AWARD NUMBER: W81XWH-17-1-0314

TITLE: Toward Novel Therapeutics for TSC and LAM: Using Mechanisms of a Bacterial Protein to Sensitize Cells to Rapamycin

PRINCIPAL INVESTIGATOR: Marcia B. Goldberg, M.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Cambridge, MA 02139

REPORT DATE: July 2018

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2018	Annual	1 Jul 2017 – 30 Jun 2018

4. TITLE AND SUBTITLE

Toward Novel Therapeutics for TSC and LAM: Using Mechanisms of a Bacterial Protein to Sensitize Cells to Rapamycin

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0314

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

Marcia B. Goldberg, M.D.

E-Mail: marcia.goldberg@mgh.harvard.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital
Boston, Massachusetts 02114-2554

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

This project is based on our discovery that a bacterial protein (*Shigella* OspB) sensitizes mammalian cells to rapamycin-mediated inhibition of mTORC1 and that this mechanism of sensitizing cells to rapamycin appears to be conserved across divergent branches of life. The most significant finding of the current funding period is that the arginine N-end rule pathway and the inositol phosphate hexakisphosphate are required for OspB function. We isolated or generated anew, and confirmed, *Saccharomyces cerevisiae* strains with deletions in each of the genes that encode the first three steps of the arginine N-end rule pathway (*nta1, ate1*, and *rad6*), in the gene that encodes the enzyme that synthesizes hexakisphosphate (*ipk1*), and in genes that encode the enzyme that convert hexakisphosphate to pyrophosphate molecules (*vip1* and *ksc1*). We found that each of the genes that encode the first three steps of the arginine N-end rule pathway and *ipk1* are required for OspB function, but that *vip1* and *ksc1* are not. In follow-up to these results, we will test the role of these pathways in the function of OspB in mammalian cells. These findings provide new insight into OspB function.

15. SUBJECT TERMS
rapamycin, signaling, TSC, LAM, OspB

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	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION

This funded project is based on our discovery that a bacterial protein (*Shigella* OspB) sensitizes mammalian cells to rapamycin-mediated inhibition of mTORC1 and that this mechanism of sensitizing cells to rapamycin appears to be conserved across divergent branches of life. Sensitization of cells to rapamycin-mediated inhibition of mTORC1 is an effect on rapamycin activity that to our knowledge has not been described previously. Our overall objectives are to uncover the mechanism of this activity of OspB and to define the extent to which this mechanism improves specific inhibition of mTORC1 in TSC and LAM cells.

2. KEYWORDS

rapamycin
signaling
TSC (tuberous sclerosis)
LAM (lymphangioleiomyomatosis)
OspB

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goals and objectives for the project:

1. To identify genes and pathways required for OspB-induced sensitivity to rapamycin in *S. cerevisiae* and investigate in mammalian cells the role of pathways identified by this approach;

2. To characterize the protease activity of OspB; and

3. To identify potential mammalian substrates of OspB protease activity.

4. Determine the extent to which OspB sensitizes TSC cells to rapamycin.

5. Test whether OspB exposure of TSC cells in the presence of rapamycin results in inhibition of mTORC2, by quantifying Akt Ser-473 phosphorylation, including at prolonged times.

6. Test whether factors shown in Aim 1 to be required for OspB-induced sensitization of cells are potential therapeutic targets in TSC cells by testing whether depletion of factors shown in Aim 1 to be required for OspB-induced sensitization of cells alters sensitization of TSC cells to rapamycin.

The project's major goals and objectives for this reporting period are the following:

1. To identify genes and pathways required for OspB-induced sensitivity to rapamycin in *S. cerevisiae* and investigate in mammalian cells the role of pathways identified by this approach. [50% complete.]

2. To characterize the protease activity of OspB. [75% complete.]

3. To begin to identify potential mammalian substrates of OspB protease activity. [60% complete.]

What was accomplished under these goals?

1. Major activities:

1.A. (Corresponds to sub-aim 1.A.) Identify genes and pathways required for OspB-induced sensitivity to rapamycin in *S. cerevisiae* and investigate in mammalian cells the role of pathways identified by this approach.

Table 1. Host pathways potentially required for OspB activity, based on hits in yeast deletion library and overexpression library screens.

Biological pathways	Screen hits	
	Deletion library	Overexpression library[a]
Arg N-end rule	*nta1, ate1, rad6, ubr1*	*Bre1*
Amino acid biosynthesis and transport	*gcn4, gln3, bul1, npr1, stp1, sak1, gal83, avt5*	*Dal81*
Inositol phosphate biosynthesis	*ipk1, vip1*	*Ddp1*[b]
TORC1 pathway	*fpr1, tip41*	

[a] Assignment of *Ddp1, Bre1,* and *Dal81* to biological pathways is based exclusively on our postulates of how each one may suppress OspB inhibition of yeast growth (see text for details).

