AWARD NUMBER:

W81XWH-19-1-0411

TITLE:

Targeting Glutaminase Isoforms for Therapy-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR:

Jiaoti Huang

CONTRACTING ORGANIZATION:

Duke University, Durham, NC

REPORT DATE:

January 2023

TYPE OF REPORT:

Final

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**
January 2023	Final	30Sep2019-29Sep2022

4. TITLE AND SUBTITLE

Targeting Glutaminase Isoforms for Therapy-Resistant Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0411

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jiaoti Huang

E-Mail: Jiaoti.huang@duke.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Duke University
2200 W Main ST STE 710
DURHAM NC 27708-4677

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

Our preliminary study demonstrates that advanced prostate cancer is addicted to glutamine, and a glutaminase isoform switch contributes to the development of therapy resistance and disease progression. The major goal of the project was to study the molecular mechanisms of GLS1 isoform switch and explore the possibility of targeting glutamine metabolism as a novel therapeutic approach. In addition to the achievements reached in Year 1 and Year 2, this year, we have made the following Key Research Accomplishments:

1. We have demonstrated a potential mechanism how c-Myc and N-Myc up-regulate GAC expression.
2. We have demonstrated a dramatic phenotype that advanced PCa cells consume more glutamine utilization.
3. We have demonstrated that the GLS1 selective inhibitor, CB-839, preferentially inhibits tumor growth of advanced PCa cells where GAC predominates. Thus, GLS1/GAC could be an ideal therapeutic target for patients who have run out of treatment of chioce.

15. SUBJECT TERMS

None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	13	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			

TABLE OF CONTENTS

	Page
1. Introduction	4
2. Keywords	5
3. Accomplishments	5
4. Impact	9
5. Changes/Problems	10
6. Products	10
7. Participants & Other Collaborating Organizations	10
8. Special Reporting Requirements	12
9. Appendices	12

1. Introduction:

Prostate Cancer (PCa) leads to ~30,000 deaths yearly in the US. Although androgen receptor (AR)-targeted therapies, including conventional androgen deprivation therapy (ADT) and the second generation of antiandrogen drugs (e.g abiraterone and enzalutamide), remain the standard management for metastatic PCa, the therapeutic failure and tumor recurrence are inevitable. This is because tumor cells develop various resistance mechanisms to overcome the consequences of AR inhibition. Therefore, instead of targeting AR which regulates numerous signaling pathways and plays critical physiologic roles, identifying AR's independent effectors that are directly responsible for tumor biology, can yield better therapeutic targets to achieve more durable therapeutic responses and reduce side effects associated with AR targeting.

The rewired metabolism is one of the most significant cancer hallmarks, and targeting metabolism has become an important therapeutic strategy for many tumors. In PCa, our preliminary studies have revealed a dramatic reprogramming of glutamine metabolism during disease development. Basically, ADT inhibited glutamine being utilized by tumor cells, which led to tumor regression. However, an isoform switch of the key enzyme of glutaminolysis, GLS1, enables tumor cells to get rid of ADT management and become extremely dependent on glutamine instead of androgen. Mechanistically, AR regulates GLS1 gene and mediates GLS1 splicing towards KGA, the relatively weaker isoform. However, evidence are lacking on how GAC becomes predominant in the late-stage of the disease. Thus, in this year, we put great effort on studying the potential mechanism of GAC

modulation. Furthermore, we performed tons of drug-related experiments to demonstrate that GLS1/GAC is an ideal therapeutic target in replacement of AR for the advanced patients, and the selective inhibitor, CB-839, showed promising therapeutic values.

2. **Key Words: Prostate cancer, Glutamine, Glutaminase, KGA, GAC**
3. **Research accomplishments associated with Task 1: In this task, we will demonstrate that ADT-induced inability to utilize glutamine mediates the inhibition effects of hormonal therapy on PCa.**

Subtask 1: Test if ADT-induced inability of glutamine utilization is general for all androgen sensitive PCa cell lines (completed, see first-year annual report).

Subtask 2: Test if GLS1 (GAC) overexpression rescues the cell proliferation under the presence of ADT (completed, see second-year annual report).

Subtask 3: Examine how AR regulates GLS1 expression (Time frame: Months 1-24).

