B. Distribution		Primary ULMS				Recurrent ULMS				Metastatic ULMS					
Marker	Total n=	absent	low	high	Average % (+/- SD)	Total n=	absent	low	high	Average % (+/- SD)	Total n=	absent	low	high	Average % (+/- SD)
<i>mTOR pathway componentry</i>															
pS6RP	19	0 (0%)	5 (28%)	13 (72%)	53% (+/-22)	62	0 (0%)	15 (24%)	47 (76%)	62% (+/-21)	120	0 (0%)	27 (23%)	93 (78%)	61% (+/-19)
Cyt. p4EBP1	16	0 (0%)	2 (13%)	14 (88%)	66% (+/-17)	59	0 (0%)	4 (7%)	55 (93%)	73% (+/-16)	118	0 (0%)	3 (3%)	115 (97%)	73% (+/-13)
Nuc. p4EBP1	16	0 (0%)	2 (13%)	14 (88%)	64% (+/-13)	59	1 (2%)	3 (5%)	55 (93%)	71% (+/-18)	118	0 (0%)	4 (3%)	114 (97%)	72% (+/-11)
pAKT	16	0 (0%)	5 (31%)	11 (69%)	59% (+/-22)	58	1 (2%)	9 (16%)	48 (83%)	66% (+/-22)	113	0 (0%)	16 (14%)	97 (86%)	68% (+/-19)
<i>Tyrosine kinase receptors/ligands</i>															
EGFR	18	7 (39%)	5 (28%)	6 (33%)	32% (+/-30)	66	15 (23%)	28 (42%)	23 (35%)	34% (+/-29)	117	17 (15%)	59 (50%)	41 (35%)	36% (+/-28)
IGF-1R	18	12 (67%)	1 (6%)	5 (28%)	22% (+/-33)	64	31 (48%)	7 (11%)	26 (41%)	35% (+/-38)	117	76 (65%)	22 (19%)	19 (16%)	16% (+/-27)
PDGFR- α	18	2 (11%)	10 (56%)	6 (33%)	33% (+/-25)	63	9 (14%)	26 (41%)	28 (44%)	44% (+/-28)	120	14 (12%)	45 (38%)	61 (51%)	44% (+/-27)
PDGF- α	18	4 (22%)	9 (50%)	5 (28%)	29% (+/-25)	65	14 (22%)	37 (57%)	14 (22%)	28% (+/-24)	120	39 (33%)	57 (48%)	24 (20%)	24% (+/-26)
PDGFR- β	18	0 (0%)	10 (56%)	8 (44%)	42% (+/-26)	61	2 (3%)	12 (20%)	47 (77%)	59% (+/-22)	120	5 (4%)	28 (23%)	87 (73%)	59% (+/-23)
PDGF- β	18	0 (0%)	7 (39%)	11 (61%)	53% (+/-27)	64	0 (0%)	19 (30%)	45 (70%)	57% (+/-24)	121	1 (1%)	33 (27%)	87 (72%)	58% (+/-24)
MET	17	0 (0%)	1 (6%)	16 (94%)	82% (+/-14)	64	0 (0%)	0 (0%)	64 (100%)	86% (+/-7)	123	0 (0%)	1 (1%)	122 (99%)	85% (+/-8)
AXL	18	8 (44%)	7 (39%)	3 (17%)	21% (+/-28)	61	15 (25%)	13 (21%)	33 (54%)	45% (+/-34)	118	29 (25%)	26 (22%)	63 (53%)	40% (+/-32)

Appendix Table A10. Biomarker distribution correlation to outcome.

A. Biomarker distribution expression univariate correlation to recurrence-free (RFS) and metastasis-free (MFS) survival				
RFS (univariable)			MFS (univariable)	
Marker	<i>P</i>	Hazard Ratio (95% CI)	<i>P</i>	Hazard Ratio (95% CI)
pS6RP	0.2674	-	0.2294	-
cyt. p4EBP1	0.8992	-	0.3479	-
nuc. p4EBP1	0.9492	-	0.9846	-
pAKT	0.3285	-	0.8661	-
PTEN	0.9087	-	0.9308	-
EGFR	0.5005	-	0.7279	-
IGF-1R	0.1337	-	0.2411	-
PDGFR- α	0.1903	-	0.0946	-
PDGF- α	0.2263	-	0.0215	0.98 (0.97-1.00)
PDGFR- β	0.0557	-	0.1556	-
PDGF- β	0.6641	-	0.3909	-
c-KIT	0.9570	-	0.1340	-
MET	0.1222	-	0.0774	-
AXL	0.3480	-	0.1671	-
B. Biomarker distribution expression univariate correlation to disease specific survival				
DDS (univariable)				
Marker	<i>P</i>	Hazard Ratio (95% CI)		
pS6RP	0.8796	-		
cyt. p4EBP1	0.0793	-		
nuc. p4EBP1	0.0494	1.03 (1.00-1.06)		
pAKT	0.4645	-		
PTEN	0.7488	-		
EGFR	0.9942	-		
IGF-1R	0.9531	-		
PDGFR- α	0.3775	-		
PDGF- α	0.3824	-		
PDGFR- β	0.0394	1.02 (1.00-1.04)		
PDGF- β	0.5717	-		
c-KIT	0.0284	5.28 (1.19-23.35)		
MET	0.4062	-		
AXL	0.7929	-		

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