**AWARD NUMBER:** W81XWH-18-1-0370

**TITLE:** Mapping the Routes to Tumor Cell Death in TSC

**PRINCIPAL INVESTIGATOR:** Brendan Manning

**CONTRACTING ORGANIZATION:** President and Fellows of Harvard College, Boston, MA

**REPORT DATE:** November 2021

**TYPE OF REPORT:** Final

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

**DISTRIBUTION STATEMENT:** Approved for public release; distribution is unlimited.

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**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)**

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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Individuals with tuberous sclerosis complex (TSC) are at risk of developing a variety of tumors, primarily affecting the brain, kidneys, skin, lung, heart, and eyes. Rapamycin/sirolimus and its analogs, such as everolimus, are effective at shrinking the variety of tumors arising in TSC patients and appear to halt further tumor growth. However, these drugs do not eliminate tumors, and upon withdrawal of treatment, tumors regrow at a remarkably rapid rate. Thus, there is an unmet need for treatments that elicit a more complete and durable response that allows TSC patients to avoid continuous therapy with rapalogs. This current study uses cutting edge new techniques to study the molecular nature of tumor cell death and survival in TSC and represents a comprehensive and novel strategy to tackle this problem. The study will identify and test, in multiple preclinical tumor models, new therapeutic approaches to induce tumor cell death in TSC, thereby eliminating existing tumors. We will focus on an emerging and promising class of drugs, called BH3 mimetics, which are being developed and FDA approved for use in cancer therapy. We anticipate that a subset of these drugs, when used in combination with rapamycin or its analogs, will safely induce cell death specifically in the tumors of TSC patients, thereby eradicating these lesions. Through rigorous testing in multiple preclinical models, it is our hope that the completion of our study in the next three years will provide the preclinical evidence needed to advance these treatment strategies into the clinic to benefit TSC patients and offer an improved response to that currently achieved with rapamycin.

**15. SUBJECT TERMS**

Tuberous sclerosis complex, tumors, therapy, rapamycin, apoptosis, BCL2 family, BH3 mimetics, preclinical mouse models.

| **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 | 20 | **19b. TELEPHONE NUMBER** *(include area code)* |

**TABLE OF CONTENTS**

## 1. INTRODUCTION:

The research under this proposal will determine the molecular effects of mTORC1 activation and inhibition in TSC on the key regulators and effectors of apoptosis to reveal novel approaches to induce tumor cell death. Rapamycin and its analogs (rapalogs) are effective at shrinking the variety of tumors arising in TSC patients and appear to halt further tumor growth. However, these drugs do not eliminate tumors, and upon withdrawal of treatment, tumors regrow at a remarkably rapid rate. Therefore, despite blocking the uncontrolled mTORC1 signaling that is a hallmark of TSC, tumor cell survival is unaffected by rapalogs, and these cells are poised to accelerate their growth when released from therapy. To understand and overcome this property of rapalogs in the TSC setting, we directly examine the status of pro- and anti-apoptotic proteins influencing mitochondrial membrane permeability and cytochrome C release, the initiating event in programmed cell death. We are measuring transcript and protein levels of these factors, the BCL2 family, in cell and tumor models of TSC, as well as available TSC patient samples. We are using functional assays to determine the effects of TSC1 and TSC2 mutations and mTOR inhibitors on mitochondrial apoptotic priming and apoptosis. Finally, we are using mouse tumor models to test the effectiveness of targeting these factors alone or in combination with mTOR inhibitors for durable anti-tumor responses that are sustained upon removal of treatment.

