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CONTRACTING ORGANIZATION: UT Southwestern Medical Center

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14. ABSTRACT 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-2 α has revealed that HIF-2 α is dispensable for some ccRCC tumors. Moreover, the rate of response in patients in clinical trials was 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.						
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1. INTRODUCTION:

The VHL/HIF-2 α 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-2 α has revealed that HIF-2 α 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 α , HIF2 α , ZHX2, SFMBT1

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Research-Specific Tasks:	Completion status
Specific Aim 2: Determine non-HIF related roles of pVHL in ccRCC tumors	67%
Major task 4: Generate PDX lines in which ZHX2 and SFMBT1 can be conditionally knocked down	100%
Subtask 1: Examine the expression level of ZHX2 and SFMBT1 in all HIF2-independent XP lines	60%
Subtask 2: Generate constructs for shRNA mediated knockdown of ZHX2 and SFMBT1 using TET-ON system	100%
Subtask 3: Establish PDX lines harboring constructs for ZHX2 and SFMBT1 knockdown and evaluate the efficiency of knockdown	100%
Major Task 5: Establish a cohort of mice harboring tumors using cells generated in subtask 4.3	100%

Milestone Achieved: A cohort of mice harboring tumors in which ZHX2 and SFMBT1 can be conditionally knocked down	
Major Task 6: Characterize the role of ZHX2 and SFMBT1 in tumor growth	75%
Milestone Achieved: Understanding the role of ZHX2 and SFMBT1 in tumor growth in HIF2-independent tumors	
Major Task 9: Identify VHL-regulated molecular pathways independent of canonical (HIF) and non-canonical (ZHX2 and SFMBT1) substrates	70%
Subtask 1: Evaluate changes in tumor growth upon providing drinking water with or without doxycycline to mice from subtask 8.1	100%
Subtask 2: Collect tumor tissue from mice in subtask 9.1 and evaluate efficiency of VHL overexpression in vivo	100%
Subtask3: RNA-Seq using tissue collected in subtask 9.2 and analyze differentially expressed genes and molecular pathways	30%
Milestone Achieved: Understanding the non-HIF related role of VHL in ccRCC tumors	

What was accomplished under these goals?

Specific Aim 2: Determine non-HIF related roles of pVHL in ccRCC tumors

Major task 4: Generate PDX lines in which ZHX2 and SFMBT1 can be conditionally knocked down.

2D *in vitro* cell lines were generated from PDX lines (which are independent of HIF2 α and inactivated

pVHL) -XP258, XP289 and XP490. These cells lines are reported to have inactivated pVHL. In these

cell lines, we have analyzed expression levels of SFMBT1 and ZHX2 by qRT-PCR and compared them to that of 786-O cell line. 786-O cell line is reported to have inactivated pVHL and is HIF2 α dependent (sensitive to HIF2 α inhibitors). It was observed that XP258, XP296 had a higher expression of ZHX2 (Figure 1a). We also investigated the level of SFMBT1 in these cell lines. The mRNA

expression of SFMBT1 has not increased in the cell lines in comparison to 786-O. In future, we are

also going to check the level of ZHX2 and SFMBT1 in tumor grafts which do not respond to HIF2 α inhibitors and compare it sensitive tumorgrafts.

Next, we sought to investigate whether ZHX2 and SFMBT1 affect tumor growth of HIF2 α independent tumors. ZHX2 and SFMBT1 have been reported to promote tumor growth in pVHL inactivated ccRCC. So, we wanted to check does ZHX2 and SFMBT1 have the same effect in VHL inactivated HIF2 α independent ccRCC lines. So, we knocked down ZHX2 and SFMBT1 in XP258 cell line using a doxycycline-inducible systems. sgRNAs targeting the gene of our interest was cloned in TLCV2 vector. Then the stable XP258 cells lines were generated using this vector. With doxycycline, the level of ZHX2 and SFMBT1 decreased validating the knockout of the respective genes(Figure 1b).

Then we implanted these cell lines subcutaneously in mice. Around 5 million cells were implanted. The tumor growth was monitored over time. It was observed that knocking out of ZHX2 and SFMBT1 lead to slower development of tumor. However, no prominent difference in tumor growth (as determined by the slope of the curve once tumors became detectable) was observed (Figure 1c,d). ZHX2 and SFMBT1 are known to be involved in tumor growth. However, no such observation is made by us. Intriguingly we observed the tumor development is delayed in HIF2 α independent lines is observed. In future it will be interesting to understand the mechanism.

As we have not observed prominent difference in tumor growth on knocking out ZHX2 and SFMBT1 we did not carry forward with RNA seq of the tumor as we believed that this will not help in fulling the aim of this grant.

