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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Using the genetically tractable fission yeast as a model, we sought to exploit recent advances in gene interaction biology to identify novel drug targets for use in the fight against tuberous sclerosis. Our ongoing study has identified two genes, *fft3* (a SMARCAD1 family ATP-dependent DNA helicase) and *ypa1* (protein phosphatase type 2A regulator) as excellent candidates for continued analysis. While deletion of either gene has little phenotypic effect in normal cells, their loss in either *tsc1* or *tsc2* gene deletion mutants profoundly inhibits growth. Significantly, we show that a subset of *tsc2* mutants (bearing mutations orthologous to those found clinically) also display synthetic lethal interactions with *fft3* and *ypa1*. Furthermore, we show that the loss of *fft3* ATPase activity, through the creation of an *fft3-K418R* mutant, is sufficient to confer a synthetic growth defect. These data suggest that inhibition of either *ypa1* or *fft3* (through targeting the ATPase domain) may represent an "Achilles' heel" of cells defective in hamartin or tuberin function.

15. SUBJECT TERMS
Tuberous Sclerosis, Genetic Buffering, Fission Yeast, Recombinase-Mediated Cassette Exchange, protein phosphatase type 2A regulator, ATP-dependent DNA Helicase

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1. INTRODUCTION

Tuberous sclerosis complex (TSC) is an inherited disorder characterized by the appearance of benign tumors in the brain, heart, lung, kidney, eyes, and/or skin. The disease affects as many as 40,000 individuals in the United States and is caused by mutations in one of two genes, *TSC1* (encoding a protein called hamartin), and *TSC2* (encoding a protein called tuberin). While research of the past two decades has provided a wealth of information regarding the genetic origins of TSC, the complexity of the molecular pathways involved, together with their intricate temporal and spatial interactions, has made it difficult to elucidate the full molecular pathology of the disease. Fortunately, recent technical and conceptual advances with respect to the importance of "genetic buffering" on phenotypic variation have provided novel avenues of exploration regarding this goal.

Using the genetically tractable fission yeast as a model, our USAMRAA funded research has sought to exploit recent advances in genetic interaction network biology to analyze previously uncharacterized genes that modulate the phenotypic effects of hamartin and tuberin loss of function mutations. Of particular interest are genes displaying negative genetic interactions with either *tsc1* or *tsc2*. Since tumor formation in TSC patients arises from loss of heterozygosity, this characteristic identifies the human orthologs of these genes as potential therapeutic targets i.e. drugs inhibiting a negative interactor would presumably suppress only the growth of tumor cells (which bear two mutant copies of the affected TSC gene: the inherited mutant germline copy, and the copy affected by the "second-hit") while leaving phenotypically normal cells (carrying only the mutant germline copy) unaffected.

Our research has clearly identified two genes, *fft3* (encoding a SMARCAD1 family ATP-dependent DNA helicase) and *ypa1* (encoding a PTPA family protein phosphatase regulator) as excellent candidates for continued analysis. While deletion of either gene has little phenotypic effect in normal cells, their loss in either *tsc1* or *tsc2* gene deletion backgrounds profoundly inhibits growth. Significantly, we show that a subset of *tsc2* mutants (bearing mutations orthologous to those found clinically) also display synthetic lethal interactions with *fft3* and *ypa1*. Furthermore, we show that the loss of *fft3* ATPase activity, through the creation of an *fft3-K418R* mutant, is sufficient to confer a synthetic growth defect. These data suggest that inhibition of either *ypa1* or *fft3* (through targeting the ATPase domain) may represent an "Achilles' heel" of cells defective in hamartin or tuberin function.

In addition to our bench-top analyses, we have also developed a formal mathematical methodology for the quantitation of genetic buffering strength (this was necessitated by the need for a system that could be used to unambiguously compare genetic interaction data). This methodology provides a simple, general, and rigorous mathematical paradigm with which to exactly quantitate the buffering strength of any genetic determinant. The methodology was published in the peer-reviewed "*The Mathematica Journal*" and is freely available to the public at "www.mathematica-journal.com/2015/03/the-quantitation-of-non-classical-buffering/". In the past year we have further developed these concepts into a comprehensive framework for classifying and quantifying gene interactions in general. We plan to submit this work, entitled "A formal and general method for classifying, quantifying, and comparing gene interactions" to the journal *Theoretical Biology and Medical Modelling* in the near future.