Genes that when deleted rescue OspB suppression of growth in *S. cerevisiae* in the presence of rapamycin (and/or in the presence of the surrogate caffeine) may encode proteins that are required for OspB function. We completed analysis of a screen on rich media of an ordered array of ~5,000 yeast haploid gene-deletion mutants (YKOv2, http://www-sequence.stanford.edu/group/yeast_deletion_project/ykov2.html), including nearly all non-essential genes, for those deletions that suppressed OspB-induced inhibition of growth in the presence of 6 mM caffeine. 85 deletions were associated with suppression of OspB-induced growth inhibition; these mapped to several conserved cellular pathways (Table 1). Of note, among the hits were each of the first four genes in the arginine (Arg) N-end rule pathway (Fig. 1), two downstream genes in the inositol phosphate pathway (Fig. 2), and two genes in the TORC1 pathway. The presence of multiple hits within single pathways suggest that these pathways are important for the phenotype and potentially for OspB function.

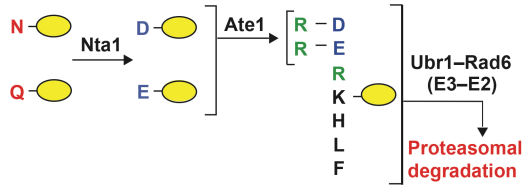

Fig. 1. Arg N-end rule pathway.

We already showed that mTORC1 is required for OspB activity. During the reporting period, we validated the findings of the screen for the Arg N-end rule pathway and the inositol phosphate pathway. The Arg N-end rule pathway targets for degradation cytosolic proteins (N-degrons) that have Asn or Gln at the N-terminus (Fig. 1). We speculate that a potential mechanism for OspB is that its protease activity mediates the cleavage of a host protein that normally inhibits TORC1, and OspB processing of the inhibitory protein targets it to the Arg N-end rule pathway, leading to its degradation and relieving TORC1 inhibition. We plan to test this.

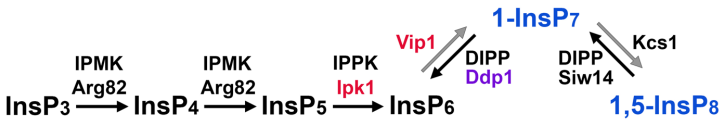

Fig. 2. Inositol phosphate biosynthetic pathway. Red, yeast deletion screen hits; purple, yeast overexpression screen hit; blue; inositol pyrophosphate species predicted to be required for OspB activity. Yeast enzymes below; mammalian enzymes above and in all caps.

The inositol phosphate $InsP_6$ is required for activation of the *Vibrio cholerae* RtxA cysteine protease domain, which is highly structurally similar to OspB, as well as closely related toxins, such as TcdA and TcdB, that are also highly structurally similar to OspB.

Validation of the Arg N-end rule pathway: We isolated and confirmed by PCR mutants in each of the four gene in the Arg N-end rule pathway. For each, we retested the growth phenotype, verifying that each gene deletion rescued *S. cerevisiae* growth in the presence of OspB and caffeine (Fig. 3). We also directly demonstrated that each gene deletion rescued *S. cerevisiae* growth in the presence of