We continued to explore the mechanism by which AR regulates GLS1. As stated in last year's annual report, AR is able to up-regulate GLS1 expression and at the same time preferentially promotes GLS1 splicing towards KGA, rather than GAC. However, loss of AR may not be the only reason that GAC is up-regulated in the late-stage prostate cancer (PCa). It is possible that some other factors or co-drivers may contribute to the emergence of GAC in advanced PCa. Numerous ligand-independent mechanisms of AR function have been implicated in the development of castration resistant PCa (CRPC) and/or small cell neuroendocrine carcinoma (SCNC). Interestingly, c-Myc and N-Myc, members of MYC family proteins, appear to drive different histological transdifferentiation in PCa. c-Myc has been found to be highly expressed in CRPC with adenocarcinoma histology and luminal differentiation. In contrast, N-Myc is frequently amplified in SCNC. Our IHC staining on human tissues verified the above (Fig. 1A). Since c-Myc has been reported to affect GAC expression via microRNA 23a/23b, we hypothesized that c-Myc might be responsible for the up-regulation of GAC in CRPC tissues. Indeed, knocking down c-Myc in LNCaP95 cells, an enzalutamide-resistant PCa cell line that overexpresses GAC, resulted in significant reduction of GAC transcript levels (Fig. 2A and C). Interestingly, KGA mRNA level was not significantly affected (Fig. 2C). Similar observation was made in another enzalutamide-resistant PCa cell line, C4-2MDVR (Fig. 2B and D). Conversely, when we overexpressed c-Myc in two hormone-sensitive PCa cell lines, LNCaP and LAPC4, where

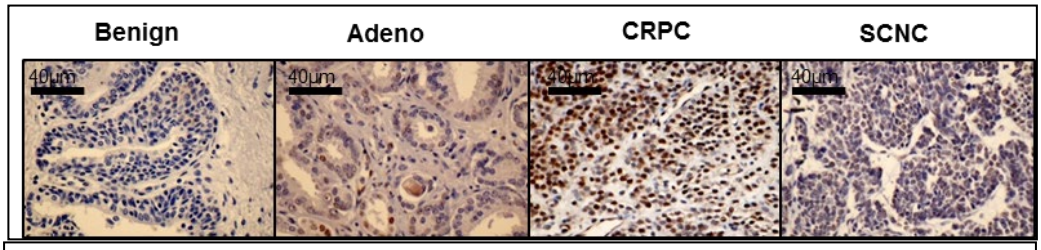

Fig. 1 IHC staining showing c-Myc expression across a panel of prostatic samples with different histology. Specifically, CRPC-adeno displays the highest expression of c-Myc in comparison to other tumors. Scale bar, 40um.

endogenous c-Myc and GAC levels are low, we found that GAC mRNA level was dramatically elevated while this manipulation has little effect on KGA (Fig. 2E-H). Taken together, these results suggest that c-Myc promotes the expression of the enzymatically potent GAC which may contribute to therapy resistance and disease progression.