2. KEYWORDS

Tuberous Sclerosis, Genetic Buffering, Fission Yeast, Recombinase-Mediated Cassette Exchange, PTPA family protein phosphatase regulator, ATP-dependent DNA Helicase

3. ACCOMPLISHMENTS

Our research accomplishments are described below in the context of the Specific Aims outlined in the approved Statement of Work (SOW).

SPECIFIC AIM 1: Establish an *S. pombe* model of TSC by replacing the endogenous *S. pombe* hamartin and tuberin genes with human *TSC1* and *TSC2*, respectively.

Task 1: Create tsc1::ura4$^+$ and tsc2::ura4$^+$ "base" strains.

Predicted Completion Date: August 31st, 2014

Progress: Gene deletion cassettes for both the *tsc1* and *tsc2* genes were created by PCR amplifying the *ura4*$^+$ selectable marker (together with the *loxP* and *loxM3* recombination sites) from the pAW1 vector using primers oTsc11 (5'-tta tca atg ctg cca aga ctt gct atc agt ata atg tcg cat agt tgt ata tca acg ttg act ttg cca act ttg tac gac gga tcc ccg ggt taa tta a-3'), and oTsc12 (5'-aat tat ttt ata tgg aat gag caa gta tgt ttt atc ata att gac cag ttc att tca agg acc ttc aaa aat ata cct acg aat tcg agc tcg ttt aaa c-3'), or oTsc13 (5'-tta aga gtt cag att tgc ttt atg tgg tta ttc tgc tga agg tcc taa ttt att gac gtt gaa aaa taa agg cca cat agc gga tcc ccg ggt taa tta a-3'), and oTsc14 (5'-ata aaa aaa att aat taa tga tgg caa ggc aca atc gta atc aat ctt tta att tag gac ttt tta tat gcc ctt atg gcg aat tcg agc tcg ttt aaa c-3'), respectively. Strain ED666 (*ura4-D18 leu1-32 ade6-210 h*$^+$) was then transformed with either the *tsc1* specific or *tsc2* specific cassette. Ura$^+$ transformants were isolated and the respective gene deletions confirmed by colony PCR using primers oTsc15 (5'-atg tgg cag act acg cta tcc t-3') and oTsc17 (5'-atg ctt ccc cta att cat agc a-3') for the *tsc1* deletion, and oTsc16 (5'-agc aac cta cga gag gaa gat g-3') and oTsc18 (5'-gcg cat aac cct ttc tac att c-3') for the *tsc2* deletion.

Status: Complete.

Task 2: Obtain full length cDNA clones of human *TSC1* (Accession BC167824) and *TSC2* (Accession BC150300) from ThermoScientific. PCR amplification.

Predicted Completion Date: August 31st, 2014

Progress: Recent advances in DNA synthesis technology altered our strategy with respect to the original SOW document (see Section 5: Problems/Changes). Instead of purchasing cDNA clones and using standard amplification/cloning techniques, it proved more economical (in terms of both time and money) to synthesize the desired sequences. Full length clones were thus obtained through Genscript's DNA synthesis service. All

constructs were confirmed by DNA sequencing.

Status: Complete.

Task 3: Molecular cloning of *TSC1* and *TSC2* into "exchange" plasmids.

Predicted Completion Date: September 30th, 2014

Progress: As part of the service provided by Genscript, it was possible to have the synthetic sequences cloned directly into the pAW8X "exchange" plasmids upon synthesis.

Status: Complete.

Task 4: Transform exchange plasmids into *S. pombe* strain ED666. Select for clones that have exchanged the *ura4*$^+$ cassette with *TSC1*/*TSC2*. Verify genotypes by colony PCR.

