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TITLE: Loss of ZDHHC-Mediated Scribble Palmitoylation Disrupts Cell Polarity and Promotes Prostate Cancer Progression

PRINCIPAL INVESTIGATOR:
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CONTRACTING ORGANIZATION:
Massachusetts General Hospital

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13. SUPPLEMENTARY NOTES						
14. ABSTRACT Progression and metastasis of prostate cancers (PCs) are major therapeutic challenges, with the underlying mechanisms remaining unclear. The rise of AR-negative neuroendocrine prostate cancer (NEPC) cells is recently recognized as a major mechanism. The apical-basal polarity of epithelial cells plays critical roles in regulating normal cell migration and proliferation in prostate. Loss of cell polarity leads to tissue disorganization, uncontrolled proliferation and migration, hallmarks of prostate cancer progression and metastasis. ZDHHC7-mediated palmitoylation of Scribble is critical for cell polarity and metastasis. In the second year of the study, we found that loss of ZDHHC7 is significant in NEPC samples. We have generated ZDHHC7 knockout cell lines with various prostate cancer progression stages. We found that loss of ZDHHC7 led to loss of SCRIB palmitoylation, and activation of YAP signaling, and up-regulation of NEPC markers. We will further test the hypothesis that ZDHHC7 loss and SCRIB depalmitoylation contributes to prostate cancer cell progression through converting castration-resistant prostate cancer cells to NEPC phenotype.						
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1. INTRODUCTION:

Prostate cancer (PCa) is a commonly diagnosed cancer in American men. However, a majority of these cancers recur and develop resistance to treatments. The apical-basal polarity of epithelial cells plays critical roles in regulating epithelial cell functions, including differentiation, migration, proliferation, and apoptosis, and is essential for normal development and tissue homeostasis. Loss of cell polarity leads to tissue disorganization, uncontrolled proliferation, epithelial-to-mesenchymal transition (EMT), and migration, which are hallmarks of progression of PCa. SCRIB has been characterized as an essential regulator of cell polarity, tumorigenesis and metastasis. SCRIB is frequently amplified and overexpressed in multiple human cancers, including PCa. Amplified, but mislocalized SCRIB could function as an oncogenic factor. Therefore, the mechanism that regulates SCRIB membrane localization might be an important molecular switch, critical for PCa progression. We identified that ZDHHC7 is the major palmitoyl acyltransferase regulating SCRIB. Loss of ZDHHC7 decreases SCRIB palmitoylation and lead to its mislocalization, activation of the oncogenic YAP pathway, and cell invasion. The overall objective of this project is to define the roles of cell polarity regulator SCRIB in PCa cell progression, and how misregulation of SCRIB palmitoylation contributes to the disease. We hypothesized that: loss of cell polarity plays major roles in prostate cancer progression, and the signal transduction network involving ZDHHC7, SCRIB and the downstream YAP, MAPK or PI3K/AKT pathways promotes prostate cancer progression. ZDHHC7 functions as a potential tumor suppressor in PCa cells, and restricts the downstream oncogenic factors. Loss of ZDHHC7 in PCa promotes SCRIB mislocalization. We will elucidate the mechanisms of ZDHHC7-mediated SCRIB palmitoylation in regulating SCRIB mislocalization and cell polarity in prostate cancers, determine the roles of the ZDHHC7-mediated SCRIB palmitoylation in prostate cancer progression using preclinical *in vitro* and *in vivo* models, and evaluate their expression in primary specimens, and identify the regulator(s) of SCRIB de-palmitoylation in prostate cancer cells, and to validate it as new therapeutic target for prostate cancer therapeutics.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Cell polarity, palmitoylation, Scribble, ZDHHC7, prostate cancer, metastasis, neuroendocrine prostate cancer (NEPC)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The overall objective of this project is to define the roles of cell polarity regulator SCRIB in PCa cell progression, and how mis-regulation of SCRIB palmitoylation and loss of ZDHHC7 contributes to the progression of the disease. As shown below in the proposed SOW and our last annual report, we have elucidated the regulation of ZDHHC7 in Scribble palmitoylation, localization in prostate cancer cell lines. In the second year of funding, we have made new progresses to show that loss of ZDHHC7 in prostate epithelial cells and prostate cancer cells leads to loss of SCRIB palmitoylation, enhance YAP, p-MEK activation, and induce the expression of neuroendocrine prostate cancer (NEPC) markers. NEPCs are recently recognized

as progression from AR-resistant prostate cancers with strong invasive and metastatic potentials. We will further investigate with loss of ZDHHC7 lead to NEPC conversion and prostate cancer progression.