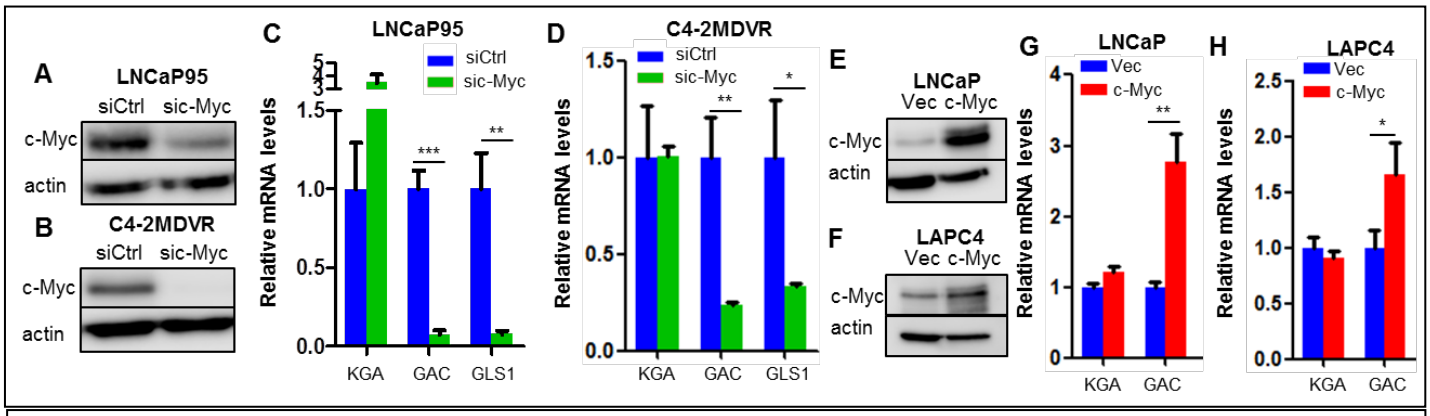

Fig. 2 c-Myc promotes GAC expression in PCa. **A and B** Western blot validating the knock down efficiency of siRNA-targeting c-Myc in LNCaP95 and C4-2MDVR cells. **C and D** qPCR determining the transcript levels of KGA, GAC and GLS1. **E and F** Western blot validating the overexpression of c-Myc in LNCaP and LAPC4. **G and H** qPCR determining the transcript levels of KGA and GAC.

Similar to the effect caused by c-Myc, overexpression of N-Myc consistently promotes the expression of GAC, not KGA, in LNCaP and C4-2 cells (Fig. 3A). AR inhibition has been shown to promote the emergence of SCNC through lineage plasticity. We found that instead of inducing GAC expression, AR inhibition alone reduced GAC expression in LNCaP cells which does not express c-Myc or N-Myc, a finding consistent with the previous results (Fig. 3B). However, when N-Myc is overexpressed, ADT led to an 8-fold increase of GAC (Fig. 3B), suggesting that ADT and N-Myc cooperate to induce the expression of GAC, resulting in increased glutamine utilization and aggressive behavior of SCNC.

Therefore, the above results, together with previous findings, have firmly established how AR, c-Myc and N-Myc function to induce the isoform switch of GLS1 in PCa during the process of hormonal therapy, treatment resistance and disease progression.

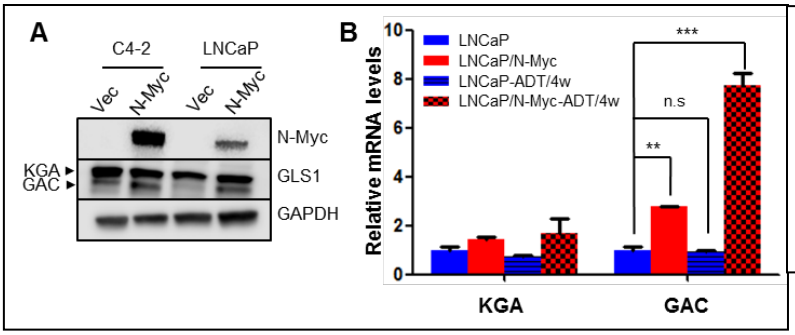

Fig. 3 N-Myc is also able to up-regulate GAC expression. **A** Western blot showing ectopic N-Myc overexpression and the resultant KGA and GAC expression in two hormone-sensitive PCa cell lines, LNCaP and LAPC4. **B** qPCR determining KGA and GAC transcript levels upon either ADT treatment alone for four weeks or N-Myc overexpression only or in combination.

Research accomplishments associated with Task 2: In this task, we will demonstrate that the GLS1 isoform switch from KGA to GAC drives castration resistance.

Subtask 1: Test if glutamine utilization is efficient initially in the therapy-sensitive stage, inhibited after treatment and efficient again after development of therapy resistance (Time frame: Months 24-36).

Our previous data indicates that in comparison to hormone-sensitive PCa cells, advanced PCa cells, such as PC3 and C4-2MDVR, are much more addicted to glutamine. Thus, we wanted to know if advanced PCa consumes more glutamine to support their aggressive tumor biology. To answer this question, we employed mass spectrometry to analyze intracellular glutamine level as well as glutamine concentration in cell culture medium to determine glutamine uptake in different PCa cell lines. We employed two pairs of cell lines for comparison, LNCaP (hormone-sensitive) vs PC3 (AR-null, hormone-independent) and C4-2 (enzalutamide-sensitive) vs C4-2MDVR (enzalutamide-resistant). In the two pairs, PC3 and C4-2MDVR represented more advanced PCa. Interestingly, we found that both PC3 and C4-2MDVR cells consumed more glutamine from the culture medium, but the intracellular glutamine concentration was dramatically lower than that of their counterparts (Fig. 4A-B and Fig. 5A-B), indicating that advanced PCa consumes more glutamine and can efficiently utilizes it to generate downstream metabolites. To further test this hypothesis, we performed 13C5-glutamine isotopomer tracing experiments. We found that advanced PCa cells generated more glutamine-derived metabolites, such as intermediates involved in the TCA cycle (Fig. 4C and Fig. 5C), which suggests that the catabolism activity of glutamine is quite hyper in those therapy-resistant PCa cells.