Predicted Completion Date: October 31st, 2014

Progress: The *tsc1::ura4/tsc2::ura4* base strains were transformed with the pAW8X-TSC1/pAW8X-TSC2 vectors containing the human *TSC1* and *TSC2* genes. As controls, the base strains were also transformed with the pAW8X-tsc1 and pAW8X-tsc2 vectors containing the fission yeast *tsc1* and *tsc2* genes. Cells in which the *ura4*$^+$ gene was exchanged with *TSC1*/*TSC2*/*tsc1*/*tsc2* were selected by growth on media containing 5-fluoroorotic acid (a drug counter selectable to Ura$^+$ cells). This created strains in which human *TSC1* or *TSC2*, or fission yeast *tsc1* or *tsc2*, were expressed from the endogenous fission yeast *tsc1/tsc2* promoters at the native *tsc1/tsc2* loci. These strains have been denoted as *tsc1::TSC1*^{Hs}, *tsc2::TSC2*^{Hs}, *tsc1::tsc1*^{Sp}, and *tsc2::tsc2*^{Sp}. Strains bearing both substitutions were then created by crossing *tsc1::TSC1*^{Hs} and *tsc2::TSC2*^{Hs} strains of opposite mating type and screening progeny by colony PCR to identify *tsc1::TSC1*^{Hs} *tsc2::TSC2*^{Hs} cells. A complete catalog of strains created during the course of this work appears in Section 6.5.1, Research Material.

Status: Complete.

Task 5: Complementation Assays.

Predicted Completion Date: December 31st, 2014

Progress: The ability of human *TSC1/TSC2* to complement the loss of the fission yeast *tsc1/tsc2* genes was determined by assaying the growth of wild-type, *tsc1Δ*, *tsc2Δ*, *tsc1::TSC1*^{Hs}, *tsc2::TSC2*^{Hs}, *tsc1::TSC1*^{Hs} *tsc2::TSC2*^{Hs}, *tsc1::tsc1*^{Sp}, and *tsc2::tsc2*^{Sp} strains on minimal media containing 60 μg/ml of canavanine. As expected *tsc1Δ* and

tsc2Δ base strains, unlike wild-type controls, were resistant to the drug and were able to form colonies. Also as expected, *tsc1::tsc1*^{Sp} and *tsc2::tsc2*^{Sp} strains behaved as wild type and were unable to form colonies (indicating that the recombinase mediated cassette exchange methodology correctly integrated the synthetic constructs). Unfortunately, *tsc1::TSC1*^{Hs}, *tsc2::TSC2*^{Hs}, as well as *tsc1::TSC1*^{Hs} *tsc2::TSC2*^{Hs} strains were able to form colonies (indicating that the expression of the human orthologs was incapable of complementing the loss of the fission yeast *tsc1* and *tsc2* genes).

Status: Complete.

SPECIFIC AIM 2: Create "*TSC* mutant allele" array.

Task 1: Construction of TSC mutant alleles via direct synthesis or site-directed mutagenesis techniques.

Predicted Completion Date: February 28th, 2015

Progress: As discussed above, due to the lack of complementation, the remainder of the project will be conducted using the fission yeast *tsc1* and *tsc2* genes. To this end, site-directed mutagenesis was employed to incorporate a total of five mutations into the *tsc2* gene. Each of these mutations (G296E, R927W, N1199S, P1223L, R1296P) affects residues analogous to mutations identified clinically. New England Biolab's Q5 Site-Directed Mutagenesis Kit was used to generate pUC57-tsc2_G296E, pUC57-tsc2_R927W, pUC57_N1199S, pUC57_P1223L, and pUC57_R1296P plasmids. DNA sequence analysis confirmed the incorporation of the desired changes.

Status: Complete

Task 2: Molecular cloning of mutant alleles into exchange plasmids.

Predicted Completion Date: March 31st, 2015

Progress: As of June 30th, 2016, all of the clones (pUC57-tsc2_G296E, pUC57-tsc2_R927W, pUC57_N1199S, pUC57-tsc2_P1223L, and pUC57-tsc2_R1296P) have been cloned into the pAW8X exchange plasmids using the *XhoI* and *SacI* restriction sites and standard molecular techniques.

Status: Complete

Task 3: Transform exchange plasmids into S. pombe strain ED666. Select for clones that have exchanged the *ura4*$^{+}$ cassette with TSC1/TSC2. Verify genotypes by

colony PCR.

Predicted Completion Date: April 31st, 2015

Progress: Recombinase mediated cassette exchange was used to integrate the respective mutant *tsc2* alleles into the tsc2::ura4 base strain. Integration has been verified by colony PCR.

Status: Complete

Task 4: Confirm lack of complementation.

