AWARD NUMBER: W81XWH-21-1-0631

TITLE: Identifying the Molecular Mechanisms Underlying HIF2-Independent Tumorigenesis in RCC

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CONTRACTING ORGANIZATION: UT Southwestern Medical Center, Dallas, TX

REPORT DATE: August 2022

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2022	Annual	01Aug2021-31Jul2022

4. TITLE AND SUBTITLE

Identifying the Molecular Mechanisms Underlying HIF2-Independent Tumorigenesis in RCC

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-21-1-0631

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development 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

Inactivation of pVHL in ccRCC leads to accumulation of HIFα and activation of pathways that play key roles in tumor development, including induction of angiogenesis and cell proliferation, regulation of apoptosis and altered metabolism. hOWEVER, not all tumors are sensitive to inhibition of HIF-2α indicating that only some ccRCC tumors are HIF-2 dependent (with 40-60% of patients failing to show disease control upon treatment). While the oncogenic role of HIF-2α in a subset of ccRCC tumors is well established, the exact role of HIF-1α in tumor development has remained elusive. Here, we study the role of HIF-1α in a novel context that has not been previously possible. In addition, the precise role of non-canincal targets of VHL including ZHX2 and SFMBT1 in ccRCC are not well understood. Here, we used state of the art PDX lines that have previously been established as HIF2-independent lines, evaluate the role of HIF1A in tumor growth in these lines, examine expression of ZHX2 and SFMBT1, and pinpoint unidentified novel VHLtargets.

15. SUBJECT TERMS

ccRCC, VHL, HIF1, HIF2, ZHX2, SFMBT1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	18	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

The VHL/HIF-2 axis is considered the fundamental oncogenic pathway in ccRCC. Indeed, VHL is inactivated in over 80% of ccRCC. However, the advent of highly specific and effective inhibitors of HIF-2α has revealed that HIF-2α is dispensable for some ccRCC tumors. Moreover, the rate of response in patients in clinical trials have been 15-25%. Here, we propose to identify the main oncogenic drivers and molecular pathways that promote tumorigenesis downstream of VHL in a HIF-2-independent manner.

2. KEYWORDS:

ccRCC, VHL, HIF1, HIF2, ZHX2, SFMBT1

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aims, major tasks and subtasks for this reporting period are below:

Research-Specific Tasks:	**Completion status**
Specific Aim 1: Determine the role of HIF-1alpha in tumorigenesis in HIF2-independent tumors	
Major Task 1: Conditional knockdown of HIF-1α in HIF2-independent PDX lines upon doxycycline administration	
Subtask 1: Local Institutional Review Board (IRB) and US Army Medical Research and Development Command (USAMRDC) Human Research Protection Office (HRPO). Of note, while the proposal uses human tissues, it does not require their being identifiable, and the tissues are already collected through an IRB-approved protocol.	100%
Milestone Achieved: Obtain IACUC and ACURO *approval*	100%
Subtask 2: Establish *in vitro* tissue culture for PDX lines (XP506 and XP530) (The rest are already established) Cells used: primary cells obtained from HIF2-independent PDX lines including XP169, XP258, XP296, XP490, XP506, XP530 (Source: Brugarolas lab)	50%
Subtask 3: Using primary cells available and generated in subtask 1.2, generate	50%

stable cell lines harboring constructs for doxycycline inducible shRNA-mediated HIF-1α knockdown using low passage number PDX primary cells and a short selection period (~two weeks)	
Subtask 4: Evaluate the efficacy of HIF-1α knockdown in cells generated in subtask 1.3 and choose the most efficient shRNA-mediated knockdown lines	50%
Milestone Achieved: generation of primary PDX lines with efficient knockdown of HIF-1α	50%
Major Task 2: Establish a cohort of mice harboring tumors using cells generated in Task1.4	
Subtask1: Submitting documents for Local Institutional Animal Care and Use Committee (IACUC) and USAMRDC Animal Care and Use Review Office (ACURO) approval.	100%
Milestone Achieved: Obtain IACUC and ACURO *approval*	100%
Subtask 2: Implant cells generated in task 1.4 subcutaneously and orthotopically in mice and evaluate tumor growth for at least two PDX lines; [10 mice for experimental shRNA per treatment arm per PDX line (10*2*2) + 2 mice for control shRNA per treatment arm and per PDX line (2*2*2) , 48 mice total]	50%
Milestone Achieved: A cohort of mice harboring tumors in which HIF-1α can be conditionally knocked down HIF-1α	50%
Specific Aim 2: Determine non-HIF related roles of pVHL in ccRCC tumors	
Major task4: Generate PDX lines in which ZHX2 and SFMBT1 can be conditionally knocked down	
Subtask1: Examine the expression level of ZHX2 and SFMBT1 in all HIF2-independent XP lines. Cells used: primary cells obtained from HIF2-independent PDX lines including XP169, XP258, XP296, XP490, XP506, XP530 (Source: Brugarolas lab)	100%
Subtask2: Generate constructs for shRNA mediated knockdown of ZHX2 and SFMBT1 using TET-ON system	100%
Subtask3: Establish PDX lines harboring constructs for ZHX2 and SFMBT1 knockdown and evaluate the efficiency of knockdown	50%
Major Task 7: Establish PDX lines harboring constructs for conditional overexpression of VHL	
Subtask 1: Generate PDX lines harboring constructs for doxycycline inducible overexpression of VHL	50%