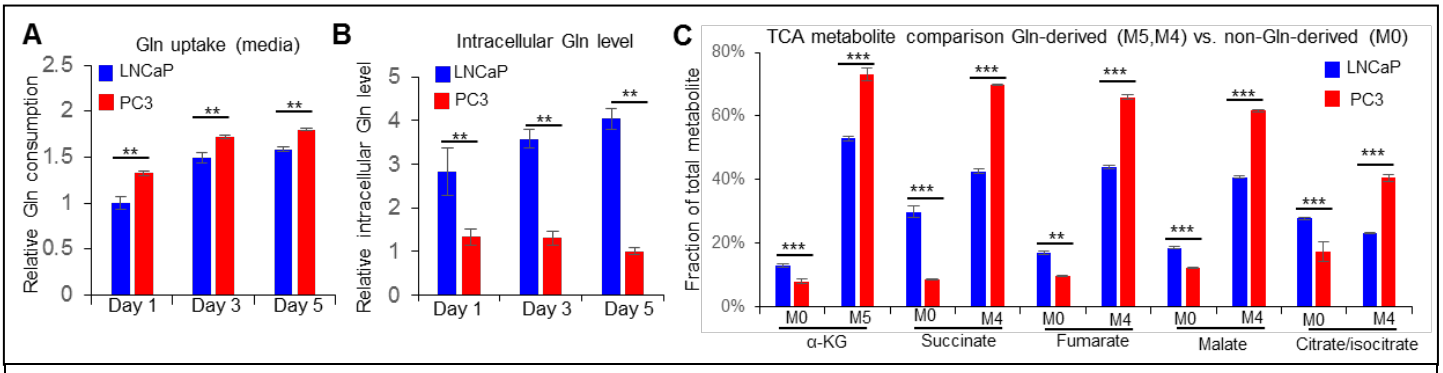

Fig. 4 Advanced PCa consumes and utilizes more glutamine. **A and B** Mass spectrometry determining glutamine concentration in medium and in cells. **C** 13C5-glutamine tracing analysis showing the fraction of glutamine-labeled intermediates.

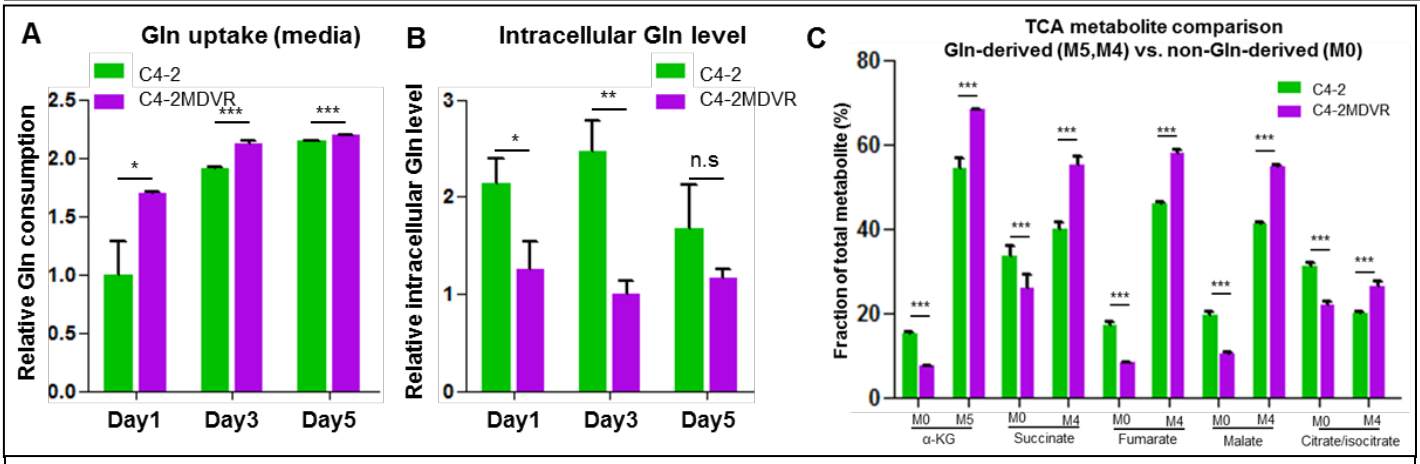

Fig. 5 Advanced PCa consumes and utilizes more glutamine. **A and B** Mass spectrometry determining glutamine concentration in medium and in cells. **C** 13C5-glutamine tracing analysis showing the fraction of glutamine-labeled intermediates.