Predicted Completion Date: June 30th, 2015

Progress: Complementation assays based on canavanine resistance were performed as described in Specific Aim 1, Task 5. As expected the tsc2 point mutation strains were able to grow on media containing canavanine indicating that they indeed represented true loss of function mutants.

Status: Complete.

SPECIFIC AIM 3: Intercross strains of the TSC mutant allele panel with strains of the "putative interactor" panel.

Task 1: Intercross "TSC mutant allele" array with "interactor" panel.

Predicted Completion Date: October 31st, 2015

Progress: Control experiments in which the fission yeast interactor panel was crossed to either the *tsc1Δ* or *tsc2Δ* gene deletion mutants have been completed. These control experiments were crucial in that they provided a proper baseline of comparison for future assays conducted with the TSC mutant allele panels. We have also now crossed all five *tsc2* point mutants to both the *ypa1Δ* and *fft3Δ* strains (see below).

Status: Complete

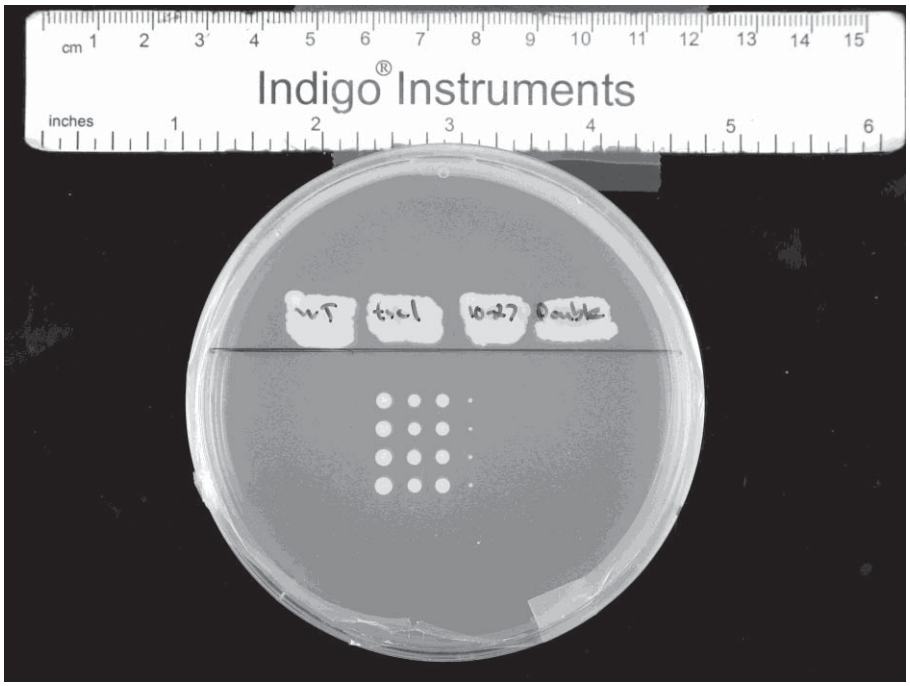
Task 2: Isolation of double mutant progeny. Growth Assays.

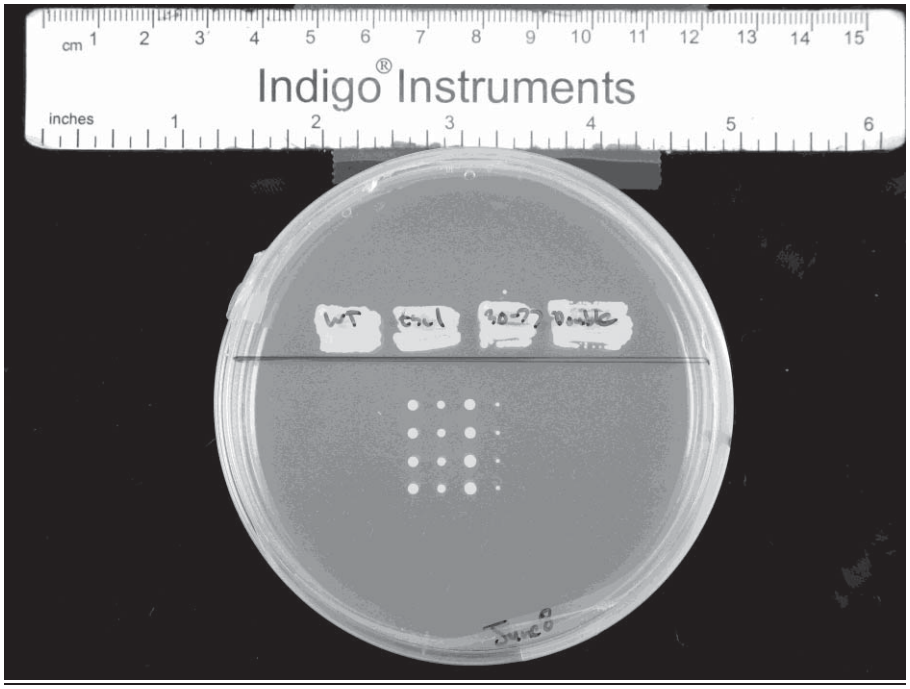
Predicted Completion Date: October 31st, 2015