## 2. KEYWORDS:

Tuberous sclerosis complex; Tumors, Therapy, Rapamycin, Apoptosis, BCL2 family, BH3 mimetics, Preclinical Mouse Models

## 3. ACCOMPLISHMENTS:

**What were the major goals of the project?**

**Specific Aim 1:** Define the status of pro- and anti-apoptotic proteins of the BCL-2 family and apoptotic priming in TSC. *Mos 1-24 – 80% complete*

**Task 1** Characterization of BLC-2 family members and BH3 profiling in TSC cell culture models. *Mos 1-12 – 100% complete*

***Milestone*** *– ACURO approval complete*

**Task 2** Characterization of BLC-2 family members and BH3 profiling in TSC mouse tumor models. *Mos 8-24 – 80% complete*

**Task 3** Characterization of BCL-2 family member in human TSC and LAM tumor samples. *Mos 18-24 – 50% complete*

**Specific Aim 2:** Preclinical studies to enhance apoptotic priming in TSC cell and tumor models with BH3 mimetics developed for clinical use. *Mos 8-36 – 90% complete*

**Task 1** Test the effects of inhibiting anti-apoptotic BCL-2 family members on the induction of apoptosis in TSC cell culture models, alone and in combination with mTOR inhibitors. *Mos 8-20 – 100% complete*

**Task 2** Testing the effects of inhibiting anti-apoptotic BCL-2 family members on the induction of apoptosis in TSC mouse tumor models, alone and in combination with mTOR inhibitors. *Mos 20-36 – 80% complete*

**Specific Aim 3:** Determine the control mechanisms downstream of the TSC complex and mTORC1 altering apoptotic priming in TSC. *Mos 1-36 – 100% complete*

**Task 1** Characterization of BLC-2 family members in multiple TSC cell culture models over time course of treatment with mTOR inhibitors. *Mos 1-12 – 100% complete*

**Task 2** Protein stability and mRNA translation will be measured for those BCL-2 family proteins showing changes with TSC gene loss and/or mTOR inhibitors that are not accompanied by changes in mRNA levels. *Mos 13-24 – 100% complete*

**Task 3** The precise molecular mechanisms regulating those BCL- 2 family members found to most strongly influence TSC-deficient cell survival and death decisions in TSC settings will be defined, with focus on potential post-translational modifications and transcription factors involved. *Mos 25-36 – 100% complete*

**What was accomplished under these goals?**

1) Major Activities: We made steady progress toward the completion of the stated research aims in final year of this award. While we have completed nearly all tasks outlined in this grant, there are two ongoing experiments. First, due to access restrictions in 2020, we had a delay in generating the *Tsc2+/-* cohorts needed for Task 2, Subtask 2 for both Aims 1 and 2. Second, while we have obtained slides with paraffin-embedded sections of archived human TSC and LAM specimens from Dr. Elizabeth Henske (Task 3), we are still troubleshooting appropriate antibodies for immunohistochemical analyses of these samples. Many commercially available antibodies are proving insufficient for this task. Representative data are presented in the two figures below, which cover specific experiments/tasks under Aim 2 (Figure 1) and Aim 3 (Figure 2).

2) Specific Objectives: Our efforts focused on measuring the selective induction of apoptosis in mouse TSC-related tumors upon short-term systemic treatment with rapamycin plus the BCL-2/BCL-xL inhibitor ABT-263 (Figure 1, Aim 2) and defining the molecular mechanisms by which mTORC1 activation and inhibition in TSC cells influence the levels of anti-apoptotic proteins (Figure 2, Aim 2).

3) Significant Results / Key Outcomes: *See two figures summarizing new data below.*

4) Other achievements: A manuscript describing the complete results of this study is now being prepared for submission by the end of 2021.

**3) Significant Results / Key Outcomes:**

**Figure 1: Data from Aim 2, Task 2.** (A) Schematic of experiment: 105K tumor-bearing mice were treated for 24 h with vehicle or rapamycin (1 mg/kg, i.p.), followed by two cotreatments 24 h apart with vehicle (i.p.) plus vehicle (oral)(n=3), rapamycin (i.p.) plus vehicle (oral)(n=3), vehicle (i.p.) plus the BCL-2/BCL-xL inhibitor ABT-263 (100 mg/kg, oral) (n=4), or rapamycin plus ABT-263 (n=4), with mice sacrificed 6 h after the last dose and tumor resected for histological analyses. (B) Representative images of paraffin-embedded tumor tissue, from mice treated as indicated, stained (brown) with antibodies to markers of mTORC1 signaling (p-S6, 20X), proliferation (Ki67, 20X), and apoptosis (cleaved caspase 3 (CC3), 40X) and counterstained with hematoxylin (blue). (C) Cleaved-caspase 3-positive cells per field were quantified in a blinded fashion in 4 representative, non-overlapping fields per tumor and are graphed as the mean percent of cells ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The combination of rapamycin and ABT-263 enhance apoptosis within this TSC tumor model relative to single-agent treatments.