Based on these new observations, we have slightly revised the SOW to include new experiments to exam NEPC features in ZDHHC7 loss, and SCRIB amplified prostate cancer cells as shown in the SOW table below.

Aim 1. To elucidate the mechanisms of ZDHHC7-mediated SCRIB palmitoylation in regulating SCRIB mislocalization and cell polarity in prostate cancers	Timeline	Site 1	Site 2	% of completion	Actual complete dates
Major Task 1: Evaluate the palmitoylation levels of SCRIB in prostate cancer cells	Months				
Subtask 1: evaluate the palmitoylation levels of SCRIB in different prostate cancer cell lines	1-3	Dr. Wu		100	Nov.2017
Subtask 2: evaluate the localization of SCRIB in different prostate cancer cell lines	1-6	Dr. Wu	Dr. Yu	100	Jan. 2018
Major Task 2: Establish that loss of ZDHHC7 leads to loss of SCRIB palmitoylation and its mislocalization in prostate cancer cells.					
Subtask 1: evaluate the ZDHHC7 expression levels in prostate cancer cell lines by western blot	3-6		Dr. Yu	100	Aug. 2018
Subtask 2: generate ZDHHC7-stably knockdown or knockout cell lines using shRNA or CRISPR/Cas9-mediated knockout methods	6-10	Dr. Wu	Dr. Yu	100	April 2019
Subtask 3: evaluate the SCRIB palmitoylation levels in ZDHHC-deleted PC cells	9-12	Dr. Wu		100	May 2019
Subtask 4: evaluate the SCRIB localization status in prostate cancer cell lines at different stage and in ZDHHC-deleted cell lines	9-12	Dr. Wu	Dr. Yu	100	April 2019
Major Task 3: Determine that SCRIB mislocalization leads to loss of cell polarity in prostate cancer cells					
Subtask 1: examine the expression of cell junction markers in prostate cancers	12-15		Dr. Yu	50	
Subtask 2: examine cell junction markers in ZDHHC7-stably knockdown or knockout PC cells	12-15	Dr. Wu		50	PrEC cells established LnCap cells are still in selection ZDHHC7 KO leads to NEPC cell marker upregulation

Subtask 3: express SCRIB C4/10S mutant in prostate epithelial cell lines or benign cells and evaluate the cell polarity markers	15-18	Dr. Wu		50	Stable cell lines in selection
Aim2: To determine the roles of the ZDHHC7-mediated SCRIB palmitoylation in prostate cancer progression using preclinical in vitro and in vivo models, and evaluate their expression in primary specimens.					
Major Task 4: Evaluate the expression levels of ZDHHC7 and SCRIB in localized and metastasized prostate cancer specimens					
Subtask 1: perform IHC of SCRIB in PC samples	12-18		Dr. Yu	50	RNA analysis done Antibody validation Plan to test in NEPC cells
Subtask 2: Perform IHC of ZDHHC7 in PC samples	12-18		Dr. Yu	50	RNA analysis done Antibody validation
Major Task 5: Determine the tumor suppressor roles of ZDHHC7 in prostate cancer cell lines in vitro and in vivo					
Subtask1: will examine the effects of re-expression of ZDHHC7 in prostate cancer cell lines	18-24	Dr. Wu		80	Re-expression in PC3 done
Subtask2: test the tumorigenesis potential of these cell lines in vivo in tumor initiation (sub-cu xenograft model) and metastasis models	20-26		Dr. Yu	0	PC3 cells die upon ZDHH7 expression
Subtask 3: generate stable knockdown or knockout cell lines of ZDHHC7 in benign cell line; as well as androgen dependent cell lines	18-24	Dr. Wu	Dr. Yu		
Subtask 4: evaluate cell growth, colony formation, cell invasion in ZDHHC7-deleted cells	24-28	Dr. Wu		30	PrEC cells with ZDHHC7 KO tumor sphere done
Subtask 5: test ZDHHC7-deleted cells in vivo	24-30		Dr. Yu	0	Plan to test the

