AWARD NUMBER: W81XWH-20-1-0736

TITLE: Dysregulation of Sphingolipid Metabolism and Actions in Tuberous Sclerosis Complex

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CONTRACTING ORGANIZATION: University of Cincinnati, Cincinnati, OH

REPORT DATE: October 2021

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2021	Annual	01Sep2020-31Aug2021

4. TITLE AND SUBTITLE

Dysregulation of Sphingolipid Metabolism and Actions in Tuberous Sclerosis Complex

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-20-1-0736

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Cincinnati, Goodman Drive, P.O. Box 210222, Cincinnati, Ohio, 45221-0222

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
TSC is an autosomal dominant disorder with multi-system manifestations including tumors in the brain, heart, kidneys, and lung. TSC is caused by germline inactivating mutations in the tuberin genes, TSC1 or TSC2, that inhibit the activation of mTORC1. Recent advancements have demonstrated that treatment with mTORC1 inhibitors (rapamycin or everolimus) are effective in arresting the growth of symptomatic tumors. The tumors regrow and symptoms resume when treatment is discontinued, suggesting a cytostatic rather than a cytocidal effect, and therefore, the need for continuous therapy with all the concerns associated with chronic treatment to consider. A major breakthrough is needed to advance therapy for TSC. A long-term strategy our laboratory has been to discover novel molecular mechanisms responsible for the survival advantage of TSC tumor cells. For this proposal, we found that dihydroceramide desaturase (DEGS1) and acid ceramidase (ASAH1), enzymes critical to regulating the cellular concentration of the cell survival sphingosine-1-phosphate (S1P), and S1P receptor (S1PR1) that mediates S1P function, were all aberrantly upregulated in TSC tumor cells, but not in normal cells. Our own preliminary data not only confirms this differential expression but also silencing of ASAH1 and S1PR1 in vitro promotes the death of TSC cells. These results suggest that sphingolipid metabolism maybe a previously unappreciated driver of cell survival in TSC and are potential biomarkers for disease severity and therapeutic response in TSC.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **Introduction.** Tuberous sclerosis complex (TSC) is a rare genetic disease that causes benign tumors to grow in many different organs of the body. Although TSC tumors are not metastatic, these tumors can still cause a wide variety of clinical problems, some critical, when the growth is located in vital organs such as the brain, kidney, and lung. Currently, there is no cure for TSC and treatment is largely devoted to surgical removal of tumors. Sometimes drugs such as rapamycin or related agents are used to keep tumors from growing. Unfortunately, the current available drugs at best only stop the tumor growth and do not make the tumors go away. As a result, the tumors enlarge further as soon as rapamycin is discontinued. In addition, long- term treatment with these drugs can lead to side effects such as diabetes. Moreover, in some cases, patients can develop resistance to the treatment. In other words, TSC is an incurable lifelong disease, and the current treatments available only affect disease progression. There is no cure. It is imperative to develop new therapeutic approaches that can not only eliminate, rather than merely block tumor growth, but also exert actions without major side effects.

2. **Keywords**

   Tuberous Sclerosis Complex (TSC), S1PR inhibitors, rapamycin, sphingolipid metabolism

3. **Accomplishments**

   Aim 1. Determine molecular mechanisms of aberrant regulation of sphingolipid biosynthesis and function in cells lacking TSC2 in vitro. Our hypothesis is that TSC2 loss alters sphingolipid production and promotes the survival of renal angiomyolipoma-derived cells.

   Major Task 1. Analyzing the functional significance of sphingolipids in TSC2-null cells

   <u>Subtask 1:</u> In collaboration with Dr. Setchell (CCHMC), we developed the quantification methodology for measure plasma levels of sphingosine, ceramides and S1P, using mass spectrometry. The method will be applied to the proposed study in this task.

   Major Task 2: Determining the molecular and biochemical mechanisms underlying TSC2 regulation of the expression of sphingolipid metabolism and response genes and sphingolipid production.