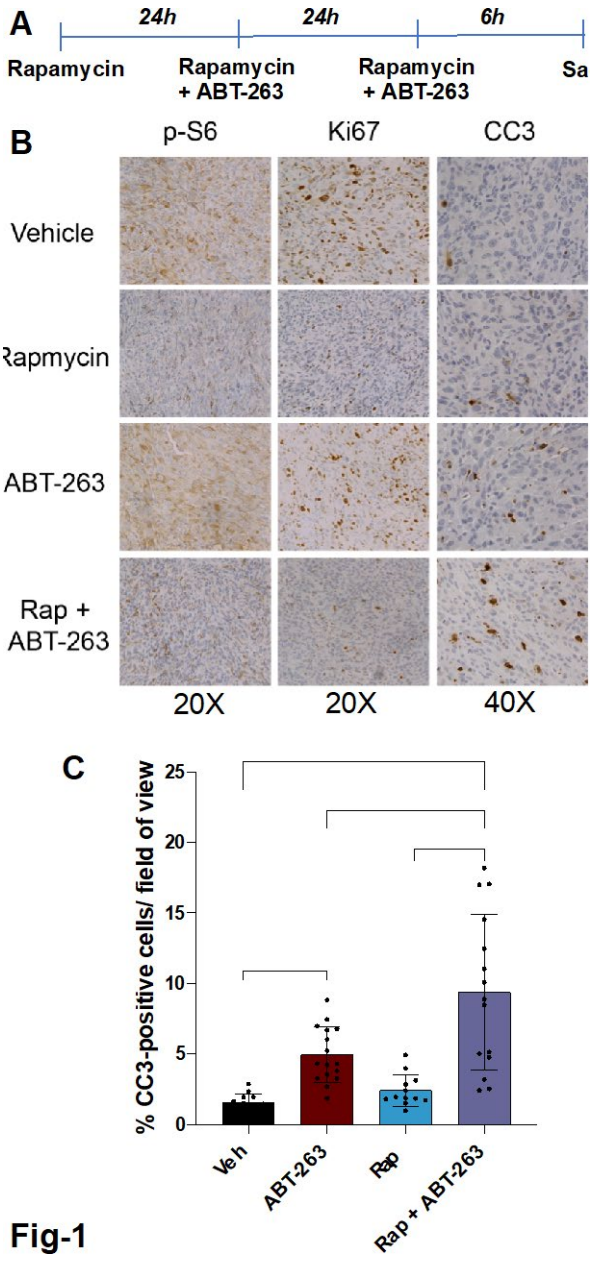


Fig-1

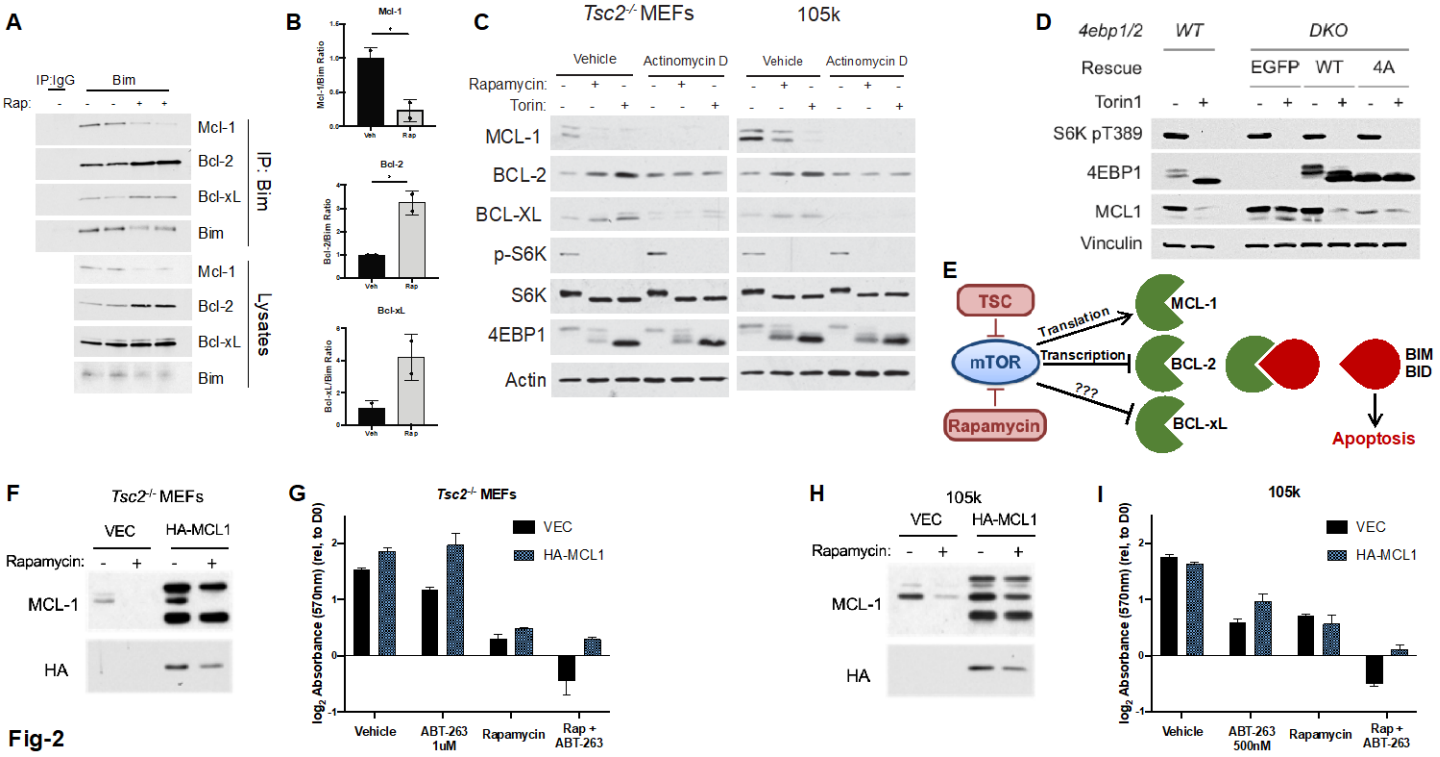


Fig-2

**Figure 2: Data from Aim 3, Tasks 2 and 3.** (A,B) Interaction of anti-apoptotic BCL-2 family members with BIM in *Tsc2-/-* MEFs treated with vehicle or rapamycin (20 nM) for 24 h. BIM was immunopreciptated from lysates of cells treated, as indicated, with biological duplicates shown in (A) immunoblots and (B) graphs as the mean ratio of MCL-1, BCL-2, or BCL-xL to BIM in the immunoprecipates ± SD. *p < 0.05. Rapamycin decreases BIM interaction with MCL-1 and increases its association with BCL-2 and BCL-xL. (C) Effects of transcription on the ability of mTOR inhibitors to change the levels of anti-apoptotic BCL-2 family members. *Tsc2-/-* MEFs and 105K tumor-derived cells were treated with vehicle, rapamycin (20 nM), or Torin1 (250 nM) for 24 h in the presence of vehicle or actinomycin D (100 ng/ml). mTOR inhibitors require transcription to increase BCL-2 protein levels. (D) Effects of Torin1 on MCL-1 protein levels in wild-type (WT) or *4ebp1/2* double knockout (DKO) cells reconstituted with control vector (EGFP) or constructs encoding either WT 4EBP1, or 4EBP1-4A, which cannot be phosphorylated and inhibited by mTORC1. Cells were treated with vehicle