					hypothesis of NEPC conversion
Major Task 6: Determine the activation of downstream oncogenic pathways upon ZDHHC7 knockdown or expression of SCRIB palmitoylation deficient mutant, and the effects result in EMT, cell migration and metastasis in vitro and in vivo. (revised plan: also test NEPC markers)					
Subtask 1: generate prostate cancer cells (LNCAP, C4-2B, 22RV1 and PC3) with expression of SCRIB WT or C4/10S mutant	24-30	Dr. Wu		0	
Subtask 2: examine the downstream signaling activities of MAPK, AKT and YAP. Western blots of p-MEK, p-ERK, p-AKT, and p-YAP will be studies (will also include NEPC markers)	30-33	Dr. Wu		0	
Subtask 3: YAP nuclear localization and transcriptional activities will be evaluated by co-focal imaging or qRT-PCR of downstream target genes (CTGF, Cyr61 etc.) (will also test NEPC factors)	33-36	Dr. Wu		0	
Subtask 4: evaluate the effects of EMT, cell migration and metastasis in vitro and in vivo (also evaluate NEPC conversion in vivo)	30-36	Dr. Wu	Dr. Yu	0	
Aim3: To identify the regulator(s) of SCRIB de-palmitoylation in prostate cancer cells, and to validate it as new therapeutic target for prostate cancer therapeutics.					
Major Task 7: Identify the de-palmitoylating enzymes that regulate SCRIB in prostate cancer	Months				
Subtask 1: clone these genes into a retroviral vector, and carry out a gain of function screen for SCRIB depalmitoylation in HEK293 and C4-2B cells (recently published literature suggest APT2 is regulating SCRIB. Will validate in prostate cancers)	24-28	Dr. Wu	Dr. Yu	0	
Subtask 2: reconfirm their activities by co-IP and mutagenesis studies	28-30	Dr. Wu		0	
Subtask 3: examine whether overexpressing of the candidates will decrease the half-life of SCRIB palmitoylation	30-33	Dr. Wu		0	
Subtask 4: generate the shRNA constructs of the candidate depalmitoylating enzymes	30-33	Dr. Wu		0	
Major Task 8: Validate the effects of the de-palmitoylating enzymes in restoring SCRIB palmitoylation and blocking prostate cancer progression using shRNA knockdown or CRISPR/Cas9-mediated knockout.	Months				
Subtask 1: examine its expression levels in different stages of prostate cancer samples	28-32		Dr. Yu	0	

Subtask 2: analyze its expression at protein level in various prostate cancer cells	30-32		Dr. Yu	0	
Subtask 3: examine its expression in primary prostate cancer specimen using IHC.	30-33		Dr. Yu	0	
Subtask 4: generate lentiviral shRNA constructs for stable knockdown or tet-inducible shRNA constructs for inducible knock-down	28-32	Dr. Wu		0	
Subtask 5: evaluate whether it can inhibit EMT, cancer cell proliferation, cell migration and induces apoptosis in these cells. Synthesize tool inhibitors and test compound in vitro	32-36	Dr. Wu	Dr. Yu	0	
Subtask 6: carry out in vivo experiments to validate that knock down or knockout of the depalmitoylating enzyme could inhibit prostate cancer cell growth in vivo	32-36		Dr. Yu	0	

What was accomplished under these goals? As shown in the above SOW table, our work has been focused on Aim 1 and 2 in the last funding period. We have developed ZDHHC7 knockout methods using CRISPR/Cas9 system. We have generated stably ZDHHC7 KO cells using PrEC, DU145 and BPH cells. We found that loss of ZDHHC7 leads to SCRIB depalmitoylation in prostate cancer cell lines. More importantly, we identified that ZDHHC7 loss leads to conversion to neuroendocrine prostate cancer cells (NEPC), which was a novel and unexpected finding. NEPCs are developed from castration resistant prostate cancers with high metastasis and drug resistance. Our finding is highly important, showing that loss of ZDHHC7 might contribute to NEPC development, which is also consistent with our initial hypothesis.