   <u>Subtask 1:</u> To delineate the impact of signaling events including mTORC2 on the expression of acid ceramidase (ASAH1) in TSC2-null cells, we first treated TSC2-null cells with Torin1, a potent and selective mTOR inhibitor that suppresses both mTORC1/2 activity.

Next, we have generated three independent lines of TSC2-null 621-101 cells with stable Raptor or Rictor knockdown. As expected, Raptor knockdown led to increased activation of Akt at S473 and decreased phosphorylation of S6K1 at T389 (**Fig. 1A**). Rictor knockdown resulted in reduction of Akt phosphorylation at S473 and 4EBP1 (**Fig. 1B**). Importantly, protein levels of ASAH1 were markedly reduced in both Raptor-shRNA and Rictor-shRNA cells, relative pLKO cells, suggesting that both mTORC1 and mTORC2 regulate ASAH1 expression in TSC2-null cells (**Fig. 1**).

It has been reported that the expression of ASAH1 is regulated via CREB-mediated transcriptional machinery. cAMP regulates gene expression via the cAMP response element binding protein (CREB), a nuclear factor that is regulated by protein kinase A (PKA) phosphorylation. Forskolin activates the enzyme adenylyl cyclase (AC) and increases intracellular levels of cAMP. We treated cells with escalating concentration of Forskolin (10-50 μM) for 24 hr, and then examine the protein levels of ASAH1. Forskolin treatment resulted in elevated protein levels of ASAH1, concomitant with increased levels of phospho-CREB (S133) (**Fig 2A**). Moreover, confocal microscopy showed nuclear localization of phospho-CREB (S133) in TSC2-null 621-101 cells but not in TSC2-addback 621-103 cells (**Fig. 2B**). Together, these data suggest that cAMP-CREM activation in TSC2-null cells.

<u>Subtask 2:</u> To determine whether ASAH1 is a key mediator of the growth of TSC2-null cells using molecular approaches, we depleted ASAH1 using siRNA. 621-101 cells transfected with ASAH1 siRNA had 90% reduction of the transcript levels of ASAH1 measured by qRT-PCR (**Fig. 3A**). MTT assay showed that ASAH1 siRNA-transfected 621-101 cells exhibited 50% reduction of cell viability compared with control siRNA (siControl) (**Fig. 3B**), further supporting a critical role of ASAH1 in enhancing TSC tumor cell viability.

4. **Impact.** Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder with multi-system manifestations including development of benign neoplasms in the brain, heart, lung, and kidneys. TSC is caused by TSC1 or TSC2 gene mutations, resulting in constitutive activation of the mechanistic target of rapamycin complex 1 (mTORC1). mTOR is a protein kinase that regulates gene transcription, protein synthesis, ribosome biogenesis, autophagy, cell metabolism, and cell growth. Recent clinical trials in

TSC demonstrate that mTORC1 inhibitors (rapamycin or everolimus) decrease the volume of TSC tumors, subependymal giant cell astrocytomas and renal angiomyolipomas, and stabilize pulmonary function decline. However, tumors regrow and symptoms resume when treatment is discontinued. This cytostatic rather than a cytocidal effect accounts for need for continuous therapy. Therefore, there is a critical need to identify novel molecular targets for development of remission-inducing therapy in patients with TSC. Our proposal directly addresses the following FY19 focus areas: 1) Eradicating tumors associated with TSC angiomyolipomas and gaining a deeper mechanistic understanding of TSC signaling pathways. Our study primarily focuses on identifying the novel cellular metabolic pathways that mediate the survival of TSC tumor cells. Our preliminary data strongly implicate sphingolipids well known in other cancers to play a role cell survival/death. We will elucidate the molecular mechanisms by which TSC2 negatively regulates the biosynthesis and actions of these key bioactive lipid molecules critical for regulating cell survival. 2) Advancing clinical trial readiness through development of biomarkers and testing efficacy of FDA-approved drugs. Effect of mTOR inhibitors on TSC tumors in these experiments has been consistently cytostatic rather than cytotoxic; tumors typically regrow upon the cessation of treatment. This proposal aims to identify novel molecular mechanisms that not only account for these responses to inhibitor therapy but also contribute to the growth properties of TSC tumors independent of inhibitors treatments. These basic studies will ultimately lead to transformative clinical trials with new combination therapies involving the **FDA-approved** drugs, Fingolimod and Sirolimus and/or its analogs. In summary, understanding the mechanism of dysregulation of bioactive sphingolipid biosynthesis and actions is highly likely to lead within a two to five year period to improved therapy for patients with TSC and other neoplasms.