or Torin1 (250 nM) for 24 h and markers of mTORC1 signaling and MCL-1 were immunoblotted. The mTORC1-dependent regulation of translation through 4E-BP1 is essential for its stimulatory effects on MCL-1 protein levels. (E) Model of mTORC1-mediated activation of MCL-1 and inhibition of BLC-2 and BCL-xL, thereby differentially influencing which anti-apoptotic BCL-2 family member engages with BIM to inhibit its induction of apoptosis when mTORC1 is activated in TSC or inhibited with rapamycin. (F-I) *Tsc2-/-* MEFs (F,G) or 105K cells (H,I) stably expressing empty vector (VEC) or HA-MCL-1 lacking its 5"UTR were treated for 24 h with vehicle or rapamycin and endogenous and exogenous MCL-1 protein levels were examined via immunoblot (F,H). Exogenously expressed HA-MCL-1 is partially resistant to rapamycin, consistent with its control by translation acting through its 5'UTR. (G,I) The indicated cells were treated for 72 h with vehicle, ABT-263, rapamycin, or both ABT-263 and rapamycin and stained with crystal violet, which was measured as a readout of viable cells and is graphed as mean $\log_2$ absorbance relative to Day 0 ± SD. Expression of partially rapamycin-resistant MCL-1 partially rescues the loss of viability observed with the combination treatment.