What was accomplished under these goals?

Specific Aim 1: Determine the role of HIF-1alpha in tumorigenesis in HIF2-independent tumors

Major Task 1: Conditional knockdown of HIF-1α in HIF2-independent PDX lines upon doxycycline administration

We have previously established cell lines for XP169, XP258. XP296. We are currently working on establishing lines for XP506 and XP530. Both lines have been grown in vitro using patient derived tumorgrafts. We are currently validating these lines by performing immune-staining experiments to verify no mouse tissue contamination as well as matching histological features with that of the original tumorgraft. In addition, constructs for HIF1A knockout have been built and validated in established cell lin HEK293T as well as the PDX line XP258, XP296 and XP169. The efficacy of knockout was evaluated by western blot and further selection for high knockout efficiency was performed using FACS sorting

Major Task 2: Establish a cohort of mice harboring tumors using cells generated in Task1.4

A cohort of mice was established using XP258+ HIF1A knockout that was validated in vitro. The rate of tumor growth was evaluated by measuring tumor sizes subcutaneous using caliber. The results indicated no significant difference in the rate of tumor growth in XP285 ctrl vs XP258 HIF1A knockout.

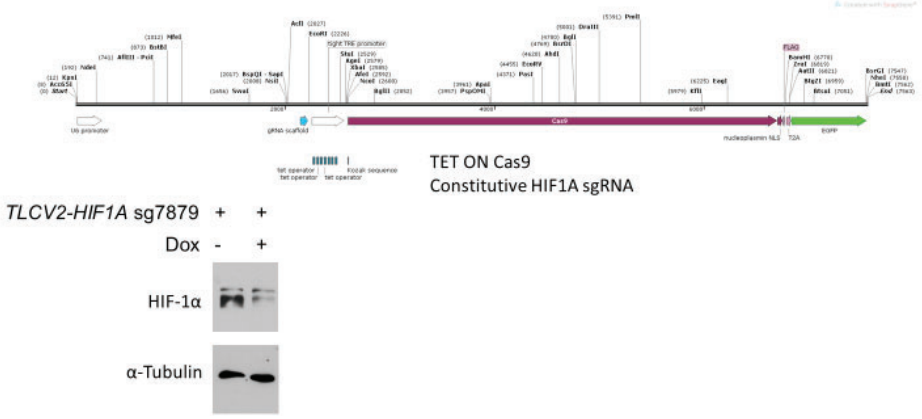


Figure 1. HIF1A knockout construct and validation. Map of the construct used for Doxycycline induced expression of Cas9 and constitutive expression of HIF1A-specific sgRNAis shown on top and validation by western blot is shown on the bottom.