Specific Aim 1. To elucidate the mechanisms of ZDHHC7-mediated SCRIB palmitoylation in regulating SCRIB mislocalization and cell polarity in prostate cancers

Major Task 2: Establish that loss of ZDHHC7 leads to loss of SCRIB palmitoylation and its mislocalization in prostate cancer cells.

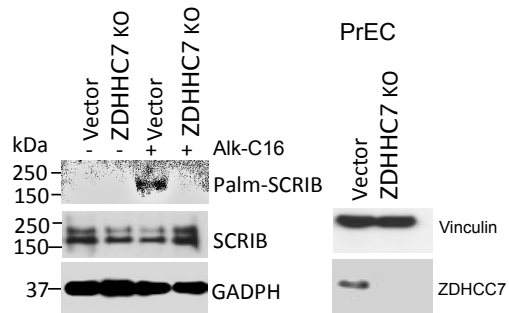


Figure 1. CRISPR/Cas9 mediated knockout (KO) of ZDHHC7 in PrEC cells leads to abolishment of SCRIB palmitoylation.

Subtask 2 : generate ZDHHC7-stably knockdown or knockout cell lines using shRNA or CRISPR/Cas9-mediated knockout methods

Subtask 3: evaluate the SCRIB palmitoylation levels in ZDHHC-deleted PC cells

Methods: We have used CRISPR/Cas9 system to generate stably ZDHHC7 knockout cell lines. Two guide-RNA sequences were used, and the knockout efficiency is evaluated by western blot (**subtask 2**).

We picked one clone with the best KO efficiency for follow up studies. To evaluate the levels of SCRIB palmitoylation (**subtask 3**), we used palmitoylation reporter (16-CYA and 18-CYA), which

metabolically label palmitoylated proteins in cells, followed by Click reaction with biotin azide, pull-down by streptavidin, and western blotting of Scribble antibody. We can detect the palmitoylation levels of endogenous Scribble in cells.

Results: As shown in **Figure 1**, in benign prostate epithelial cell line PrEC, we have successfully knocked out ZDHHC7. In addition, we have also knocked out ZDHHC7 in DU145 cell lines. Similarly, loss of ZDHHC7 led to loss of SCRIB palmitoylation.

Conclusion: This result is consistent with our hypothesis that loss of ZDHHC7 leads to loss of Scribble palmitoylation.

Subtask 4: evaluate the SCRIB localization status in prostate cancer cell lines at different stage and in ZDHHC-deleted cell lines

Methods: We have used anti-Scribble antibody to detect endogenous Scribble, and carried our confocal immunofluorescent staining. We detected the localization of Scribble in different prostate cancer cell lines.

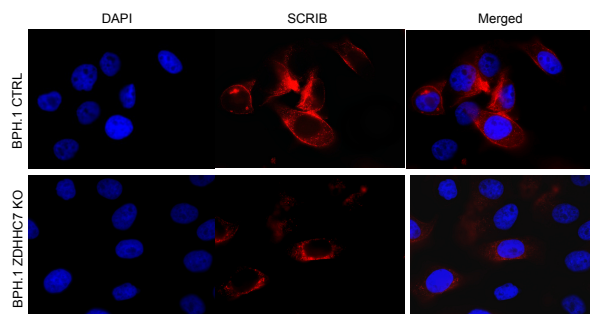


Figure 1. Immunofluorescent staining of Scribble in BPH1 cells with or without ZDHHC7 KO. Loss of Scribble membrane localization and loss of cell-cell junctional structures are observed in ZDHHC7 KO cells.

Results: As shown in **Figure 2**, we have successfully detected the localization of Scribble in cell lines with or without ZDHHC7 knockout. In benign BPH1 cells, Scribble is mainly localized at the cell-cell junction, consistent with its functions in maintaining cell polarity and epithelial cell structures. However, ZDHHC7 KO cell line, we have observed enhanced cytoplasmic localization of SCRIB.

Conclusion: Scribble is mislocalized in prostate cancer cells. Loss of cell-cell junctional structure and Scribble membrane localization is characteristic to the progression of prostate cancer cells.

Major Task 3: Determine that SCRIB mislocalization leads to loss of cell polarity in prostate cancer cells.

Subtask 2: examine cell junction markers in ZDHHC7-stably knockdown or knockout PC cells.