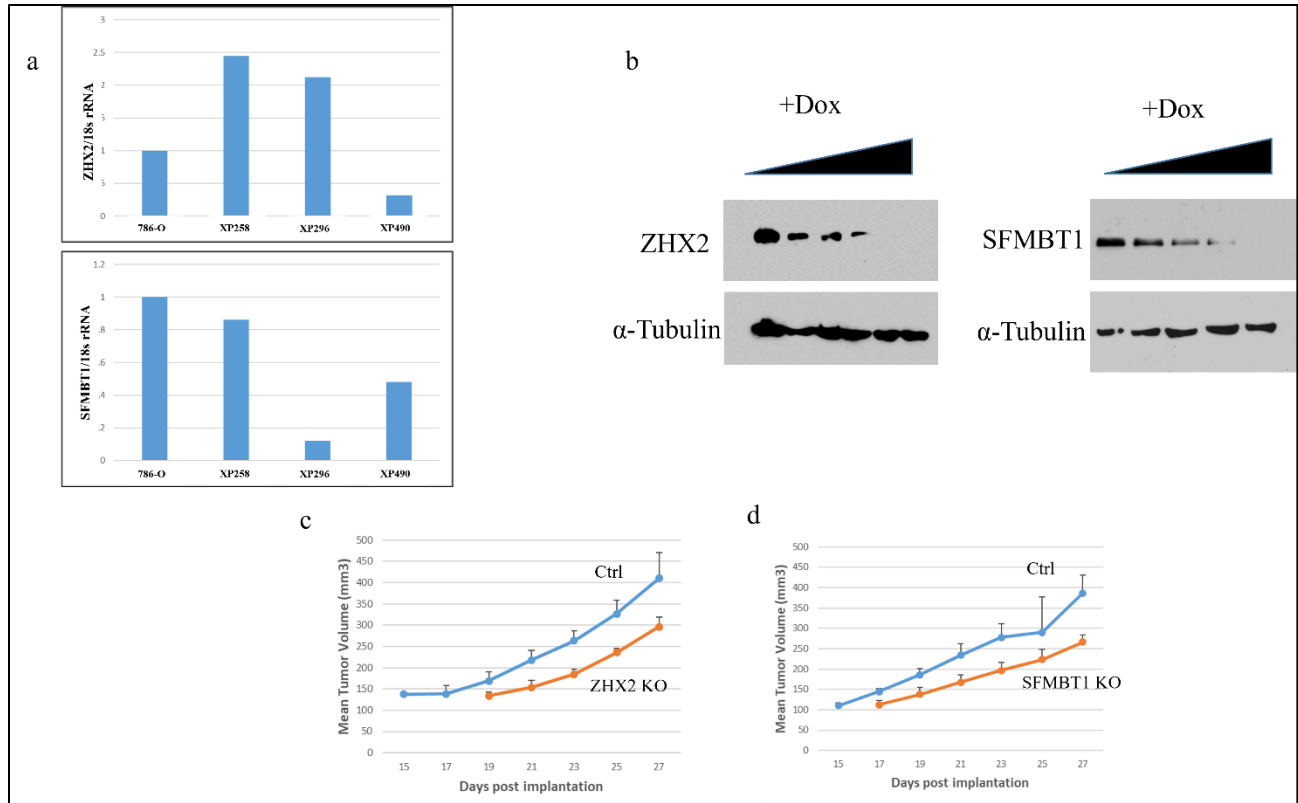


Figure 1- Effect of knocking out ZHX2 and SFMBT1 on tumor growth. **a-** Graph showing the fold change of mRNA of ZHX2 (upper panel) and SFMBT1 (lower panel) in different PDX derived cell lines . **b-** Immunoblot showing the level of ZHX2 and SFMBT1 on doxycycline treatment of XP258 stably expressing the cloned vector from panel a. **c, d-** Graph showing tumor growth on ZHX2 knockout (c) and SFMBT1 knockout (d).

Major Task 9: Identify VHL-regulated molecular pathways independent of canonical (HIF) and non-canonical (ZHX2 and SFMBT1) substrates.

Inactivation of pVHL is the initial step for development of ccRCC. Loss or inactivation of VHL subsequently led to accumulation of many oncoproteins like HIF2 α , ZHX2, SFMBT1 and other oncoproteins, which eventually leads to development of ccRCC. We have already observed that HIF2 α is not crucial for tumor growth in certain cohort of ccRCC. In these HIF2 α independent ccRCC tumors we have shown that ZHX2 and SFMBT1 plays minor roles. Now, we also sought to identify other factors that might be crucial for growth of these HIF2 α independent tumors. For that purpose, we stably overexpressed VHL in XP258 cell lines using a doxycycline inducible system (pCW-eGFP vector, map shown in Figure 2a). This cell was implanted subcutaneously in mice. After tumors

developed and reached size of 300mm³, doxycycline was added to drinking water. As expected, tumor kept growing in control mice (where no doxycycline was added), while tumor growth was repressed in the doxycycline group (Figure 2b). Thus, VHL loss or inactivation is crucial for tumor growth. However, we reported that VHL loss alone is insufficient for development of ccRCC. Combinatorial loss of other genes like BAP1 and PBRM1 along with VHL is necessary for development of ccRCC. However, our study showed that if VHL is reintroduced (overexpressed) then tumor development can be halted, showing that VHL loss is necessary but not sufficient.

