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Award Number: W81XWH-07-1-0358

TITLE: A search for New Therapeutic Targets: Using Yeast to Find the GEF for Rheb

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REPORT DATE: July 2008

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;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 13-07-2008		2. REPORT TYPE Final		3. DATES COVERED 14 JUN 2007 - 13 JUN 2008	
4. TITLE AND SUBTITLE A search for New Therapeutic Targets: Using Yeast to Find the GEF for Rheb				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-07-1-0358	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Janet Leatherwood, Ph.D. Email: jleatherwood@ms.cc.sunysb.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Research Foundation of SUNY Stony Brook, NY 11794-5222				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 The Tsc1/2 complex known as Hamartin/Tuberin is mutated in the human disease Tuberous Sclerosis and such mutation predisposes for cancer. Tsc1/2 complex has a clearly established chemical role as a GTPase Activating Protein or GAP for the small GTPase Rheb. Rheb in turn regulates TOR. The Tor kinases and associated proteins are large complex units that integrate signals pertaining to nutrients and proliferation potential. Tor promotes growth and proliferation and thus de-regulation of Tor is implicated in carcinogenesis and disease. We have worked toward development of a simple genetically tractable model system for understanding of the Tsc1/2 pathway. Our particular interest is in finding factors that work in opposition to Tsc1/2. Typical GTPases such as the Tsc1/2 target Rheb are controlled by both negative regulators (GAPS) and positive regulators known as guanine nucleotide exchange factors or GEFS. Our most important progress has been to establish functional screens for GEF type activators of the Rheb signalling factor in the simple yeast Schizosaccharomyces pombe.					
15. SUBJECT TERMS Rheb, GEF, Tor					
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
U	U	U	UU	6	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-5
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusion.....	6
References.....	6
Appendices.....	6

Introduction:

The Tsc1/2 (hamartin/tuberin) complex acts as a GTPase Activating Protein, or GAP, for the small GTPase Rheb. Rheb in turn regulates TOR (Target of Rapamycin), which promotes growth and proliferation. Small GTPases such as Rheb are typically controlled by two kinds of proteins: A GTPase Activating Protein (GAP), in this case Tsc1/Tsc2, that promotes GTP hydrolysis to GDP and a Guanine nucleotide Exchange Factor (GEF) that promotes exchange of this GDP for GTP. The GAP and GEF work in opposition, thus loss of Tsc1 or Tsc2 ordinarily allows hyperactivation of Rheb by the GEF, but this could be countered by inhibition of the GEF; that is, the hyperactivated Rheb could be deactivated by loss or inhibition of the GEF. This is a mode of therapy for Tuberous Sclerosis that has the potential to be highly effective and highly specific. However the GEF that controls Rheb has not yet been identified in any organism. Since the TSC1/2--Rheb--TOR pathway has been highly conserved in the fission yeast *Schizosaccharomyces pombe*, we will use this simple model system to most rapidly and efficiently identify the GEF for Rheb. Several genetic selections have been designed to identify factors that work in opposition to TSC1/2. While these screens are focused on discovery of the critical GEF, they will also uncover any additional novel pathways that oppose TSC1/2 function. This GEF and any novel regulators are highly promising targets for therapeutic intervention for treatment of Tuberous Sclerosis as well as a wide range of human cancers.

Body:

Task 1 – Isolate mutational suppressors of *tsc1/2* deletion mutants.

A complete deletion of the *tsc1* gene marked with *ura4* was obtained in *S. pombe* strain background *h- ura4-d18, leu1-32, his7-366, ade6-M210*. This deletion allele designated *tsc1::ura4* was confirmed as a stable integrant and then backcrossed to wild-type. We noted significant variations amongst progeny from this first backcross and so four independent isolates were again crossed to wild-type and the progeny analyzed. From this cross two independent isolates were selected for further study. Isolates GM-1 and GM-2 were screened for ability to grow on minimal medium (EMM-2) supplemented with 250mg/l each of Leucine, Histidine, and Adenine and for their ability to grow on a series of lower concentrations of supplement (100mg/l, 50mg/l, 30mg/l, 10mg/l, and 5mg/l). GM-1 grew well on 100mg/l, poorly on 50mg/l and showed no growth on 10 or 5 mg/l at 32C even after 10 days incubation. GM-2 displayed a similar profile except that papillae colonies appeared on 50 and 30 mg/l concentrations after 5 days at 32 C.