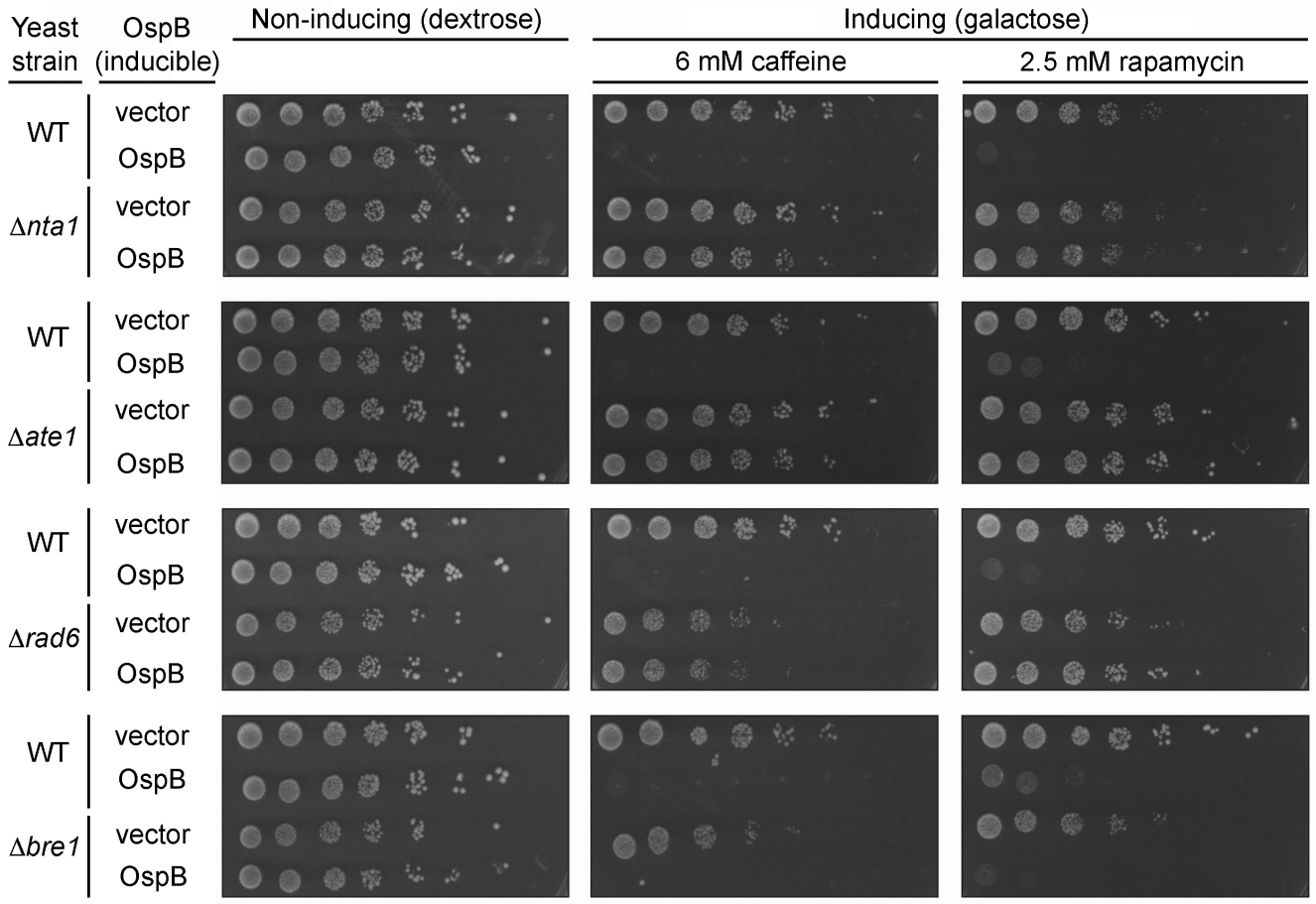

Fig. 3. Plates showing growth of serial dilutions of designated yeast strains, expressing or not expressing OspB or vector alone, in the presence or absence of caffeine or rapamycin. Deletion of each of the genes that encode the first three steps of the Arg N-end rule pathway (*nta1, ate1*, and *rad6*) suppresses OspB inhibition of yeast growth in the presence of rapamycin or caffeine. *Bre1* was not a hit in the deletion screen, but rather was a hit in the overexpression screen (see below); deletion of *bre1* does not suppress OspB inhibition of yeast growth in the presence of rapamycin or caffeine.

OspB and rapamycin (Fig. 3). These results show that in *S. cerevisiae*, the Arg N-end rule pathway is required for OspB function and that the OspB phenotype in the presence of rapamycin is generally reproduced in the presence of caffeine (N.B., caffeine is technically easier to use).

Validation of the inositol phosphate pathway: In addition to identifying *ipk1* in the deletion screen, we found that overexpression of Ddp1 rescued the OspB growth phenotype (see below). Based on these findings, we postulated that rather than utilizing $InsP_6$ as a co-factor, OspB might use the inositol pyrophosphate 1-$InsP_7$ and/or 1,5-$InsP_8$. To test this, we generated new clean mutants (verified by PCR) in genes in the inositol phosphate pathway, including *ipk1* and *vip1*. For each, we tested whether the gene deletion rescued OspB suppression of growth in the presence of caffeine and/or rapamycin. Deletion of *ipk1* rescued the OspB phenotype in the presence of rapamycin, and validating the screen, deletion of *ipk1* rescued the OspB phenotype in the presence of caffeine (Fig. 4). Deletion of *vip1* did not rescue the OspB phenotype under these growth conditions, indicating that $InsP_6$ is a co-factor for OspB function and that the inositol pyrophosphates 1-$InsP_7$ and 1,5-$InsP_8$ are not. As we predicted, deletion of *ksc1* also did not rescue the OspB phenotype. The demonstration that, like the RtxA, TcdA, and TcdB cysteine protease domains, OspB requires $InsP_6$ as a co-factor strongly supports our model that OspB is a cysteine protease.