The tumors were isolated, and it was observed that HIF1 α level was low in the doxycycline group in comparison to those controls (Figure 2c), indicating activation of VHL. We further plan to carry out RNA sequencing of the tumors where VHL is overexpressed and compare the differentially regulated genes from this tumor with those from earlier identified differentially regulated genes in HIF2 α independent tumors. This will help us to identify the genes differentially expressed in HIF2 α independent tumorgrafts that are also downregulated by VHL overexpression. The tissues have been collected and will be used for RNA sequencing.

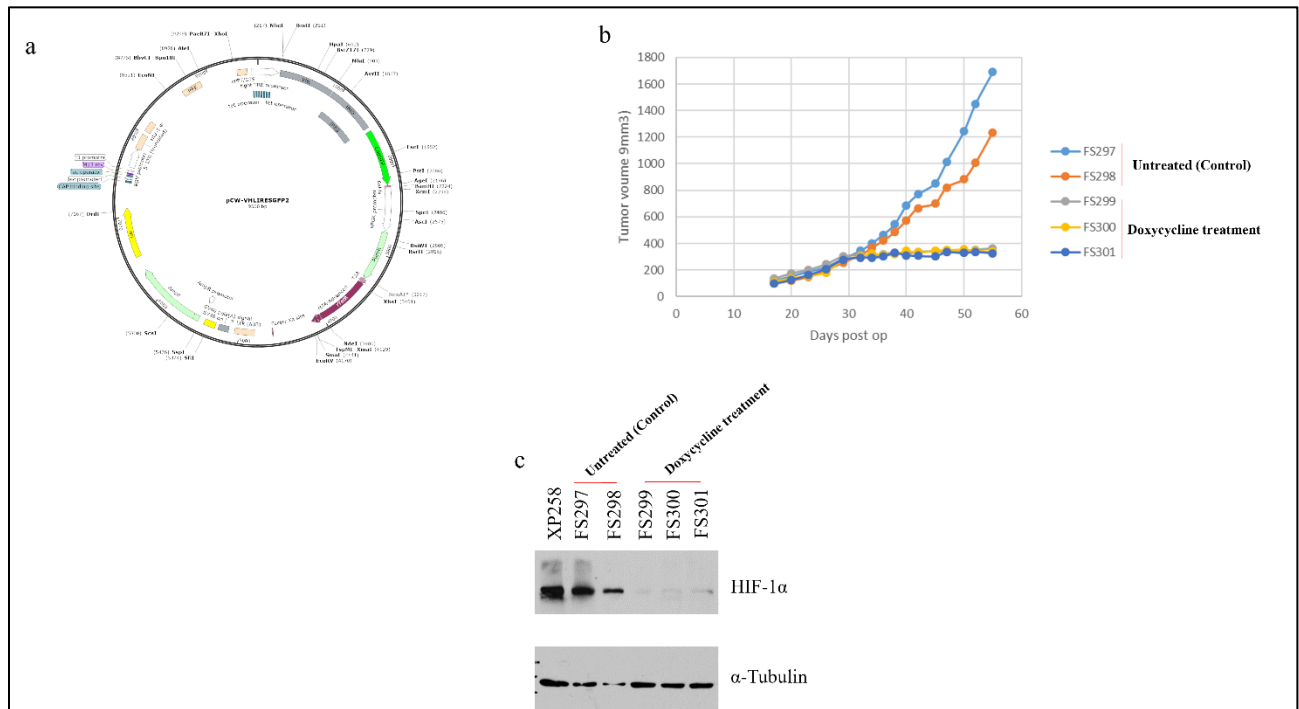


Figure 2- Effect of conditional overexpression of VHL on tumor growth. **a-** pCW doxycycline inducible vector in which the VHL was cloned. **b-** Graph showing the tumor growth. Mice FS297, FS298 are in control group, not treated with doxycycline in drinking water. Mice FS299, FS300 and

FS301 are treated with doxycycline in drinking water. c- Immunoblot showing the level of HIF1 α in tumors from figure panel b. XP258 cell line used as positive control.

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

1. Completed 2 semester of postdoctoral training program at UT Southwestern Medical Center
2. Completed professional development courses under postdoctoral training program.
3. Weekly one-on-one work with mentor
4. Presentation of research article in journal clubs as a training for review and critical analysis of data
5. Lab meeting, group meeting at UT Southwestern Medical Center helps in development of professional skills like presentation data interpretation etc.

How were the results disseminated to communities of interest?

Results were presented to members of the kidney cancer program.

This is the final report for the grant.

However, we are interested in pursuing:

1. RNA sequencing to understand the mechanism behind delayed tumor initiation on knocking out SFMBT1 and ZHX2
2. RNA sequencing of VHL overexpressing tumors to identify non-canonical VHL substrates that may be crucial in HIF2 α independent tumors.

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?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. CHANGES/PROBLEMS:

Nothing 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

Nothing to report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

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

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

(international, national, local societies, military meetings, etc.). Use an asterisk () if presentation produced a manuscript.*

Nothing to report.

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

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

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

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

- Nothing to report

- *physical collections;*
- *audio or video products;*
- *software;*

- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Nothing to report

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

The grant has been transferred from initial PI-Faeze Saatchi to New PI- Arijit Mal. Prior approval has been obtained.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: *N/A*