Subtask 2-3: Generate *GLS1* knockout cell lines by CRISPR-Cas9 system and test if the switch from KGA to GAC mediates castration resistance (Time frame: Months 1-36).

We performed various experiments to show the therapeutic value of targeting GLS1 in advanced PCa.

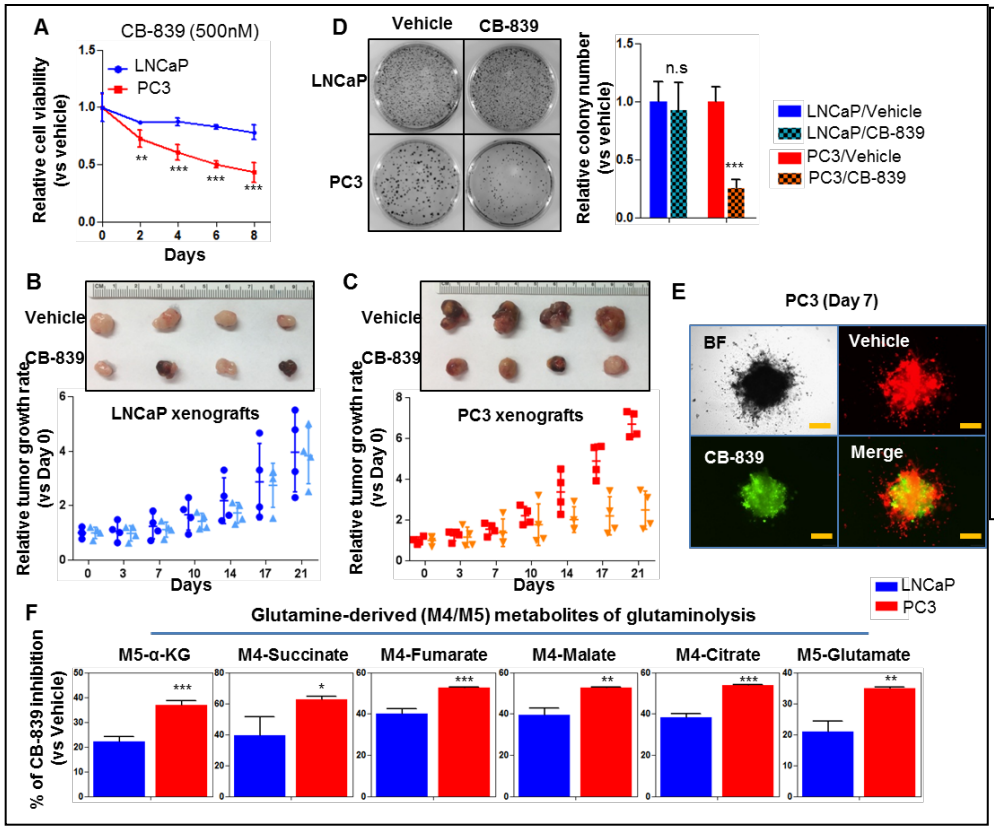

Fig. 6 CB-839 preferentially inhibits GAC-dominant tumors. **A** Cell viability assay showing inhibitory effect of CB-839 on LNCaP and PC3 cells. **B and C** Xenograft studies showing CB-839 displays more profound influence on PC3 cells. **D and E** Colony formation and three-dimensional assay showing the effect of CB-839 treatment on clonogenesis and migration ability of the indicated PCa cells. **F** 13C5-glutamine tracing analysis showing the inhibitory effect of CB-839 on the conversion of glutamine to the downstream metabolites.