**What opportunities for training and professional development has the project provided?**

Alexander Valvezan (postdoctoral fellow) and Molly McNamara (graduate student), have been working on specific elements of this project, which is the specific focus of Ms McNamara's doctoral research in the laboratory. Dr. Valvezan trained Ms McNamara in the techniques to generate and work with the TSC preclinical mouse models needed for this study and to deliver compounds by oral gavage, experiments that led to important new discoveries under Aim 2 regarding the greatly enhanced durability of combinatorial treatment with rapamycin plus the BH3 mimetic ABT-263, relative to rapamycin alone (data described in Figure 4 under accomplishments). Dr. Valvezan has recently been hired as an Assistant Professor at Rutgers University and started in September of 2020. Ms. McNamara is completing the project, the results of which will be submitted by the end of the year, with her graduating in the Spring of 2022.

**How were the results disseminated to communities of interest?**

The PI, Brendan Manning, presented the results of this study as a plenary speaker this summer at the 2021 International Tuberous Sclerosis Complex Research Conference, which was held virtually, and also recorded a related talk geared toward lay audiences for TSC patients and their families to bring this research to those communities.

**What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to Report

***4.*** **IMPACT:**

**What was the impact on the development of the principal discipline(s) of the project?**

This project has potential to change the current treatment paradigm for tumors developing in patients with tuberous sclerosis complex and lymphangioleiomyomatosis, with improved and more durable treatment responses, perhaps over a shorter treatment time. The project results provided preclinical evidence to support future clinical trials with combinations of rapamycin analogs and specific BCL-2 family inhibitors, which are in active clinical development and use.

**What was the impact on other disciplines?**

As the TSC-mTORC1 pathway is frequently dysregulated in human cancers, the study has the real potential to alter treatment paradigms across multiple lineages of human cancer, where mTORC1 inhibitors alone have exhibited little to no anti-tumor efficacy.

**What was the impact on technology transfer?**

Nothing to Report

**What was the impact on society beyond science and technology?**

Nothing to Report

## 5. CHANGES/PROBLEMS:

### Changes in approach and reasons for change

Nothing to report.

### Actual or anticipated problems or delays and actions or plans to resolve them

Due to limited access to our laboratory during the Covid-19-related shutdown of Harvard University, we were delayed in our development of a cohort of *Tsc2*$^{+/-}$ mice for a subset of the described experiments.

### Changes that had a significant impact on expenditures

Nothing to Report.

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

Nothing to Report.

**Significant changes in use of biohazards and/or select agents**

Nothing to Report.

**6. PRODUCTS:**

- **Publications, conference papers, and presentations**

  **Journal publications.**

Nothing to Report. Manuscript to be submitted in late 2021.

**Books or other non-periodical, one-time publications.**

Nothing to Report.