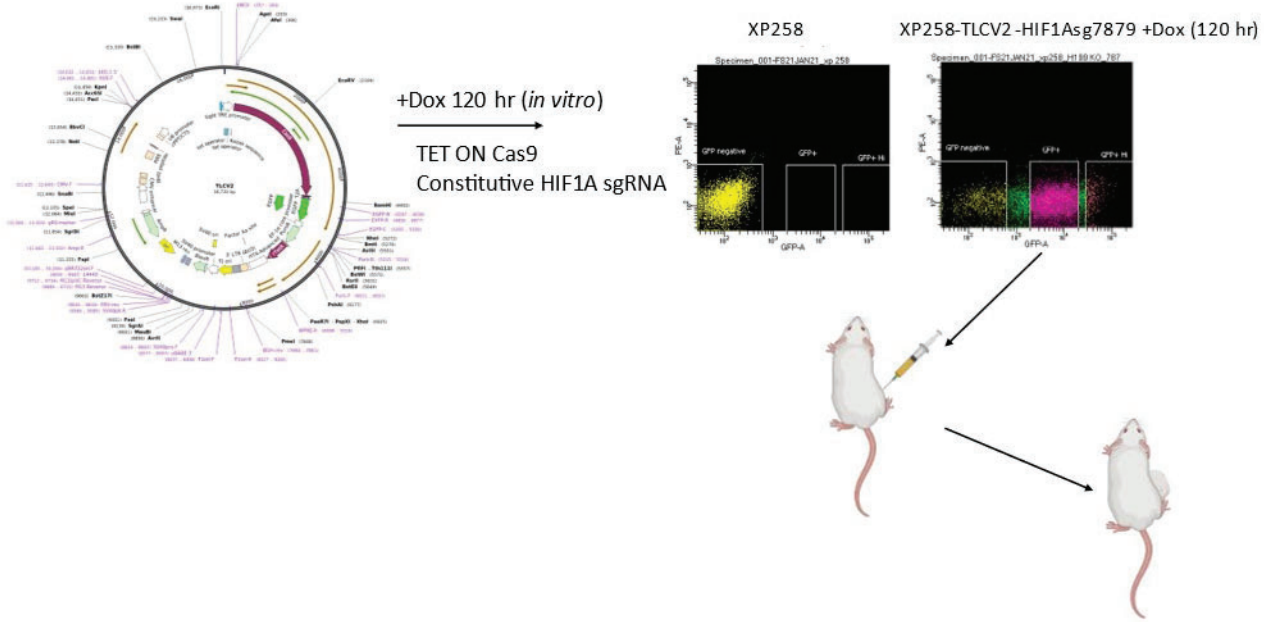


Figure 2. Selection of cells harboring HIF1A knockout construct by FACS. Cells expressing high level of Cas9 was were selected by FACS sorting and population expressing high levels of Cas9 (as measure by GFP expression) were implanted in mice subcutaneously.

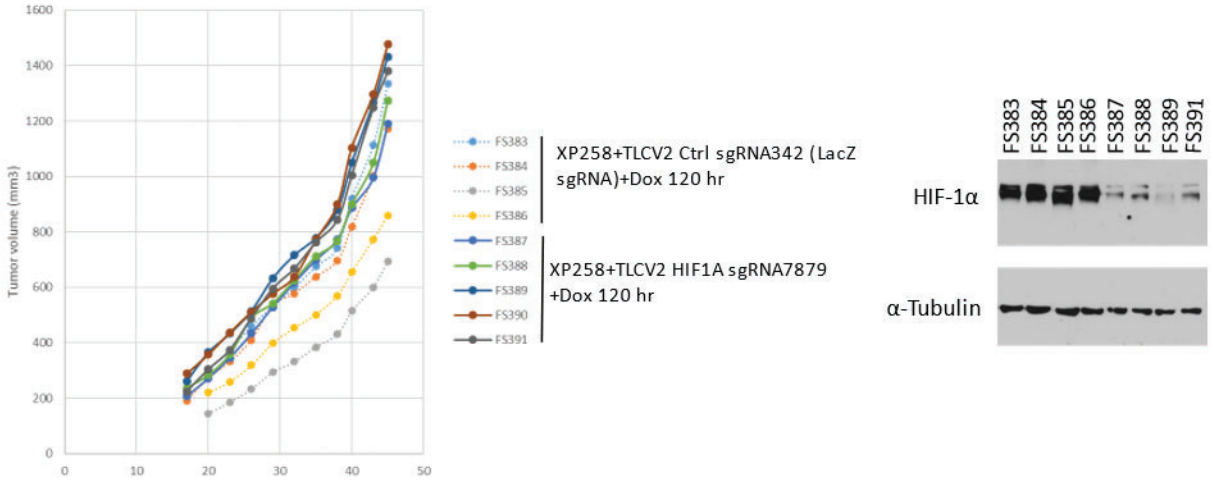


Figure 3. Effect of loss of HIF1A on tumor growth in HIF2-independent line XP258. Mice implanted with XP258 HIF1KO cells (or XP258 control cells) were monitored for tumor growth. Once tumors reached maximum size, mice were sac'd and tumorgrafts were harvested and used in immunoblots

Specific Aim 2: Determine non-HIF related roles of pVHL in ccRCC tumors
Major task4: Generate PDX lines in which ZHX2 and SFMBT1 can be conditionally knocked down
Major Task 7: Establish PDX lines harboring constructs for conditional overexpression of VHL

We have analyzed expression levels of SFMBT1 and ZHX2 by qRT-PCR in various lines. We have also built constructs for doxycycline-inducible knock out of ZHX2 and SFMBT1 as well as VHL overexpression. Constructs have been validated by western blot. In addition, the effect of doxycycline-inducible VHL overexpression on tumor growth was validated in tumorgraft models.