Both out of technical concern for the variability of phenotype and in an effort to better understand the nutritional responses of the *tsc1*-deletion mutant, we tested media varying nitrogen source(1) and titrating concentrations of supplement. Extensive analyses of different nitrogen sources led to three preferred nitrogen source substrates in order of reliability and growth being proline, glutamate, and phenylalanine.

Further tests of these nitrogen sources for effects on G1—S progression in wild-type and *tsc1*-deletion mutants are ongoing. Our working hypothesis is that at low phenylalanine levels, a switch in the MBF transcription factor from activation by Rep2 to Rep1 is achieved and this affects the balance of proliferation potential in the population. We do not know at this time to what extent Tsc1 participates in such a switch, though its suppression by *pas1* would be consistent with this hypothesis(3). We have been unable to identify GEF candidates amongst mis-regulated transcripts in the *tsc1*-deletion allele. While a GEF might reasonably be up-regulated under such conditions as part of a homeostatic mechanism, there is no strong prediction that such regulation should necessarily occur at the level of mRNA abundance.

Our original proposal for mutagenesis was to use EMS mutagen. During the course of this study, a new report was published as the first example of a feasible transposon tagging mutagenesis system for the fission yeast *S. pombe* (2). The advantage of transposon mutagenesis includes the

fact that mutagens are not involved in the procedure thus minimizing environmental hazards plus the fact that the resulting mutants can so much more easily be identified and cloned. The downside of course is that a wide range of mutants are difficult or impossible to obtain with a transposon mutagenesis approach and it is not possible to know in advance whether this will be the case.

We obtained the Hermes mutagenesis transposon from Dr. Henry Levin and re-engineered our mutant and control wild-type strains for transposon mutagenesis. To date we have failed to isolate transposon tagged mutational suppressors of *tsc1*-deletion in which the suppressor phenotype segregates cleanly 2:2 and is linked with the transposon insertion. Additional screening using this method is highly recommended because of the substantial benefits in later steps of mutant identification.

After substantial delays connected with re-training of a skilled neurobiology technician in elements of yeast genetics and molecular biology as well as substantial delays in strain constructions, media optimizations, and efforts with transposon tagging mutagenesis, we returned to the traditional EMS mutagenesis approaches. Both strains GM-1 and GM-2 were mutagenized using 5 EMS conditions to optimize survival and mutagenesis, the target goal being 80% survival given the fact that the first step is a screen not a selection. To date after mutagenesis of $>10^9$ cells of each genotype we have had difficulty isolating mutational suppressors that segregate 2:2; establishment of linkage groups is ongoing.

Task 2 – Isolation of high copy suppressors of Tsc1/Tsc2 overexpression

Task 2 was hampered by the need to train the technician in basic molecular biology methods. Creating over-expression strains took far longer than it should have in experienced hands. In retrospect, the strain construction would have been speeded by purchasing assistance from the on-campus cloning facility rather than spending the time in technician training. Because of these delays, pilot screens for high copy suppressors are at the point of initiation. The technician in charge of this project is no longer employed in my lab.

Task 3 – Identify GEF among suppressors

Suppressors are still in the stage of isolation and have yet to be characterized in depth. Nevertheless, the *tsc1*-deletion, over-expresser, and *tor1,2* alleles are now incorporated in pathway analyses for effects on meiotic entry. This represents a new research direction for my lab. While this extends beyond the time-line of the current grant, it is to be expected that substantial progress will be made in this area. The meiotic focus is the subject for a program project grant to be submitted to NIH in September 2008.

Key Research Accomplishments:

Exploration of Hermes Transposon in functional genetic screen in *S. pombe*

EMS mutagenesis and resulting isolation of *tsc1*-deletion suppressors

Incorporation of tuberous sclerosis pathway in ongoing analyses of meiotic regulation of *S. pombe*

Reportable Outcomes:

Funding will be applied for from NIH for program project grant, September 25, 2008.

Conclusions: The importance of this research is that it establishes working systems for obtaining mutants affecting potentially high value therapeutic targets for cancer therapy.

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3. **van Slegtenhorst, M., A. Mustafa, and E. P. Henske.** 2005. Pas1, a G1 cyclin, regulates amino acid uptake and rescues a delay in G1 arrest in Tsc1 and Tsc2 mutants in *Schizosaccharomyces pombe*. *Hum Mol Genet* **14**:2851-8.

Appendices: none