Methods: We have used western blotting to evaluate the levels various proteins involved in cancer progression and metastasis in ZDHHC7 KO cells. In addition, we have also evaluated the expression of neuroendocrine prostate cancer markers in ZDHHC7 KO cells using qRT-PCR.

Results: As shown in Figure 3a, we found that loss of ZDHHC7 lead to SCRIB mislocalization, and activation of p-AKT, p-MEK. YAP pathway is also activated, measured by qRT-PCR of YAP target genes (CTGF, ANKRD1, Cyr61) (Fig. 3b) and genes involved in metastasis (Fig. 3c). More importantly, we found that loss of ZDHHC7 leads to upregulation of N-Myc and other NEPC markers (Fig. 3d).

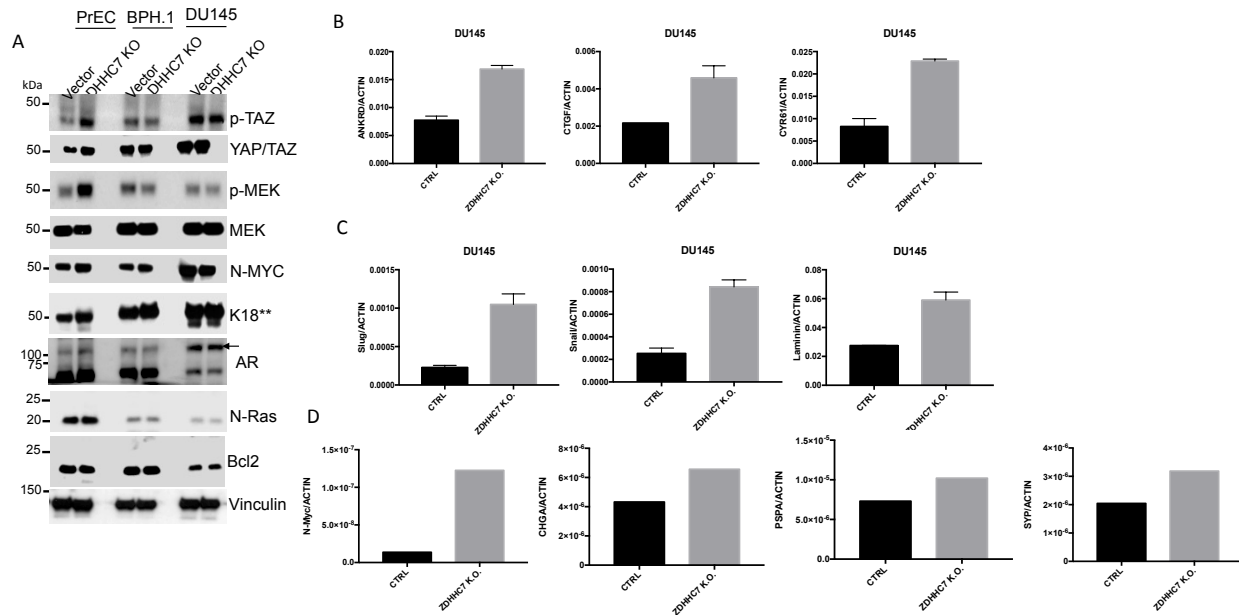


Figure 2. Effects of loss of ZDHHC7 in prostate cancer cell lines. (A). Western blot analysis of cell junction, MEK, and YAP pathway. (B). qRT-PCR showed that loss of ZDHHC7 led to upregulation of YAP target genes in DU145 cells. (C) Loss of ZDHHC7 led to upregulation of pro-metastasis genes (Slug, Snail and laminin) in DU145 cells. (D) Loss of ZDHHC7 led to upregulation of NEPC markers in PrEC cells.

Conclusion: Loss of ZDHHC7 leads to prostate cancer progression through activation of MEK and YAP pathways. In addition, loss of ZDHHC7 leads to conversion to NEPC phenotype.

Aim2: To determine the roles of the ZDHHC7-mediated SCRIB palmitoylation in prostate cancer progression using preclinical in vitro and in vivo models, and evaluate their expression in primary specimens.

Major Task 5: Determine the tumor suppressor roles of ZDHHC7 in prostate cancer cell lines in vitro and in vivo

Subtask1: will examine the effects of re-expression of ZDHHC7 in prostate cancer cell lines.