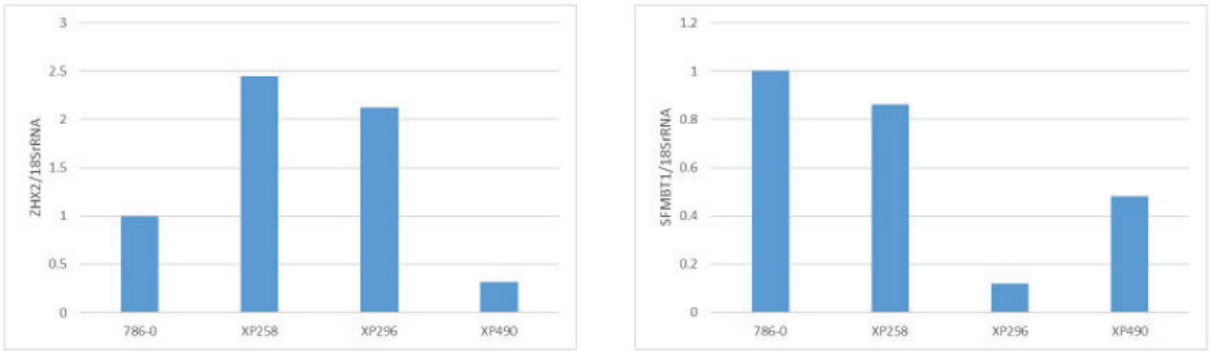


Figure 4. Expression level of ZHX2 and SFMBT1 in different XP lines. Expression of ZHX2 and SFMBT1 genes were measured in indicated XP lines and normalized to ccRCC line 786-0 previously shown to express high levels of SFMBT1 and ZHX2.

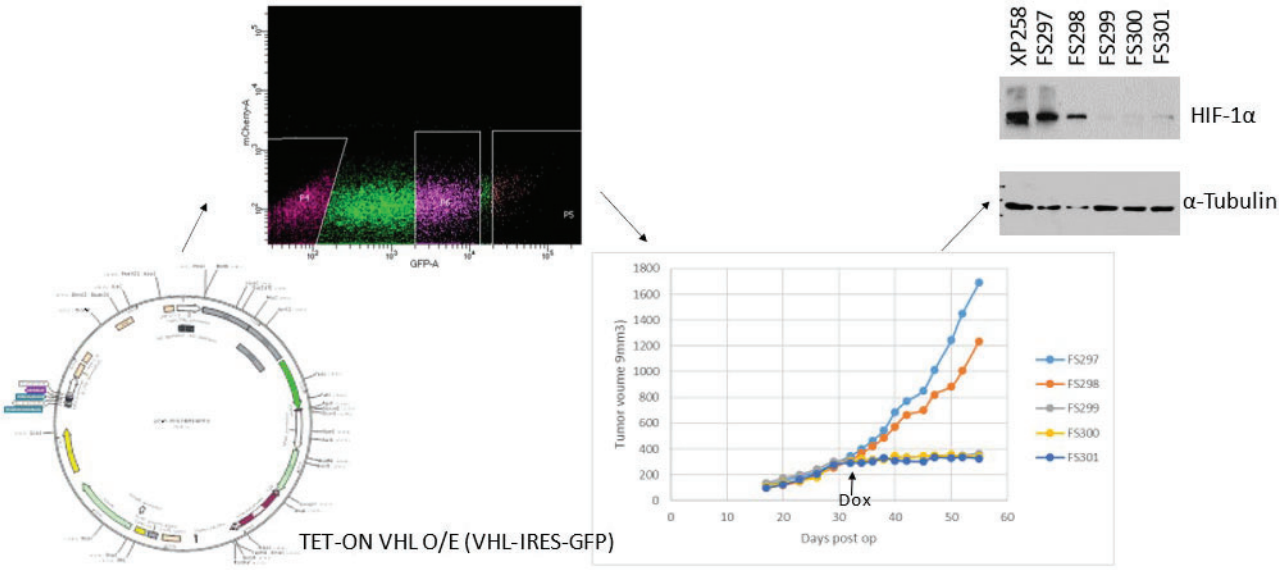


Figure 5. Effect of conditional overexpression of VHL on tumor growth. Constructs for doxycycline-inducible overexpression of VHL were tranduced in XP lines. Following selection of high expression level of VHL by FACS, cells were implanted subcutaneously and tumor growth

was measured regularly. Doxycycline was added to drinking water once tumors reached ~300 mm^3. Once tumors reached maximum size, all tumorgrafts were harvested and used in immunoblots.