5. Changes/Problems.

During the COVID19 pandemic, the recruitment of new lab members has been challenging and was much longer than we anticipated. Dr. Astreinidis joined Yu Lab on 8/30/2021 and he started to work on studies funded by this award.

6. Products.

None

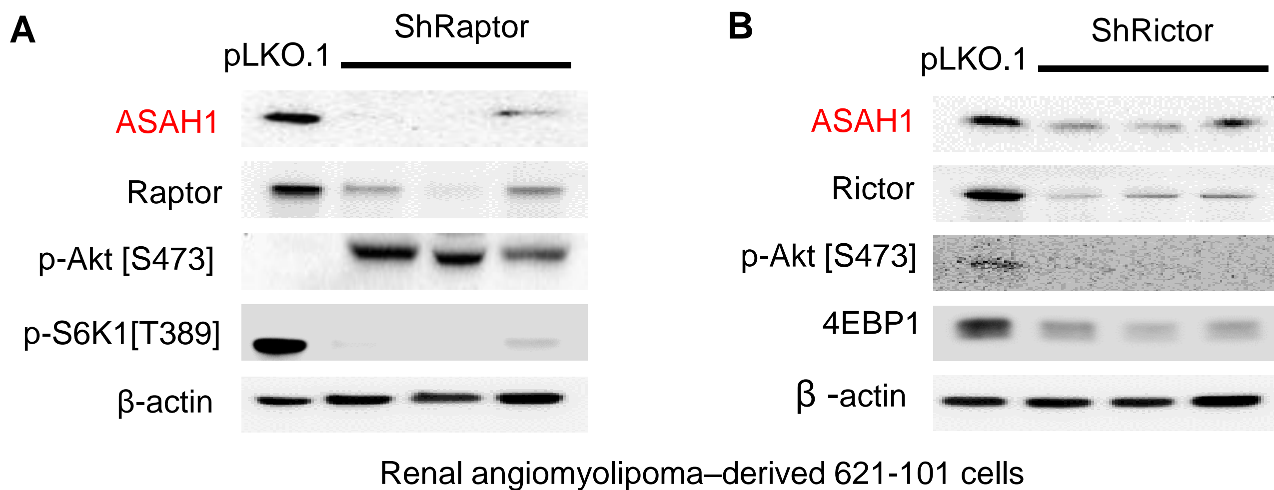
7. **Participants & Other Collaborating Organizations**

Jane Yu, Erik Zhang, Aristotelis Astreinidis, Frank McCormack, Ken Setchell (Cincinnati Children's Hospital Research Center)

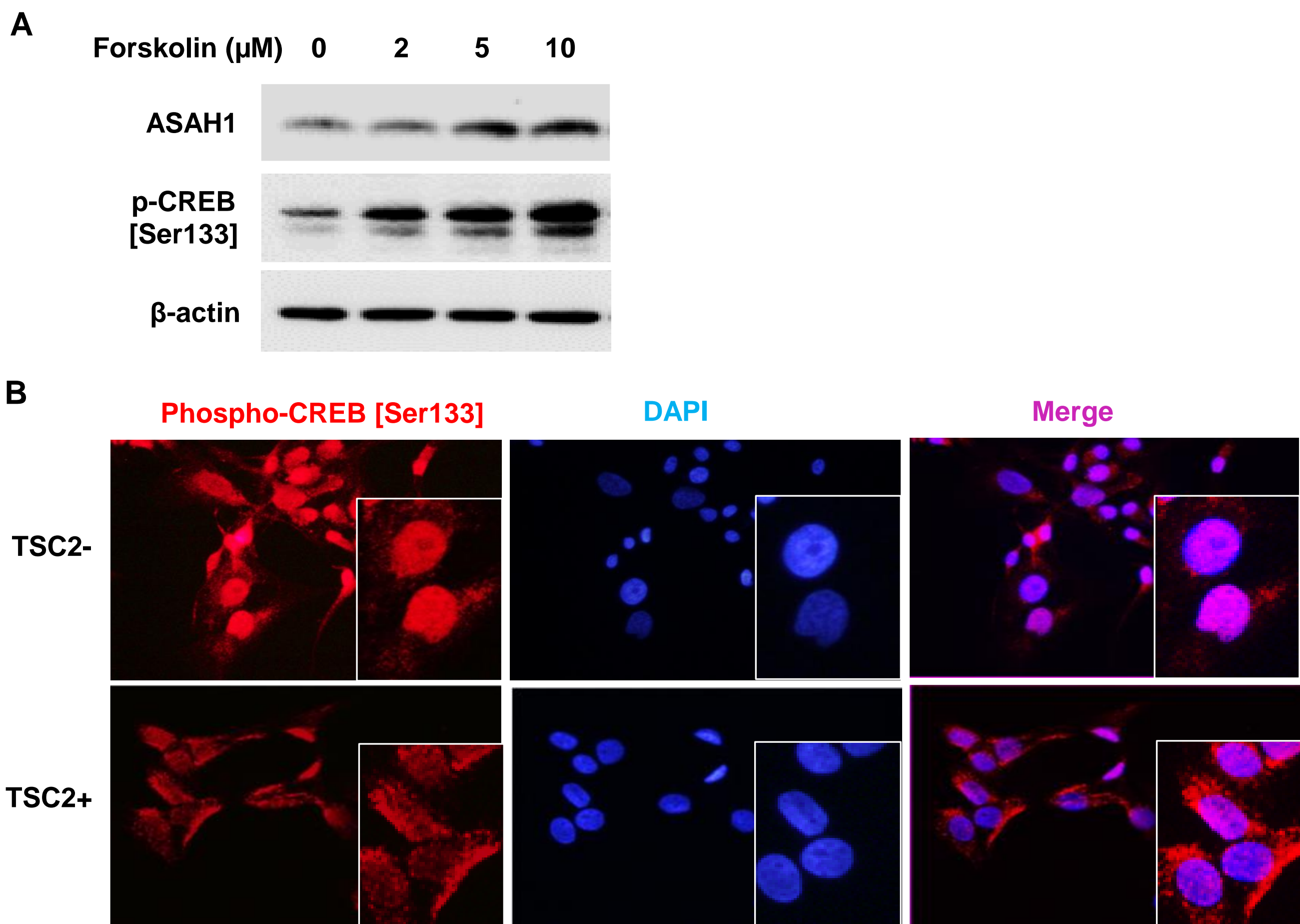
8. **Special Reporting Requirements.** N/A

9. **Appendices.**

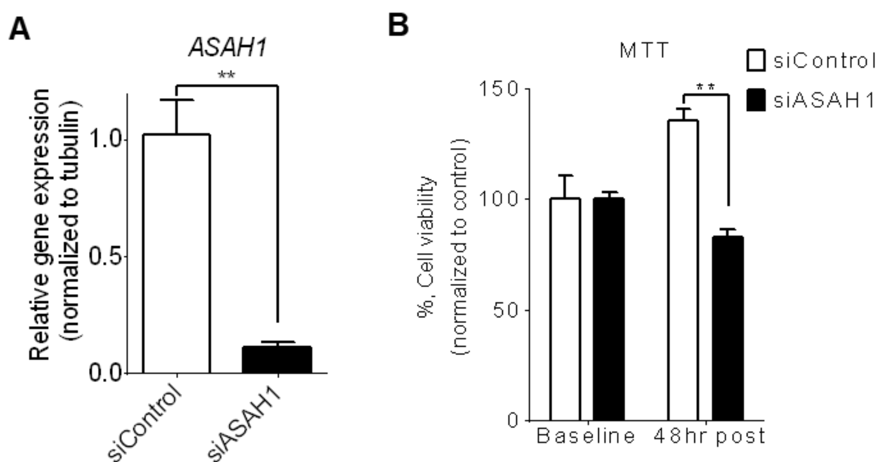
9a. Figure 1. Molecular depletion of Raptor and Rictor decreases ASAH1 protein levels in TSC2-null cells. 621-101 cells were infected with lentivirus containing shRNA-Raptor (A), shRNA-Rictor (B), or vector control pLKO.1. Three independent clones were selected with puromycin. Protein lysates were isolated using mPER lysis buffer supplemented with proteinase and phosphatase inhibitor cocktails. Immunoblotting analysis was performed for ASAH1, phospho-Akt (S473), phospho-S6K1 (T489), or 4EBP1. β–actin was used as a loading control.



9b. Figure 2. Activation of CREB is associated with increased expression of ASAH1 in TSC2-null cells. (A) 621-101 cells were treated with 2-10 μM Forskolin to activate cAMP for 24 hr. Immunoblotting analysis of ASAH1 and phospho-CREB (S133) was performed. β–actin was used as a loading control. (B) Confocal microscopic images of phospho-CREB (S133) in 621-101 (TSC2-) and 621-103 (TSC2+) cells. DAPI staining indicates cell nuclear staining



9c. Figure 3. Depletion of ASAH1 reduces the viability of TSC2-null 621-101 cells. Cells were transfected with three independent ASAH1-siRNAs or non-targeting control-siRNA for 48 hr. (A) siRNA knockdown efficiency was determined by qRT-PCR. Tubulin was used as a house-keeping gene for normalization. (B) Cell viability was assessed 48 hr post siASAH1 RNA transfection in 621-101 cells using MTT assay. **p<0.01, Student t-test.



9d. SOW and highlight progress

Statement of Work (SOW)

Site: PI: Dr. Jane Yu, email: jane.yu@uc.edu, University of Cincinnati

Specific Aim 1: Determine molecular mechanisms of aberrant regulation of sphingolipid biosynthesis and function in cells lacking TSC2 in vitro.	**Timeline (Months)**	**Site**
Obtain HRPO and ACURO approval	1-3	Dr. Yu
Major Task 1: Analyzing the functional significance of sphingolipids in TSC2-null cells	Months	
Subtask 1: Quantify the cellular levels of sphingolipids in patient plasma (The MILES samples n=30), using mass spectrometry. Plasma from age- and gender-matched normal individuals (UC-TPSC Bank n=30) will be used as controls.	1-9	Drs. Yu, McCormack, and Setchell
Subtask 2: Conduct cell-based assays for measuring: (1) cell growth and (2) cell death (apoptosis and necroptosis) of human renal angiomyolipoma-derived 621-101 vs. TSC2 re-expressing cells, Tsc2-deficient rat uterine leiomyoma-derived ELT3 vs. TSC2-reexpressing cells, and Tsc2-/- MEFs vs. Tsc2+/+ MEFs, in response to sphingolipid treatment.	10-18	Drs. Yu and Davidson
Major Task 2: Determining the molecular and biochemical mechanisms underlying TSC2 regulation of the expression of sphingolipid metabolism and response genes and sphingolipid production.		
Subtask 1: Delineate the impact of signaling events including Rheb and mTORC2 on the expression of acid ceramidase (ASAH1) and sphingolipid production in TSC2-null cells vs. TSC2-addback cells.	13-18	Drs. Yu and Blenis
Subtask 2: Test the consequence of molecular and pharmacologic suppression of DEGS1, ASAH1 and S1PR1 on cell survival and signaling pathway activation in TSC2-null cells. Three independent shRNAs will be used to knockdown each gene in 621-101 cells. The vector pLKO.1 will be used as a control for gene depletion.	19-24	Dr. Yu
Milestone #1: Manuscript preparation on TSC2 regulates sphingolipid metabolism and viability in TSC progression.	22-24	Dr. Yu
Specific Aim 2: Determine the efficacy of molecular and pharmacologic suppression of S1PR1 on the growth and regrowth of xenograft tumors composed of patient-associated angiomyolipoma-derived cells.		
Major Task 3: Test the effect of pharmacologic suppression of S1PR1 on the progression and regrowth of 621-101 xenograft tumors using non-invasive bioluminescent imaging technology. A total of 64 male NSG and 64 female of 6 week-old NSG mice will be purchased from Jackson Laboratory.		
Subtask 1: Submit animal protocol amendments for testing agents in xenograft models.	1-3	Dr. Yu

Subtask 2: Establish tumors and test drug effects on tumor progression and re-growth upon drug cessation using bioluminescent imaging.	4-12	Dr. Yu
Major Task 4: Test the effect of molecular depletion of sphingolipid metabolism gene S1PR1 on the growth 621-101 xenograft tumors using bioluminescent imaging. A total of 32 male NSG and 32 female of 6 week-old NSG mice will be purchased from Jackson Laboratory.		
Subtask 1: Establish and expand 621-101-luciferase-S1PR1shRNA stable cells (at least three clones plus empty vector control pLKO.1-shRNA cells). Validate the effect of molecular depletion of S1PR1 in tumor cells.	13-20	Dr. Yu
Subtask 2: Test the effect of molecular depletion of S1PR1 on the growth 621-101 xenograft tumors using bioluminescent imaging.	21-24	Dr. Yu
Major Task 5: Determine the dysregulation of sphingolipid metabolism pathway proteins in xenograft tumors of 621-101 cells.		
Subtask 1: Collect 10-16 snap-frozen xenograft tumors from mice under drug treatment.	25-27	Dr. Yu
Subtask 2: Real-time RT-PCR, immunoblotting, and immunohistochemistry analysis of the expression of sphingolipid metabolism gene expression in xenograft tumors.	28-33	Dr. Yu
Milestone #2: Manuscript preparation: Tissue-specific expression of sphingolipid and response genes in TSC tumorigenesis.	34-36	Dr. Yu
Specific Aim 3: Determine the effect of pharmacologic suppression of S1PR1 on tumor progression and kidney functions in a genetic renal tumor model of TSC.		
Major Task 6*:* Examine the effect of pharmacologic inhibitors of S1PR1 and mTORC1, singly or in combination, on suppressing renal tumor development.		
Subtask 1: Submit animal protocol amendments for testing agents in xenograft models.	1-3	Dr. Yu
Subtask 2: Develop renal cysts and tumors in *Tsc2*$^{+/-}$A/J mice. Breeding will be set up. 64 male *Tsc2*$^{+/-}$A/J mice and 64 female *Tsc2*$^{+/-}$A/J mice age of 5-months will be used. MRI imaging will be performed monthly beginning at 2-month of age. Perform IVIS imaging for cell death.	4-12	Dr. Yu
Subtask 3: Treat *Tsc2*$^{+/-}$A/J mice (5 month of age) with FTY720, and/or rapamycin for 2 months. Analyze mice for renal tumor growth using MRI imaging. Perform IVIS imaging for cell death.	13-14	Dr. Yu

Subtask 4: Treat *Tsc2*$^{+/-}$ mice (5 month of age) with FTY720, and/or rapamycin for two months, stop drug treatments, and monitor tumor regrowth for 2 months using MRI imaging bi-weekly.	13-16	Dr. Yu
Subtask 5. Perform renal function tests in above mice.	17-24	Drs. Yu and Soleimani
Subtask 6: Perform Mass Spectrometry analysis of plasma levels of rapamycin and Fingolimod-phosphate from mice bearing renal tumors.	25-33	Drs. Yu and Setchell
Milestone #3: Manuscript preparation: Identification of altered sphingolipid metabolites as potential biomarkers for TSC renal tumor progression.	34-36	Dr. Yu