Progress: Control assays (in triplicate) comparing the growth of wild type, *tsc1Δ*, *tsc2Δ*, the interactor panel mutants, as well as the respective double mutants have been completed. Briefly, a micromanipulator was used to array single cells representing each genotype (in

quadruplicate) upon YES agar plates. Plates were incubated at 30°C for five days. Digital images were taken at 24 hour intervals. These experiments identified *fft3* (a SMARCAD1 family ATP-dependent DNA helicase) and *ypa1* (a PTPA family protein phosphatase) as strong negative interactors of both the *tsc1Δ* and *tsc2Δ* gene deletions (see Section 4.1, Impact). Representative images of plates at day 4 for one biological replicate are shown below (10-27 denotes the *ypa1Δ* mutant, and 30-77 denotes the *fft3Δ* mutant).

Growth assays using the *tsc2* mutant allele panels have also been performed. Somewhat unexpectedly, we found that only one of the five alleles (R1296P) displayed negative interactions (with both *fft3Δ*and with *ypa1Δ*). This indicates, at least in *S. pombe*, that interactions between *tsc2* and its interactors are allele specific. This, unfortunately, argues against the idea that identified interactors could be targeted to inhibit the growth of *tsc2* strains (bearing a wide range of incapacitating mutations) in general.



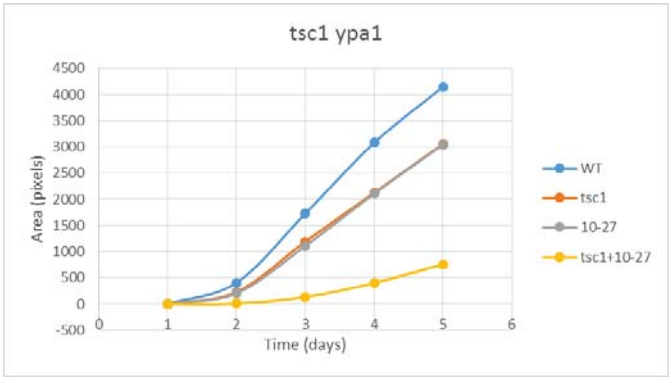


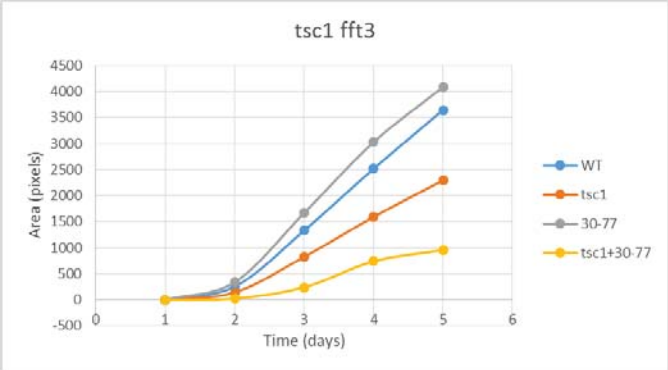
Status: Complete

Task 3: Image analysis of colony size.

Predicted Completion Date: December 31st, 2015

Progress: ImageJ (imagej.nih.gov/ij/) analysis tools were used to determine the colony size of wild type, *tsc1Δ*, *tsc2Δ*, each interactor panel mutant, and the respective double mutants (in quadruplicate) for each of three independent biological replicates. Representative data describing the interaction between *tsc1* and either *ypa1* or *fft3* is shown below. Similar analysis was performed with respect to the tsc2 mutant allele panel. As described above, only one of the five alleles (R1296P) displayed a growth defect in combination with either *fft3Δ* or *ypa1Δ*.





Status: Complete

SPECIFIC AIM 4: Physiological analysis of TSC modifiers

Task 1: Test effect of pharmacological inhibitors of "negative" interactors (if available) in strains expressing wild-type and mutant TSC alleles.

Predicted Completion Date: December 30th, 2016

Progress: These assays are currently underway.

Status: Ongoing.

Task 2: Molecular/physiological/genetic analysis of interactors. Specific analyses will be determined based on the molecular identity of the genes identified from the interactor panel.

Predicted Completion Date: June 30th, 2017

Progress: These assays will be performed upon completion of Specific Aims 4, Task 1.

Status: Pending Completion of Specific Aims 4, Task 1.

4. IMPACT

4.1 Impact on the Development of the Principal Disciplines of the Project

Our ongoing study has clearly identified *fft3* (a SMARCAD1 family ATP-dependent DNA helicase) and *ypa1* (a PTPA family protein phosphatase) as strong negative interactors of both the *tsc1Δ* and *tsc2Δ* gene deletions. While deletion of either gene has little phenotypic effect in normal cells, their loss in either *tsc1* or *tsc2* mutants profoundly inhibits growth. Thus, the chemical inhibition of the encoded Fft3 or Ypa1 proteins (both of which have clear orthologs in humans) may represent a novel means with

which to specifically inhibit the growth of cells defective in hamartin or tuberin function. In other words, the inhibition of either Fft3 or Ypa1 may represent an "Achilles' heel" of cells diminished in *tsc1* or *tsc2* activity. It is also important to note that neither of these genes have been identified using traditional genetic analysis. Thus, their continued study represents a novel avenue of exploration with regards to the molecular pathology of TSC.

Unfortunately, we also found that interactions between *tsc2* and *fft3Δ* and *ypa1Δ* are allele specific. This is disappointing in the sense that it argues against the idea that identified interactors could be targeted to inhibit the growth of *tsc2* strains (bearing a wide range of incapacitating mutations) in a general way. In other words, while true that *fft3* and *ypa1* could be targeted as an Achilles' heel, this will only be true for a subset of *tsc2* alleles.

4.2 Impact on other Disciplines

During the course of analyzing our genetic interaction data, it became apparent that there was no established method with which to formally and unambiguously quantitate the strength of "genetic buffering". To address this issue we developed a formal, axiomatic methodology – using the Wolfram Programming Language – to achieve this goal. The method is described in our recent publication "The Quantitation of Non-Classical Buffering: Applying the Formal and General Approach to Problems in the Biological Sciences (this article is freely available and can be downloaded at "www.mathematica-journal.com/2015/03/the-quantitation-of-non-classical-buffering/". While we will use this methodology to quantitate and compare the genetic interactions observed between the interactor panel and TSC mutant allele array, the methodology can also be applied to any scenario (and in any discipline) where a quantity partitions between two compartments or states.

In the past year we have further developed these concepts into a comprehensive framework for classifying and quantifying gene interactions in general. This is to say, we have created an axiomatic mathematical framework that can be used – provided a quantitative phenotypic measure exists – to classify, quantify, and compare buffering relationships between genes (using angular units of radians). We also extend this framework to unambiguously define and compare gene interactions in a general way. We plan to submit this work, entitled "A formal and general method for classifying, quantifying, and comparing gene interactions" to the journal Theoretical Biology and Medical Modelling in the near future.

4.3 Impact on Technology Transfer

Nothing to Report

4.4 Impact on Society Beyond Science and Technology

Nothing to Report.

5. CHANGES/PROBLEMS

5.1 Changes in Approach

5.1.1. Cloning of human *TSC1* and *TSC2*

Instead of purchasing cDNA clones of *TSC1* and *TSC2* and subsequently employing standard amplification and cloning techniques to create the exchange plasmids, we chose to use a commercial entity to synthesize the desired sequences. Recent advances in DNA synthesis technology have made such direct DNA synthesis approaches the most economical choice. Full length clones were thus obtained through Genscript's DNA synthesis service.