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

Ability to independently lead and execute a cutting-edge research project, mentoring by PI at weekly 1:1 meetings and presentations at lab meetings and group meetings at UT Southwestern.

How were the results disseminated to communities of interest?

Results were presented to members of the kidney cancer program every quarter. In addition, a presentation was made to graduate students, postdocs, research assistants and principal investigators of several research labs at UT Southwestern as a function of "floor meetings".

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

I will follow the SOW as outlined in the original application to accomplish goals and objectives.

4. IMPACT:

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

Nothing to report

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:

No changes to report

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

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

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

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

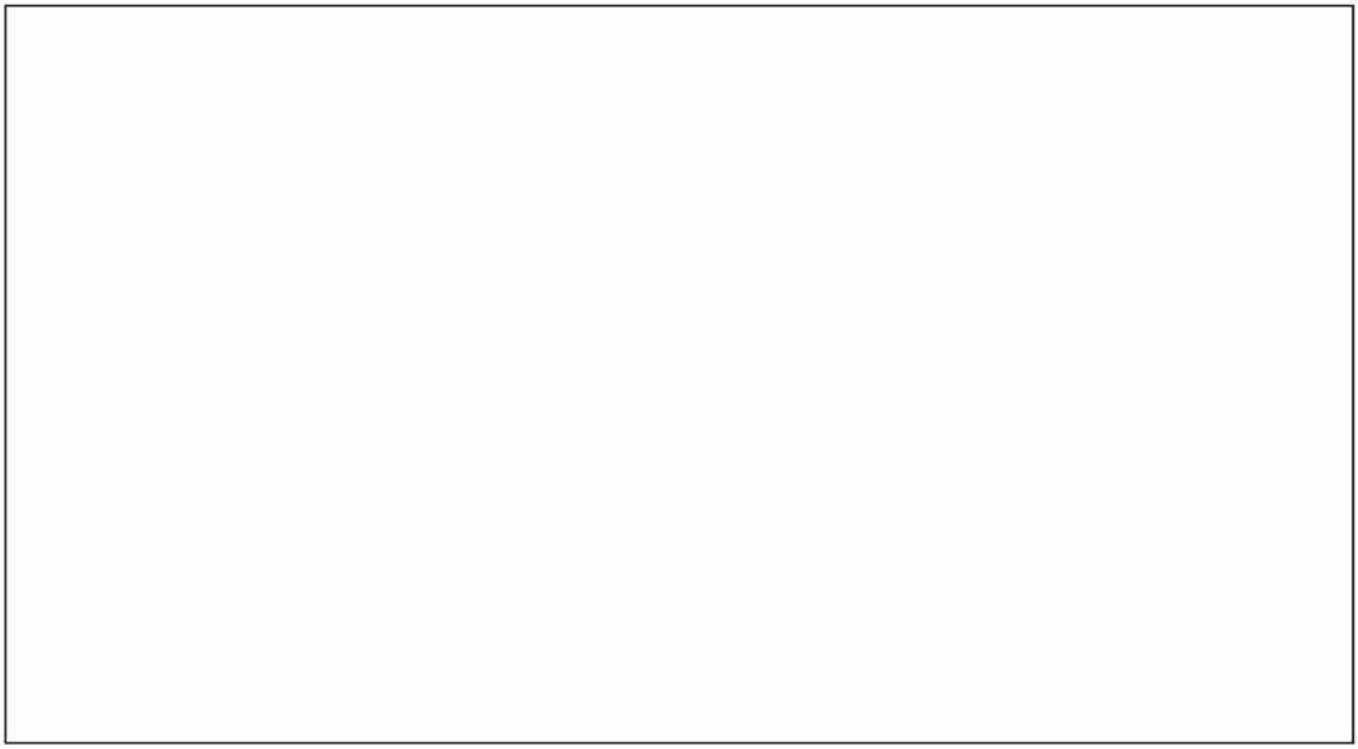
Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?



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

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: