AD_____

Award Number: W81XWH-13-1-0060

TITLE:
Targeting Amino Acid-mTORC1 Signaling Limb for TSC Suppression

PRINCIPAL INVESTIGATOR:
Do-Hyung Kim, Ph.D.

CONTRACTING ORGANIZATION:
University of Minnesota, Minneapolis, MN 55455-2070

REPORT DATE:
February 2014

TYPE OF REPORT:
Annual Report

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
February 2014	Annual Report	1 February 2013 – 31 January 2014

4. TITLE AND SUBTITLE
Targeting Amino Acid-mTORC1 Signaling Limb for TSC Suppression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0060

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Do-Hyung Kim, Ph.D.

E-Mail: dhkim@umn.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Minnesota

Minneapolis, MN 55455-2070

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 goal of this project is to define the role of SH3BP4 in TSC development and as a potential therapeutic target for TSC. This annual report provides a complete summary of the research accomplishments to date with respect to the approved statement of work. In the first year of the project, we completed the generation of SH3BP4 flox mice by gene targeting approach. The mice are the unique resource that can be broadly used in many areas of research. The TSC1 flox mice and nestin-Cre mice were also made available. The molecular mechanism studies have been on going to determine the underlying mechanism of SH3BP4 inhibition of Rag GTPases and the smaller fragments of SH3BP4 for the inhibitory function. We determined that NPF motifs are required for the interaction of SH3BP4 with Rag GTPases and mTORC1 inhibition. We identified AP2alpha as a binding protein of SH3BP4 and three candidate proteins. The interaction with other proteins may be important to better understand how SH3BP4 is localized and inhibits Rag GTPases. The brain tumor model system has been well established. We prepared constructs for the brain tumor study and established a quantitative assay method. Furthermore, the brain tissue analysis was conducted and revealed that SH3BP4 is expressed in different levels in different part of the brain, the information critical to develop tissue-specific deletion studies we proposed in specific aim 1.

15. SUBJECT TERMS
SH3BP4, Rag GTPases, TSC1, mTORC1, brain, flox mice

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

Targeting Amino Acid-mTORC1 Signaling Limb for TSC Suppression

INTRODUCTION: This goal of this project is to define the role of SH3BP4 in TSC development and as a potential therapeutic target for TSC. The central hypothesis of this project is that SH3BP4 gene deletion or its functional disruption will facilitate the development of TSC disease states, whereas activation of SH3BP4 functions will suppress TSC development. We predict that, once a cell has a reduced level of SH3BP4 activity, it may have a growth advantage over its neighbors by maintaining a higher activity of mTORC1 activity. In the first year of the project, we planned to develop mouse models to understand how SH3BP4 contributes to TSC development. We also planned to identify a minimum sized-fragment of SH3BP4 that inhibits mTORC1 signaling. We also planned to establish a new mouse model to study TSC tumor development in the brain. The first year has made the key progress for each planned experiment. The SH3BP4 flox mice, the key resource for the project, have been successfully generated. The delimination experiment for small-sized fragments of SH3BP4 has been conducted providing important insight into the mode of interaction between SH3BP4 and Rag GTPases. We established the brain tumor system with implementing a quantitative assay method to measure tumor mass. Furthermore, the brain tissue analysis showed that SH3BP4 is expressed at different levels in different part of the brain, the information critical to develop tissue-specific deletion studies we proposed in specific aim 1.

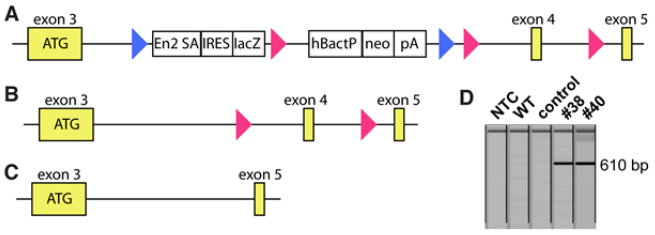

Figure 1. Construction of *SH3BP4^{loxP}* mice. (**A**) Introduction of flipped LacZ-neo cassette between exons 3 and 4, and loxP elements. Flip elements in blue; LoxP elements in red. (**B**) Deletion of flip-recombined deletion of the cassette to make the exon 4 floxed allele. (**C**) Deletion of exon 4 after Cre-mediated recombination. (**D**) PCR genotyping of mice #38 and #40 harboring the floxed allele after flip recombination.

BODY: *(1) Develop a mouse model to understand how SH3BP4 contributes to TSC development:* Following the broadly-used mouse genetic strategy and gene targeting strategy, we developed the SH3BP4 flox mouse system successfully. The targeting vector was designed as shown in Figure 1A, in which the exon 4 of SH3BP4 is floxed with loxP elements (Figure 1). If the lox-Cre recombination occurs when the flox mice are crossed with a Cre line, the loxP-flanked SH3BP4 allele will undergo deletion of the region between two loxP elements. The vector was introduced into mouse ES cells, germline transmission was confirmed, and flip elements were eliminated to make the floxed allele without flips. The deletion of the exon 4 results in the translation of the N-terminal 39 amino acids followed by off-frame 35 amino acids. SH3BP4 contains 963 amino acids in total. So, the floxed gene must completely disrupt the gene function. The mice harboring the target vector LacZ/neo cassettes are a SH3BP4 KO mice. We could obtain only het mice for the LacZ/neo cassette-harboring allele, indicating that deletion of both copies of SH3BP4 would be embryonic lethal. **The SH3BP4 flox mouse will be the first that we introduce to address the functions of amino acid-mTORC1 signaling in a broad aspect of physiological studies in mice.** Heterozygotes of SH3BP4 whole body KO and the mice harboring homo floxed alleles were viable and fertile and ready for our study. This mouse is ready for breeding with *Nestin-CreER^{T2}* line available from Jackson Lab. This line, combined with *Tsc1^{loxP}* mice, has already been demonstrated to recapitulate TSC disease states in mice. We have also tested our antibody specific to SH3BP4 that were generated using a peptide epitope in rabbits. The antibody recognized SH3BP4 in multiple brain tissues by western blotting (Figure 2). Interestingly, the expression of SH3BP4 was seen in all the brain tissues. In particular, the protein was very highly expressed in the brainstem region, the posterior part of the brain where the brain is adjoined with the spinal cord. It is known that the region is involved in motor and sensory innervation to the face and neck. This small region appears to be very important for nerve connections between the brain and the rest of the body, thus controlling

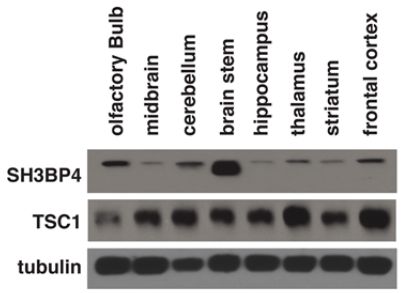

Figure 2. The expression pattern of SH3BP4 and TSC1 in different tissues of the brain. SH3BP4 is most highly expressed in brain stem.

peripheral motor neurons, fine touch, vibration sensation, and proprioception, pain, temperature, itch and crude touch, cardiac and respiratory function, and consciousness and sleep cycle, heart rate, breathing, sleeping and eating. The region has also been an area of intensive research of neurodegenerative diseases.

(2) Identify a minimum sized-fragment of SH3BP4 that inhibits mTORC1 signaling: We have generated multiple fragment constructs of SH3BP4 by PCR amplification. The fragments were expressed as myc-tagged constructs in HEK293T cells and their interaction with RagB and RagC were analyzed by myc-tag affinity chromatography. When smaller sized fragments were expressed, their expression levels were very low. We were able to express a fragment containing residues 59-139. Although the fragment contains the SH3 domain, the fragment did not interact with either RagB or RagC (Figure 3A and B). This result suggests that the SH3 domain alone might not be enough to stably interact with Rag GTPases. Given that the 1-300 fragment inhibits mTORC1 signaling as we have already shown in our proposal (Figure 3C), we think that the 300 amino acids have residues critical for mTORC1 signaling inhibition in addition to the SH3 domain. Interestingly, the 1-300 fragment was always expressed as double bands on SDS-PAGE (Figure 3A-D). We do not know yet why the fragment shows the doublet pattern. Perhaps, understanding the cause of the doublet pattern might be critical for its inhibitory effect on mTORC1 signaling. We will use the purified 1-300 fragment for its effect on Rag GTPase activity in vitro. The 1-300 fragment contains NPFs, which are putative Eps15 homology domain-binding sites. It is important to note that the 1-300 fragment contains critical residues for mTORC1 inhibition. Knowing that the 59-139 fragment does not bind to Rag GTPases, it is possible that the NPFs or Eps15 domain-containing protein(s) might play an important for the interaction with Rag GTPases and for inhibition of mTORC1 signaling. Since it must be important to understand the molecular basis of the inhibitory effect of the 1-300 fragment on mTORC1 signaling, we purified 1-300 fragment to test its effect on Rag GTPase activity in vitro. Knowing that the inhibitory effect on mTORC1 signaling depends on a broad region of SH3BP4, we considered a possibility that SH3BP4 binding proteins might also be important for mTORC1 signaling. We conducted immunoprecipitation of endogenous SH3BP4 and its binding proteins and analyzed the immunoprecipitates on SDS-PAGE. After the gel was silver stained, we detected a few bands specifically detected in SH3BP4 immunoprecipitates (Figure 4). We conducted mass spectrometry identification of the proteins, and one of the proteins turned out to be AP2alpga, a protein involved in vesicle trafficking. Since

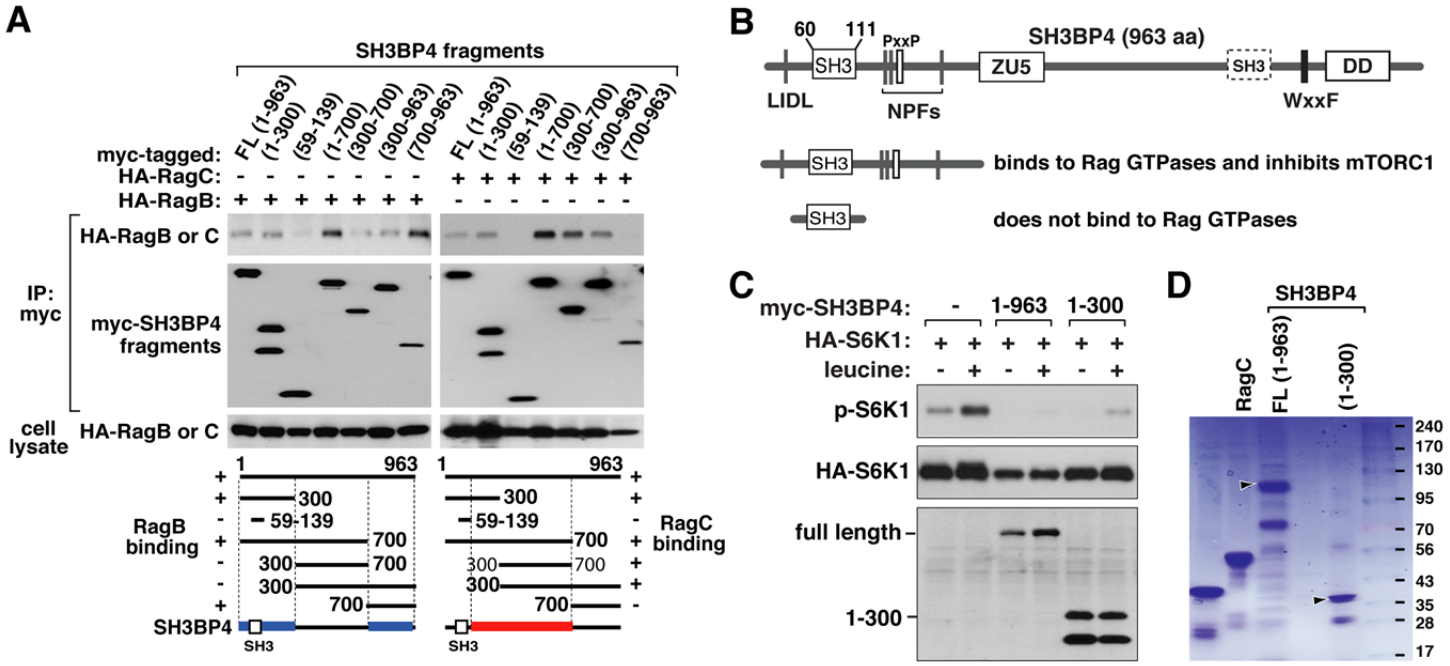

Figure 3. (A) The SH3BP4 fragment containing amino acids 59-139 is not capable of binding to Rag GTPases, indicating that NPF motifs might be important for the interaction. (B) The domain schematics of SH3BP4. SH3: Src homology 3; NPF: putative Eps15 homology domain-binding sites; PxxP: SH3 binding motif; WxxF: AP1 and AP2 binding motif; ZU5: putative binding site for DD; DD: death domain. (C) A blot we showed in our proposal. 1-300 fragment inhibits mTORC1 signaling. The 1-300 fragment always shows double bands on SDS-PAGE gels. (D) We purified recombinant SH3BP4 from bacteria to use for in vitro GTPase assay.

SH3BP4 contains a WxxF motif, AP2alpha may interact with SH3BP4. We have also identified three other candidate proteins that might interact with SH3BP4 also.

(3) Establish a new mouse model to study TSC tumor development in the brain: We have successfully established the brain tumor mouse experimental system in lab using C57BL6 mice, which allows for adding human genes to make humanized brain tumors. Since the vector-based delivery does not require using immune-deficiency mice, the delivery tools could be used to the BL6 mice. We have tested injection of well-established brain tumor models, and successfully implemented the assay condition and quantification method (Figure 5). We made a construct for SH3BP4 expression using a transposon-based vector to deliver the gene to neonatal mice. We also constructed two different shRNAs for SH3BP4.

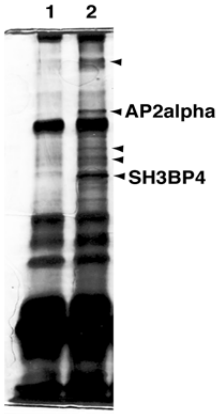

Figure 4. Identification of SH3BP4 binding proteins. Lane 1: SH3BP4 immunoprecipitation in the presence of epitope peptide; Lane 2: SH3BP immunoprecipitation in the absence of epitope peptide. AP2alpha was identified specifically only in lane 2, indicating that the protein might specifically interact with SH3BP4.

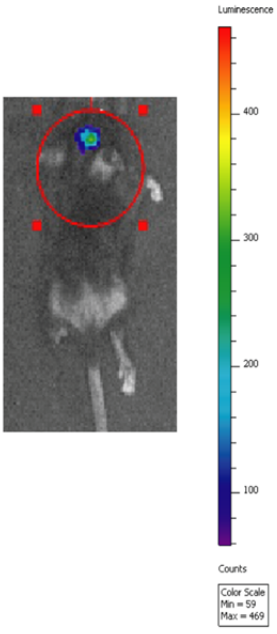

Figure 5. The brain tumor mouse model to study SH3BP4 role in tumor suppression or tumorigenesis. Two vectors that harbor sleeping-beauty (SB)-dependent transposable element (IR) combined luciferase gene were injected into the brain of neonatal mice. We first tested NRasG12V and shp53 transposons with SB100-luciferase to establish the quantitative assay protocol. The color bar is the scale we used to quantify the tumor volume.

KEY RESEARCH ACCOMPLISHMENTS IN THE FIRST YEAR

- We completed the generation of SH3BP4 flox mice by gene targeting approach and prepared them for breeding with TSC1 flox and Nestin-Cre mice. The mice are the unique resource that can be used broadly in many areas of research.
- We determined that the NPF motifs are required for the interaction of SH3BP4 with Rag GTPases and the inhibition of mTORC1 signaling.
- We identified AP2alpha as a binding protein of SH3BP4 and three other candidate binders, which will be important for us to better understand how SH3BP4 localization is regulated and how SH3BP4 inhibits Rag GTPases.
- We prepared constructs for the brain tumor mouse system and implemented a quantitative assay method based on the intensity of bioluminescence reflecting the volume of tumor mass.

REPORTABLE OUTCOMES: None

CONCLUSIONS: We have successfully completed the first year by generating the SH3BP4 flox mice and preparing TSC1 flox and Cre mice for breeding. Although we found some difficulty in identifying smaller fragments of SH3BP4, the outcome indeed provided important insight into the mode of interaction critical for the interaction between SH3BP4 and Rag GTPases. We found that NPF motifs are necessary for the interaction between SH3BP4 and Rag GTPases. The SH3BP4 binding proteins, such as AP2alpha, further indicates that the multiple domains of SH3BP4 involved in the protein-protein interaction may play a role in regulating the interaction between SH3BP4 and Rag GTPases. Characterization of the SH3BP4 binders and how they play a role in the regulation of SH3BP4 and its localization may be important for our comprehensive understanding of the nutrient-regulated pathway. Lastly, we have prepared constructs for the brain tumor mouse experiment for Aim 3 and implemented our control experiments successfully. This progress is significant in making us to achieve our overall goal. Collectively, the first year has allowed us to make the key resources and provided new insight into the interaction important for SH3BP4 inhibition of Rag GTPases. The newly-developed mouse model is critical for our study as well as for many other researches and can be broadly applicable to growth-related human diseases.