**Other publications, conference papers and presentations**.

Plenary talk at the 2021 International Tuberous Sclerosis Complex Research Conference

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

| | |
|---|---|
| Name: | Brendan D. Manning |
| Project Role: | PI |
| Researcher Identifier (e.g. ORCID ID): | eRA Commons ID: BMANNING1 |
| Nearest person month worked: | 1 |
| Contribution to Project: | No change. |
| Funding Support: | See updated Current Support below. |

| | |
|---|---|
| Name: | Alexander J. Valvezan, PhD |
| Project Role: | Postdoctoral Fellow |
| Researcher Identifier (e.g. ORCID ID): | eRA Commons ID: VALVEZAN |
| Nearest person month worked: | 1 |
| Contribution to Project: | Has developed the cell viability and apoptosis assays and trained Ms. McNamara for use of the TSC preclinical mouse models. |
| Funding Support: | DoD Award W81XWH-18-1-0659-TS170030 (PI: Manning) |
| Name: | Molly McNamara |
| Project Role: | Graduate Student |
| Researcher Identifier: | MOLLYMCNAMARA |
| Nearest person month worked: | 12 |
| Contribution to Project: | Has performed analyses of BCL2 family member expression levels by immunoblot and qRT-PCR and has carried out the BH3 profiling assays. |
| Funding Support: | |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Since the last reporting period, both Department of Defense awards have completed. Please see full, updated DoD Other Support info starting on the next page.

**KEY PERSONNEL PREVIOUS/CURRENT/PENDING SUPPORT**

MANNING, BRENDAN

**PREVIOUS SUPPORT (**last five years)

Tuberous Sclerosis Alliance 194641 (PIs: Manning & Valvezan) 12/01/2015 – 11/30/2018
**Repurposing clinically approved inhibitors of purine synthesis for the treatment of TSC**
DC/YR, 0.6 calendar months
Tuberous Sclerosis Alliance
801 Roeder Road, Suite 750, Silver Spring, MD 20910-4487
Grants Officer: Kari Luther Rosbeck
This project screened nucleotide synthesis inhibitors for selective effects in TSC1/2-deficient cells, determined the underlying mechanism, and demonstrated anti-tumor efficacy in preclinical TSC tumor models.
**Aim 1:** Characterize the response of TSC1/2-deficient cells to available inhibitors of purine synthesis
**Aim 2:** Preclinical trials of IMPDH inhibitors in mouse models of TSC
**Aim 3:** Define the mechanism underlying the selective response of TSC cells to purine synthesis inhibitors
No overlap.

Zafgen, No Award Number, (PIs: Manning & Mitchell) 09/01/2016 – 09/30/2019
**Determining the mechanism of action of derivatives of the anti-obesity drug, fumagillin**
DC/YR, 0.6 calendar months
Zafgen, Inc.
3 Center Plaza, Suite 610, Boston, MA 02108
CFO: Patricia Allen
Under this grant, we are characterizing the effects of anti-obesity drugs on cellular and systemic metabolism, and nutrient signaling pathways.
No overlap.

Department of Defense (PI: Manning) 09/01/2018 – 07/31/2021
**Mapping the Routes to Tumor Cell Death in TSC**
W81XWH-18-1-0370-TS170026
DC/YR, 1.2 calendar months
U.S. Army Medical Research Acquisition Activity
820 Chandler Street, Fort Detrick, MD 21702-5014
Grants Specialist: Christopher Meinberg
Under this grant, we will examine how TSC gene loss and mTORC1 activation influences the cell intrinsic apoptosis machinery in TSC cell and tumor models, and the therapeutic implications.
**Aim 1:** Define the status of pro- and anti-apoptotic proteins of the BCL-2 family and apoptotic priming in TSC.
Role: PI
***This award has moved from "Current" to "Completed" since the last report.**

Department of Defense (PI: Perrimon) 09/30/2018 – 09/29/2021
**An evolutionary approach to vulnerability mapping in order identify alternative and synergistic therapeutic strategies for TSC and related diseases**
DC/YR, 0.6 calendar months
U.S. Army Medical Research Acquisition Activity
Fort Detrick, Maryland 21702-5012
Grant Specialist: Mark Wilkison
This project aims to identify synergistic interactions with Rapamycin.
**Aim 1.** Elucidate the mechanism underlying the synthetic lethal interaction between CTNS and TSC1/2. To

expand our list of high-confidence candidate genes that show synthetic lethality with TSC, we recently performed genome-wide CRISPR knockout screening and RNAi screens to search for TSC vulnerabilites. A strong hit in all fly screens, which also had similar effects in mouse TSC cells, was the lysosomal cysteine transporter, CTNS. Preliminary evidence suggests altered cystine levels in TSC-mutated fly cells, hinting at a mechanistic link at the level of cystine metabolism. Therefore, we propose to determine how the levels of cystine and related metabolites affects growth rates in TSC-deficient mouse cell-lines and mice, and how and if these interface with mTOR signaling.

**Aim 2.** Use of a rapamycin-sensitized screen in Drosophila cells to identify synergistic vulnerabilities to be characterized in human or mouse TSC deficient cell-lines. A promising approach for the treatment of TSC is to identify synergistic interactions with rapamycin, as these could lead to combinatorial therapeutic approaches. Thus, we propose to capitalize on our development of CRIPSR knockout screening to perform rapamycinsensitized genome-wide screens in wild-type and TSC deficient Drosophila cells. The results will be validated in a collection of 4 different mammalian cell models of TSC (2 mouse and 2 human), prioritizing hits against which small molecule inhibitors exist. The most promising hits from the screening and validation experiments will be tested in a preclinical mouse TSC tumor model for synergistic elimination of TSC-associated tumors in combination with rapamycin. The results of this work are likely to contribute new combinatorial therapeutic options for TSC and related diseases.

Role: Co-PI

No overlap.

***This award has moved from "Current" to "Completed" since the last report.**

## CURRENT SUPPORT

NIH/NCI Outstanding Investigator Award: R35-CA197459 (PI: Manning) 07/01/2015 – 06/30/2022

**Decoding and targeting the PI3K-mTOR signaling network in cancer**

DC/YR, 6 calendar months

National Cancer Institute

BG 9609 MSC 9760, 9609 Medical Center Drive, Bethesda, MD 20892-9760

Grants Management Specialist: Marianne Galczynski

There are no specific aims in this award, but research is focused on defining the upstream regulation and downstream functions of the PI3K-mTOR network.

No overlap.

***A competing renewal application was submitted for this award on 11/01/2021**

NIH/NCI P01 CA120964 (PI: Kwiatkowski; Project leader: Manning) 08/01/2018 – 07/31/2023

**Molecular Pathogenesis of the Hamartoma Syndromes**. **Project 1 (Manning and Perrimon): Identifying new therapeutic avenues to selectively target tumors with uncontrolled mTORC1 activation.**

DC/YR, 1.2 calendar months

National Cancer Institute

BG 9609 MSC 9760, 9609 Medical Center Drive, Bethesda, MD 20892-9760

Grants Management Specialist: Rogers Gross

This project uses unbiased genomic, proteomic, and genetic approaches to reveal new components, connections, and targets within the TSC-Rheb signaling network. The co-project leaders are focused on identifying novel therapeutic strategies and biomarkers by merging high-throughput *Drosophila* studies with mechanistic biochemical and cell biological studies in mammalian systems.

No overlap.

## PENDING SUPPORT

NIH R21 (PI: Manning) 04/01/2022 – 03/31/2024

**Neurodevelopmental Function of TBC1D7: A Core Component of the TSC Complex**

DC/YR, 0.6 calendar months
National Institutes of Health
Defects in the control of neuronal growth underlie a myriad of human neurological disorders, including epilepsy, autism, brain overgrowth, neurocognitive deficits, and neuropsychiatric disorders. The protein TBC1D7 is a key component of growth control pathways that has been found to be defective in neurological disorders, but its function is unknown. This study will use a newly established genetic model to define the function of TBC1D7 as it relates to brain development and neuronal growth.
No Overlap.

**What other organizations were involved as partners?**

Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**
   **COLLABORATIVE AWARDS:**
   **QUAD CHARTS:**

9. **APPENDICES:**