5.1.2. TSC mutant allele panel

Unfortunately, human *TSC1* or *TSC2* were unable to complement the loss of their fission yeast counterparts when expressed at the native *tsc1* or *tsc2* loci. While disappointing, this was an eventuality we previously considered. As discussed in the original project narrative (page 4, paragraph 1), the project will continue using the fission yeast hamartin/tuberin genes instead of their human orthologs. This is to say alleles of the fission yeast *tsc1* and *tsc2* genes – bearing mutations affecting residues analogous to those identified clinically – will be employed.

5.2 Actual or Anticipated Problems

5.2.1 Confounding Effect of Auxotrophic Markers

Due to the confounding effect of auxotrophic markers present in the interactor panel (derived from the Bioneer gene deletion set), it was necessary to construct an alternative panel devoid of secondary genetic alterations. This delayed the initiation of Specific Aims 2 and 3. While tedious and time consuming an alternative interactor panel, devoid of any background auxotrophic markers, has now been constructed and will be employed during the remainder of the study.

5.2.2 Personnel

The initial proposal identified graduate student, Bidhan Chakraborty, as associated with the project. Unfortunately, Mr. Chakraborty, upon successfully defending his MSc thesis, returned to Bangladesh to be married and ultimately decided to pursue other interests. While Mr. Chakraborty has now been replaced by Mr. Ryan Chevalier, his initial departure caused minor delays with respect to the completion of Specific Aim 2.

6. PRODUCTS

6.1 Publications, Conference Papers, and Presentations

6.1.1 *Journal Publications*

Karagiannis J. The Quantitation of Non-Classical Buffering: Applying the Formal and General Approach to Problems in the Biological Sciences. *The Mathematica Journal* **17**, 2015. (Published)

6.1.2 *Books or Other Non-Periodical, One-time Publications*

Nothing to Report

6.1.3 *Other Publications, Conference Papers, and Presentations*

Ashyad Rayhan, Adam Faller, Jim Karagiannis. Investigation of genetic mutants displaying synthetic lethality with *tsc1* and *tsc2* loss of function mutations in *Schizosaccharomyces pombe*. Northeast Regional Yeast Meeting, June 16-17, University at Buffalo, The State University of New York, Buffalo NY.

6.2 Websites or Other Internet Sites

Nothing to Report

6.3 Technologies or Techniques

Nothing to Report

6.4 Inventions, Patent Applications, and/or Licenses

Nothing to Report

6.5 Other Products

6.5.1 *Research Material (Fission Yeast Strains)*

Genotypes of the relevant fission yeast strains generated during the course of this study are listed below:

- *tsc1Δ* "base" strain (gene deletion with flanking *loxP* and *loxM3* sites)
- *tsc2Δ* "base" strain (gene deletion with flanking *loxP* and *loxM3* sites)
- *tsc1::tsc1*^{Sp}
- *tsc2::tsc2*^{Sp}
- *tsc1::TSC1*^{Hs}
- *tsc2::TSC2*^{Hs}
- *tsc1::TSC1*^{Hs}*tsc2::TSC2*^{Hs}
- *tsc2::tsc2*^{Sp}*-G296E*
- *tsc2::tsc2Sp-R927W*
- *tsc2::tsc2*^{Sp}*-N1199S*
- *tsc2::tsc2*^{Sp}*-P1223L*
- *tsc2::tsc2*^{Sp}*-R1296P*

7. PARTICIPANTS

Name:	Jim Karagiannis
Nearest Person Month Worked:	24
Contribution to Project:	Principal Investigator
Name:	Ryan Chevalier
Nearest Person Month Worked	4
Contribution to Project	Mr. Chevalier was involved in the routine growth and maintenance of fission yeast cultures, molecular cloning, and was also responsible for all image analysis of genetic interaction data.
Name:	Adam Faller
Nearest Person Month Worked	12
Contribution to Project	Created *tsc2* mutant allele panel.
Name:	Ashyad Rayhan
Nearest Person Month Worked	8
Contribution to Project	Testing the effects of pharmacological inhibitors of "negative" interactor in strains expressing mutant *tsc2* alleles

8. SPECIAL REPORTING REQUIREMENTS

Not Applicable