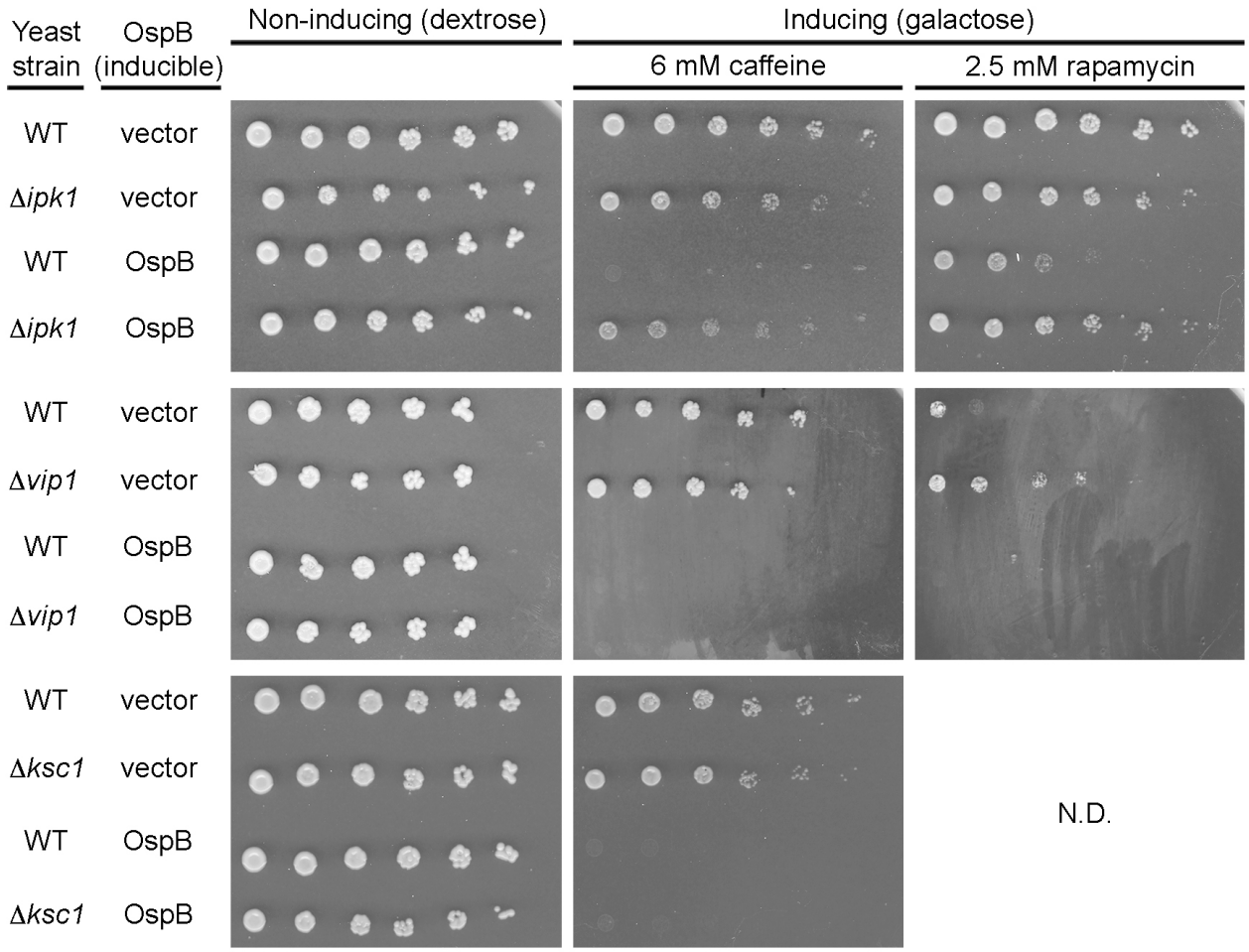

Fig. 4. Plates showing growth of serial dilutions of designated yeast strains, expressing or not expressing OspB or vector alone, in the presence or absence of caffeine or rapamycin. Deletion of *ipk1*, but not *vip1* or *ksc1*, suppresses OspB inhibition of yeast growth in the presence of rapamycin or caffeine. N.D., not done.

We obtained tools for testing the role of the Arg N-end rule pathway in mammalian cells. We established a collaboration with Alexander Varshavsky and have obtained from him murine fibroblasts that are null for Ate1. We are preparing to test whether, as in *S. cerevisiae*, Ate1 is required for the OspB phenotype.

1.B. Characterize the protease activity of OspB. (Corresponds to sub-aim 1.B.)

1.B.1. (Corresponds to sub-aim 1.B.1.) We previously showed that the sensitization of *S. cerevisiae* to OspB depended on OspB protease activity, as mutation of the putative catalytic residue (C184S) was associated with loss of the phenotype. Here, using MEFs, we tested and demonstrated that sensitization of mammalian cells to rapamycin is dependent on the protease activity of OspB (Fig. 5).

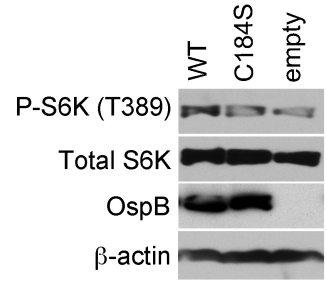

Fig. 5. OspB C184S is defective in activation of mTORC1. Transfection of MEFs with WT OspB or OspB C184S. Phosphorylation of mTORC1 substrate S6K.