Methods: We have generated lentiviral vectors carrying wild type ZDHHC7 or the catalytically inactive mutant (C160S). We evaluated the SCRIB palmitoylation levels and the growth of these cells.

Results: As shown in Figure 4A, re-express of ZDHHC7 WT, but not the C160S mutant could significantly upregulate SCRIB palmitoylation level. Expression of ZDHHC7 WT, but not C160S mutant leads to significant cell death in PC3 NEPC cells (Fig 4B). In addition, WT ZDHHC7 also blocks DU145 cell growth, while the catalytically inactive C160S mutant is inactive (Fig. 4C).

Conclusion: Our results showed that ZDHHC7-mediated SCRIB palmitoylation could significantly block cancer cell growth.

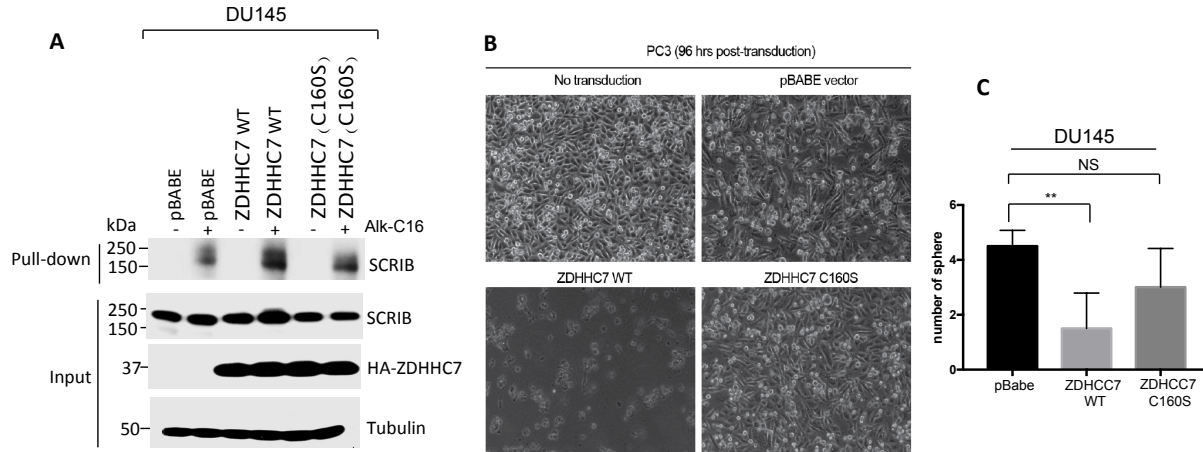


Figure 3. (A) Re-expression of ZDHHC7 WT, but not the C160S mutant could significantly rescue SCRIB palmitoylation levels in DU145 cells. (B) Expression of ZDHHC7 WT led to significant cell death in PC3 cells. (C) Expression of ZDHHC7 WT significantly blocks cell growth of DU145 cells.

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

Postdoctoral fellows and graduate students at the Cutaneous Biology Research Center (CBRC) receive in depth training within their PI's laboratory. They gain first hand experience in state of the art experimental approaches. As an important part of training, fellows are continuously challenged and prompted to prepare early written drafts of ongoing work, in which initial results are already framed within clearly formulated working hypotheses. Research goals are achieved through direct personal discussions with their PI supervisors, as well as other colleagues in the laboratory and more formal weekly lab meetings. There are a number of seminars and tutorials, held at various locations throughout the institution and Boston area that faculty, staff, pre and postdoctoral trainees, and graduate students are encouraged to attend. Each Tuesday at CBRC, a tutorial is presented by a staff member (or pre or postdoctoral fellow or graduate student) on work in progress or review of fields that are being considered for expansion. The CBRC Seminar Series is presented monthly. World-renowned scientists from a variety of specialties are invited to spend the day meeting with faculty, having a private lunch with fellows and students and giving a seminar that is attended by staff throughout the institution.

Within our research groups, the PI provides continuous supervision, advice, and guidance related to ongoing research projects in the laboratory as well as career counseling and personal development.