There are several highly selective GLS1 inhibitors. Among them. CB-839 has been used in clinical trials for several cancer types. Importantly, CB-839 has been shown to be quite efficacious in inhibiting triple-negative breast cancer, which also highly expresses the GAC isoform. Thus, we wondered if CB-839 can also inhibit tumor growth of advanced PCa where GAC is abundant. In comparison to the hormone-sensitive LNCaP cells, PC3 cells showed much more pronounced sensitivity to CB-839 treatment in vitro and in vivo (Fig. 6A-C). CB-839 also displayed dramatic inhibitory effect in cell clonogenesis and migration (Fig. 6D-E). In terms of enzymatic kinetics, CB-839 caused more inhibition of the downstream metabolite generation as determined by 13C5-glutamine tracing analysis (Fig. 6F). These results suggest that CB-839 preferentially inhibits tumors where GAC is predominant and such inhibition of glutamine catabolism is more efficacious in advanced and hormonal therapy resistant PCa than in primary PCa.

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

- Nothing to Report.

How were the results disseminated to communities of interest?

- Two publications have resulted from the DOD grant support.

1. Xu L, Zhao B, Butler W, Xu H, Song N, Chen X, Spencer Hauck J, Gao X, Zhang H, Groth J, Yang Q, Zhao Y, Moon D, George D, Zhou Y, He Y, Huang J. Targeting glutamine metabolism network for the treatment of therapy-resistant prostate cancer. Oncogene. 2022 Feb;41(8):1140-1154. doi: 10.1038/s41388-021-02155-z. Epub 2022 Jan 20. PMID: 35046532.
2. Xu L, Zhao B, Butler W, Xu H, Song N, Chen X, Spencer Hauck J, Gao X, Zhang H, Groth J, Yang Q, Zhao Y, Moon D, George D, Zhou Y, He Y, Huang J. Targeting glutamine metabolism network for the treatment of therapy-resistant prostate cancer. Oncogene. 2022 Feb;41(8):1140-1154. doi: 10.1038/s41388-021-02155-z. Epub 2022 Jan 20. PMID: 35046532

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

- This is the final report. We have successfully completed all the proposed work.

4. Impact:

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

During this study period, we have further explored the mechanism by which GAC is upregulated in advanced PCa. We have revealed that c-Myc and N-Myc, two oncogenic drivers of PCa, are able to increase GAC expression. We have also illustrated advanced PCa cells' pronounced addition to glutamine. This represents a novel mechanism by which recurrent PCa tumor cells switch their energy and nutrient sources to glutamine, leading to castration resistance. Additionally, we have obtained strong evidence showing that a selective GLS1 inhibitor, CB-839, displays profound inhibitory effect on advanced PCa tumors. Therefore, GLS1 is an important therapeutic target and targeting the metabolic vulnerability of advanced PCa can help patients who have run out of treatment options.

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:

- Nothing to Report.

6. Products

- Results of the funded study have been published:

1. Xu L, Zhao B, Butler W, Xu H, Song N, Chen X, Spencer Hauck J, Gao X, Zhang H, Groth J, Yang Q, Zhao Y, Moon D, George D, Zhou Y, He Y, Huang J. Targeting glutamine metabolism network for the treatment of therapy-resistant prostate cancer. Oncogene. 2022 Feb;41(8):1140-1154. doi: 10.1038/s41388-021-02155-z. Epub 2022 Jan 20. PMID: 35046532.
2. Xu L, Zhao B, Butler W, Xu H, Song N, Chen X, Spencer Hauck J, Gao X, Zhang H, Groth J, Yang Q, Zhao Y, Moon D, George D, Zhou Y, He Y, Huang J. Targeting glutamine metabolism network for the treatment of therapy-resistant prostate cancer. Oncogene. 2022 Feb;41(8):1140-1154. doi: 10.1038/s41388-021-02155-z. Epub 2022 Jan 20. PMID: 35046532

7. Participants & Other Collaborating Organizations

Name:	*Jiaoti Huang*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-1195-1998
Nearest person month worked:	3
Contribution to Project:	*Oversight of the entire project, Hypothesis, design, results interpretation*
Funding Support:	

Name:	*Qing Yang*

Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Experimental design, power calculation, statistics*
Funding Support:	

Name:	*Qing Cheng*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	*Experimental design, results interpretation, informatics*
Funding Support:	

Name:	*William Butler*
Project Role:	*PhD Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Performance of experiments, results interpretation*
Funding Support:	

Name:	*Hong Zhang*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*10*
Contribution to Project:	*Lab management, orders,*
Funding Support:	

Name:	*Xue Jiang*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Performance of experiments, results interpretation*
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

Nothing to Report.

9. Appendices

None