1.B.2. (Corresponds to sub-aim 1.B.2.) We tested activity-based probes that recognize OspB-like cysteine proteases (such as RtxA, TcdA, and TcdB) also recognize OspB. We obtained from our collaborator Matt

Bogyo two types of probes: (i) The probe AWP19 contains a Ser-Leu dipeptide that mimics the recognition site of the endogenous substrates of RtxA, TcdA, and TcdB, an acyloxymethyl ketone-reactive electrophile that covalently links to the enzymatic active site. The probe mimics the substrate recognition site. (ii) A library of acyloxymethyl ketone-reactive electrophile probes that contained distinct amino acids in the P1 position of the mimic of the substrate recognition site (the position immediately N terminal to the cleavage site). We obtained 12 of the 20 possible permutations (only these 12 were available). We performed a series of a assays to test whether any of these probes bound OspB upon its expression in *S. cerevisiae*. We chose *S. cerevisiae* rather than mammalian cells because OspB is produced at higher levels in *S. cerevisiae* than in mammalian cells; we used conditions that reproduced those in which we observed an OspB phenotype that is dependent on the putative active site cysteine. As a positive control, we used the *C. difficile* toxin TcdB, purified from *E. coli*, which reacted with AWY19 (not shown) and the leucine P1 library probe (Fig. 6, left panel). As a negative control, we used OspB C184S. We optimized partial purification of OspB from yeast grown under conditions in which OspB is active and used partially purified OspB on beads. We tested the AWP19 probe, which is cell-permeable, in intact yeast expressing OspB. The P1 library probes are cell-impermeable, so we tested these on permeabilized, but intact yeast expressing OspB. We added or did not add exogenous purified $InsP_6$ or inositol pyrophosphates. We tested the P1 library probes individually and in pools. We tested the probes in the presence of levamisole, which inhibits phosphatases, particularly alkaline phosphatases, or in the presence of mild reducing agents, as TcdB must be reduced to maintain activity. Under no condition did we see specific binding of probe to WT OspB (a representative experiment is shown in Fig. 6). Possible explanations for these results include (i) the P1 position of the OspB substrate recognition site contains one of the eight residues that were absent in the library we screened, (ii) some factor in *S. cerevisiae* interferes with acyloxymethyl ketone-reactive electrophile interaction with OspB, or (iii) OspB is not a cysteine protease, although given the preponderance of data, it seems likely OspB is a cysteine protease. We plan to test AWP19 and the P1 probe library in a mammalian system and to take additional approaches to examining OspB protease activity (see below).

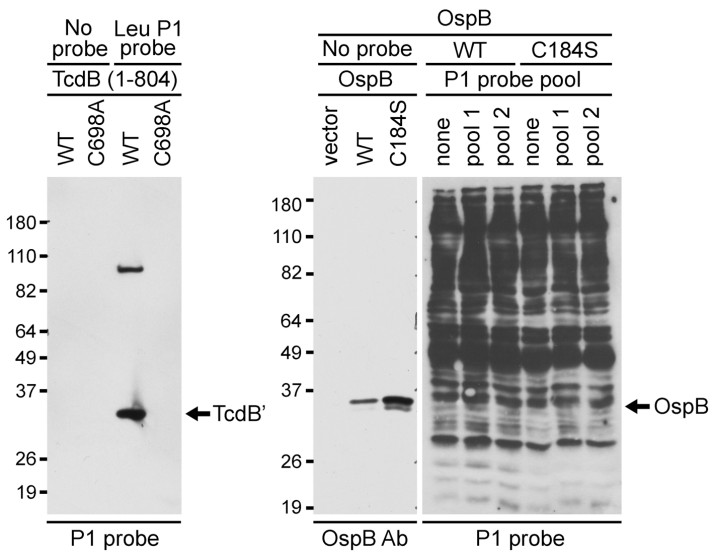

Fig. 6. Representative example of assay of cysteine protease activity-based probes looking for cysteine protease activity of OspB. *S. cerevisiae* lysates containing WT OspB, OspB C184S, or vector alone. Left panel, probe recognition of active TcdB' (aa 1-804), but not of catalytic mutant of TcdB' (C698A) as controls. Middle panel, Presence of OspB in lysates. Right panel, probe of lysates containing OpsB with no probe or two pools or the P1 activity-based probe library split into two pools (pool 1 and pool 2); no specific band was detected for WT OspB with either probe pool.

1.C. Begin to identify potential mammalian substrates of OspB protease activity. (Corresponds to sub-aim 1.C.)

We reasoned that overexpression of an OspB substrate under conditions in which OspB suppresses growth in *S. cerevisiae* might rescue the suppression of growth, and that any substrate identified in *S. cerevisiae* might facilitate the identification of a mammalian substrate. Thus, to potentially identify an OspB substrate, using an ordered array, we screened OspB-expressing *S. cerevisiae* on 6 mM caffeine in the presence of each of 5280 *S. cerevisiae* gene overexpression plasmids (>80% coverage of the genome). This screen yielded three hits (Bre1, Dal81, and Ddp1), each of which is functionally associated with one of the pathways identified in the deletion library screen (Table 1);

their identification supports the relevance of each of these pathways in OspB function in this experimental system. None of the proteins encoded by these three genes showed processing in the presence of OspB (by western blot, not shown), suggesting that none is a substrate of OspB protease activity.