This project has provided training and professional development of postdoctoral fellows in Wu lab: Baoen Chen and Carla Guarino. Both of them have gained significant progresses in their molecular and cell biology, and chemical biology. Through interactions with our collaborator Jindan Yu's lab, they have also learned techniques to culture and test prostate cancer cell lines. We focused on improving their understanding to cell polarity and prostate cancer progression, and improving their technical skills of various experiments. I believe they are becoming more independent in terms of experimental design and interpretation.

How were the results disseminated to communities of interest?

Nothing to report.

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

As we planned in the original and revised SOW, we will continue to evaluate the role of ZDHHC7 and SCRIB in prostate cancer cell progression. We will evaluate the expression of NEPC markers upon ZDHHC7 loss, and SCRIB palmitoylation deficient mutant expression. In addition, we will carry out mechanistic studies downstream of SCRIB and characterize whether SCRIB is involved in YAP activation and NEPC conversion. We will carry out in vivo studies to validate the finding. Furthermore, it was not originally proposed in the SOW, we have observed that MTDH, a critical regulator of metastasis is a potential substrate of ZDHHC7, and could bind to Scribble. Therefore, we will explore whether MTDH could also contribute to prostate cancer progression, in conjunction with Scribble mislocalization.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

We have demonstrated that a chemical approach using palmitoylation reporters to detect Scribble palmitoylation in prostate cancer cell lines, and correlated with its mislocalization. In addition, we found that loss of ZDHHC7 promotes NEPC conversion, which could have significant impact in drug discovery and cancer research.

What was the impact on other disciplines?

Nothing to report

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:**

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

As we stated in the SOW table, we found that NEPC conversion as a major mechanism downstream of SCRIB, which contributes to prostate cancer progression. We have accordingly revised some of the plans to evaluate the effects of NEPC phenotype upon ZDHHC7 loss or SCRIB depalmitoylation.

Changes that had a significant impact on expenditures

No change on the expenditure.

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

No change.

Significant changes in use or care of human subjects

No change.

Significant changes in use or care of vertebrate animals

No change.

Significant changes in use of biohazards and/or select agents

No change.

6. PRODUCTS:

None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Xu Wu
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-1624-0143
Nearest person	3

month worked:	
Contribution to project:	Dr. Wu has supervised the research, designed the experiments and interpreted the results
Funding support:	MGH Institutional fund Melanoma Research Alliance NCI NIDDK Astellas Innovation fund

Name:	Baoen Chen
Project Role:	Instructor
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to project:	Dr. Chen has carried out the studies of Scribble in prostate cancers. He has carried out imaging studies of SCRIB in prostate cancer cells, and generated ZDHHC7 KO cell lines
Funding support:	None

Name:	Carla Guarino
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to project:	Dr. Guarino has carried out the studies of Scribble in prostate cancers. She has developed the biochemical methods to detect Scribble palmitoylation in prostate cancers.
Funding support:	Italian American cancer research foundation fellowship

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

Yes.

Dr. Wu has recently been awarded the following grants, which have no overlap with this grant.

Astellas Venture Management LLC (Wu)	6-27-17 – 6-26-20	1.8 cal mths
Sponsored research agreement	\$1,336,122	
Industry collaboration project for high throughput screen and pre-clinical development of new chemotypes of TEAD inhibitors		
Overlap: None.		

1R01CA219814-01A1 (Wu, Zheng) m-PI
Targeting Hippo pathway in melanoma

4-1-19 – 3-31-24 3.0 cal mths

The goal of this study is to identify the mechanisms and functions of Hippo pathway in BRAF-mutant cutaneous melanomas which are resistant to target-based therapies (BRAF and MEK inhibitors), to target resistant melanoma with novel TEAD inhibitors, and to identify the roles of YAP-dependent recruitment of myeloid-derived suppressor cells (MDSCs) in resistant melanoma.

OVERLAP: None.

1R01CA238270-01 (Wu and Mao) m-PI

4-1-19 – 3-31-24 3.0 cal mths

Targeting dynamic palmitoylation of TEAD transcription factors

The goal of this study is to identify the mechanisms and functions of ABHD1-mediated depalmitoylation of TEAD transcription factors in vitro and in vivo, and to target YAP activated and/or ABHD1-silenced breast and liver cancers using TEAD inhibitors.

OVERLAP: None.

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Dr. Yu will submit a separate report.

9. APPENDICES:

None.