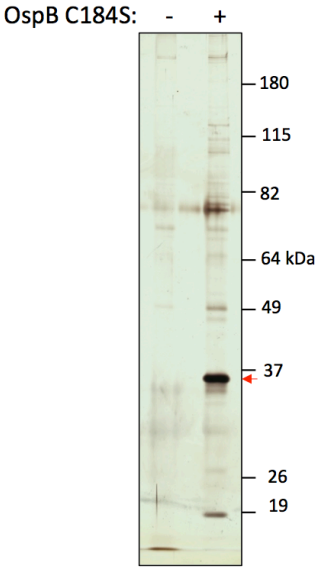

Fig. 7. Proteins that co-purify with OspB C184S from lysates of *S. cerevisiae*. OspB C184S was purified from the lysates via two-step purification using a 3xFLAG-6XHis tag. Precipitated proteins stained with silver. Red arrow, OspB C184S.

1.C.1. (Corresponds to sub-aim 1.C.1.) We identified proteins that appear to interact with OspB by performing two-step purification of OspB from *ospB*-expressing yeast grown under conditions where OspB is active and using mass spectrometry to identify co-precipitated proteins, with as a control, proteins pulled down by beads alone. We used the catalytic mutant of OspB, rather than WT OspB, because of its potential to "trap" substrates at the active site, as done successfully for other proteases. Quantitative mass spectrometry analysis of co-purifying proteins identified several proteins substantially enriched in the presence of OspB (Fig. 7), all of which have human homologs. Of particular interest is Cdc48, a segregase that removes ubiquitinated proteins from complexes and has roles in multiple cell processes, including the N-end rule pathway. Cdc48 also complexes with three proteins encoded by genes identified in our screen for suppressors of OspB-induced caffeine sensitivity: Ufd3, Ubp3, and Bre5. This further supports the hypothesis that degradation of a proteolytic substrate(s) of OspB or other host protein(s) is required for OspB function.

Also co-precipitating with OspB was Emp47, a mannose-binding lectin that is localized to intracellular vesicles. The human homolog of Emp47 binds to the lysosomal membrane protein RagC, an activator of mTORC1; active mTORC1 is localized to lysosomes. This suggests that OspB could modulate TOR function through Emp47.

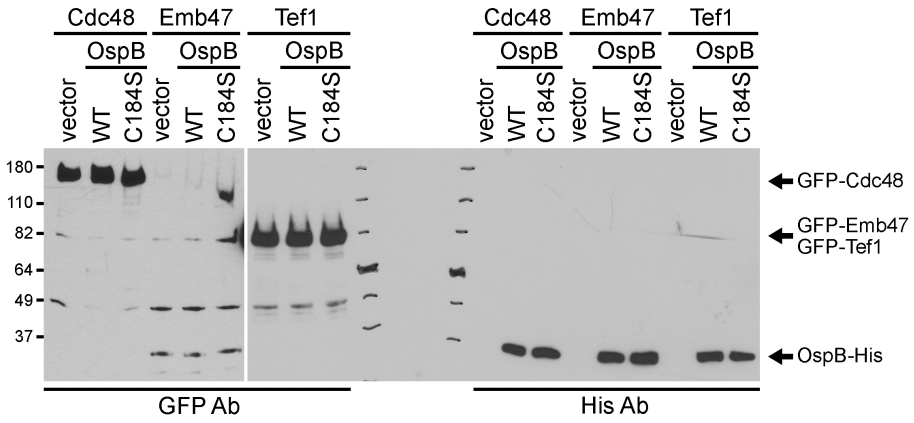

Fig. 8. Absence of evident cleavage of Cdc48 or Emp47 by OspB. No change in gel migration of GFP-Cdc48 or GFP-Emp47 in lysates from cells that produce these proteins as well as WT OspB, OspB C184S, or vector.

1.C.2. (Corresponds to sub-aim 1.C.2.) We tested whether Cdc48 and/or Emp47 is a substrate of OspB by assessing whether GFP fusions to these proteins demonstrated cleavage in the presence of OspB, using yeast Tef1 (homolog of human elongation factor 1-alpha), an abundant protein that frequently non-specifically co-purifies in pull downs and which was enriched in the OspB pull down. We observed no evidence of OspB-specific reduction in protein levels of OspB-specific cleavage products (Fig. 8), indicating that it is unlikely Cdc48 or Emp47 is a substrate of OspB protease activity.

Although we postulated that OspB was not itself a substrate of its protease activity, we completed a series of experiments that directly tested this. This was particularly important because the bacterial homologs of OspB (the cysteine protease domains of RtxA, TcdA, and TcdB) cleave between domains of larger precursor RtxA, TcdA, and TcdB multi-domain molecules. To test whether OspB cleaves itself, we examined whether under cellular conditions in which OspB is active, there is any

evidence of OspB processing or degradation compared to conditions in which it is inactive. Using OspB derivatives tagged at either the N- or C-terminus, we observed no evidence of OspB processing or instability (Fig. 9). These findings provide strong evidence that OspB is not a substrate of itself and support that the OspB substrate(s) is a host protein.

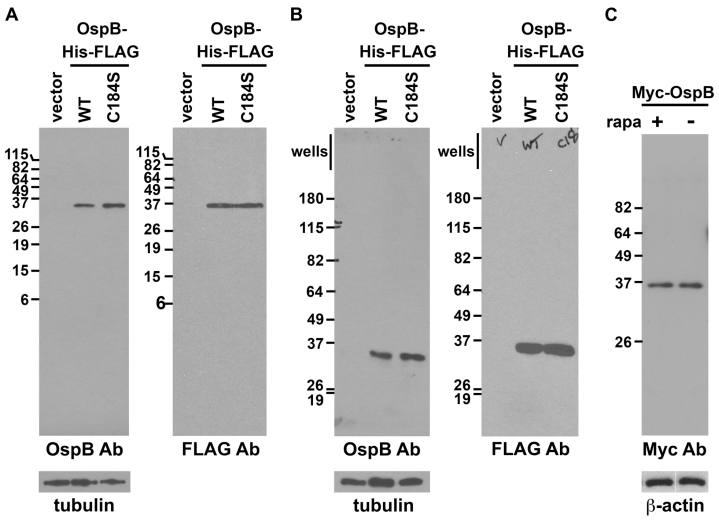

Fig. 9. Lack of evidence of auto-processing of OspB in cell lysates. A, C-terminally tagged WT OspB and OspB C184S produced in *S. cerevisiae*. 15% SDS-PAGE. B, Same as A, but 7.5% SDS-PAGE. C, N-terminally tagged WT OspB produced in MEFs ± rapamycin. 10% SDS-PAGE. OspB Ab recognizes 14-mer peptide of OspB 18 residues from the C-terminus. Beta-actin bands are from one blot.

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

The project provided instructive training for MyungSeo Yoon, an undergraduate student at Harvard University who worked on this project part-time in the laboratory, under my supervision and with one-on-one mentoring from Heather Eshleman and Thomas Wood. The project provided training for Austin Hachey, a research technician in my laboratory who worked briefly on the project, with one-on-one mentoring from me; Mr. Hachey has since enrolled in a PhD graduate program in Chemistry. The project provided training and professional development for Heather Eshleman, PhD, a postdoctoral fellow in the laboratory, with one-on-one mentoring from me. Dr. Eshleman has left the laboratory to accept a research position as a Science Analyst at Lexical Intelligence. The project is providing training and professional development for Jeffrey Duncan, a research technologist in the laboratory, with one-on-one mentoring from me; Mr. Duncan is currently applying to MD PhD programs. Lastly, the project is providing training and professional development to Thomas Wood, PhD, a new postdoctoral fellow in the laboratory, with one-on-one mentoring from me; Dr. Wood is focusing on this project for his postdoctoral research, taking over where Dr. Eshleman left off. Dr. Eshleman, Mr. Hachey, Mr. Duncan, and Dr. Wood attended numerous seminars in the field through the Harvard Medical School Department of Microbiology and Immunobiology weekly invited seminar series throughout the 2017-2018 academic year and at the Harvard Medical School Department of Microbiology and Immunobiology annual retreat. In addition, Mr. Duncan and Dr. Wood attended the Boston Bacterial Meeting in June 2018.

How were the results disseminated to communities of interest?

Nothing to report.

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

In the next reporting period, we will carry out the approaches as outlined in the Statement of Work for the 13-24-month timeline. These include determining the extent to which OspB sensitizes TSC cells to rapamycin (Major Task 4), testing whether OspB exposure of TSC cells in the presence of rapamycin results in inhibition of mTORC2, by quantifying Akt Ser-473 phosphorylation, including at prolonged times (Major Task 5), and testing whether factors shown in Aim 1 to be required for OspB-induced sensitization of cells are potential therapeutic targets in TSC cells by testing whether depletion of factors shown in Aim 1 to be required for OspB-induced sensitization of cells alters sensitization of TSC cells to rapamycin (Major Task 6).

In addition, because the approaches carried out so far have not led to the identification of a substrate(s) of OspB, we will also perform N-TAILS on proteins that co-precipitate with OspB (Major Task 3, Subtask 4).

4. IMPACT

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

Our accomplishments to date indicate that OspB is a cysteine protease that, like the cysteine protease domains of RtxA, TcdA, and TcdB, depends on the inositol phosphate $InsP_6$ for function. Although we are not yet able to mechanistically link this function of OspB to its ability to sensitize cells to rapamycin inhibition of mTORC1, these findings support our postulate that as yet undiscovered mechanisms of modulation of mTORC1 exist that could potentially be manipulated for the benefit of individuals receiving rapamycin or rapamycin-like therapeutics for the treatment of TSC and LAM.

What was the impact on other disciplines?

Our accomplishments to date validate the importance of the Arg N-end rule in the function of OspB. To our knowledge, the Arg N-end rule has not previously been mechanistically linked to modulation of the mTORC1 pathway. Insights into the linkage of these two pathways has the potential to impact our understanding not only of mTORC1, but also of eukaryotic ubiquitination and proteasome degradation processes.

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

There were no significant changes in objectives or scope.

To the methods for the identification of potential substrate(s) of OspB activity (sub-aim 1.C), we added the *S. cerevisiae* genetic approach of screening for overexpression suppressors of the OspB phenotype. Our reasoning was that overexpression of an OspB substrate might rescue OspB-dependent suppression of growth, and therefore, that this approach might lead to the identification of a substrate(s) of OspB. Although the approach did not lead to the identification of a substrate(s) of OspB, it was informative, as it generated data that complemented our identification of cellular pathways required for OspB function.

A minor change in approach was that in sub-aim 1.C.1, instead of precipitating OspB-interacting proteins from MEFs that stably express OspB and OspB C184S, we precipitated OspB-interacting proteins from *S. cerevisiae* that stably express OspB C184S. The reasons for this change in approach were (i) after passage, our MEFs that stably express OspB lose certain OspB phenotypes, raising concern that OspB-interacting partners in these cells may not represent true OspB-interacting partners, whereas *S. cerevisiae* that stably express OspB or OspB C184S do not lose the OspB phenotype; and (ii) we found that we were able to consistently precipitate OspB from *S. cerevisiae* that stably express it, whereas transient transfection of MEFs with OspB is not sufficiently efficient to precipitate OspB in the quantities that would be required for mass spectrometry. We chose to use

OspB C184S as the bait because of its potential to “trap” substrates at the active site, as done successfully for other proteases.

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

We found that after passage, our MEFs that stably express OspB lose certain OspB phenotypes, raising concern that their use to identify OspB-interacting partners may not identify true OspB-interacting partners. As described above (“Changes in approach and reasons for change”), we therefore used *S. cerevisiae* that stably express OspB for the identification of OspB-interacting partners.

There was a month and a half gap between when Dr. Eshleman left the laboratory and Dr. Wood arrived; during this time, progress on the project was slowed.

Changes that had a significant impact on expenditures

None.

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

Not applicable.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use or care of biohazards and/or select agents:

None.

6. PRODUCTS

Publications, conferences, and presentations

Journal publications. Nothing to report.

Books or other non-periodical, one-time publications. Nothing to report.

Other publications, conference papers, and presentations. Dr. Eshleman presented findings from this work in oral talks in two local settings: (i) the Harvard Medical School Department of Microbiology and Immunobiology work-in-progress meeting; and (ii) the annual Harvard Infectious Diseases training program retreat.

Website(s) or other Internet site(s). None.

Technologies or techniques. We have not developed any new technologies or techniques. The techniques we used are standard and are described above (“Accomplishments”). All of the techniques we use will be shared through peer-reviewed publication of the findings.

Inventions, patent applications, and/or licenses. None.

Other products. None.

PARTICIPANTS

What individuals have worked on the project?

Name:	Marcia B. Goldberg, M.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID)	0000-0003-4266-9733
Nearest person month worked:	1
Contribution to project:	Supervised all aspects of the project.
Funding support:	

Name:	Cammie F. Lesser, M.D., Ph.D.
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID)	0000-0002-1702-6333
Nearest person month worked:	0.1
Contribution to project:	Provided advise on all genetic approaches in *S. cerevisiae*.
Funding support:	

Name:	Heather Eshleman, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID)	N/A
Nearest person month worked:	10
Contribution to project:	Conducted all experiments relating to activity-based probes, identification of OspB-interacting proteins, testing whether OspB-interacting proteins are OspB substrates, and testing whether OspB is

	autoproteolyzed. Supervised MyungSeo Yoon in carrying out the overexpression genetic screen. Also supervised Austin Hachey, and Jeffrey Duncan.
Funding support:	T32 AI007061-39 and F32 AI131582-01A1

Name:	Thomas Wood, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID)	N/A
Nearest person month worked:	2
Contribution to project:	Conducted experiments that validated that the Arg-N-end rule pathway genes are required for OspB suppression of growth of *S. cerevisiae* in the presence of rapamycin or caffeine. Supervised MyungSeo Yoon in testing genes in the inositol phosphate pathway.
Funding support:	

Name:	Jeffrey Duncan
Project Role:	Research technologist
Researcher Identifier (e.g. ORCID ID)	N/A
Nearest person month worked:	11
Contribution to project:	Conducted experiments that showed that the catalytic site mutant of OspB is defective in activation of mTORC1 in mammalian cells. Maintained cell lines, maintained stocks, and ordered reagents for all experiments.
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?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS. N/A.

9. APPENDICES. None.