AWARD NUMBER: W81XWH-19-1-0411

TITLE: `Targeting Glutaminase Isoforms for Therapy-Resistant Prostate`

`Cancer`

PRINCIPAL INVESTIGATION: Jiaoti Huang

CONTRACTING ORGANIZATION: Duke University
2200 W Main ST STE 710
DURHAM, NC 27708-4677

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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, this year, we have made the following Key Research Accomplishments:

1.   We have demonstrated a potential mechanism by which AR regulates GLS1 gene expression coupled with its mRNA splicing.
2.   We have demonstrated a role of GAC, the more potent isoform of GLS1, for driving therapeutic resistance and disease progression in vitro and in vivo.
3.   By establishing KGA-/GAC-specific expression cell models, we have demonstrated that GAC displays higher enzymatic activity and its expression leads to more aggressive biological behavior of PCa tumor cells.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
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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 behavior may yield better therapeutic targets and 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 using LNCaP cells have revealed that androgen withdrawal causes a significant accumulation of intracellular glutamine by blocking glutamine utilization, and this finding was confirmed in another androgen-sensitive cell line, LAPC4, which

was reported in last year's annual report. More interestingly, after cancer recurs, glutamine becomes the major nutrient instead of androgen to support growth and proliferation of recurrent tumor cells. Mechanistically, we have obtained evidence that this glutamine metabolism reprogramming is due to AR's regulation of the rate-limiting enzyme, glutaminase 1 (GLS1). Interestingly, GLS1 has two isoforms, KGA and GAC, which possess different enzymatic capabilities. We hypothesize that in addition to regulating the transcription of GLS1 (as demonstrated in last year's report), AR may also regulate GLS1's different splicing contributing to therapy resistance and disease progression. Functionally, GAC is much more potent than KGA in catalyzing glutamine usage. However, the ability for GAC to confer hormonal therapy resistance needs to be validated in PCa. More importantly, although we have obtained strong evidence showing the differential expression of KGA and GAC in different stage of PCa from human tissue microarray staining which is the basis for the hypothesis that an isoform switch of GLS1 may occur during disease development, direct evidence is still lacking. Thus, we worked to confirm our findings in animal models longitudinally by recapitulating the natural history of PCa in human patients.

In summary, in addition to what was accomplished in the first year, in this period we demonstrated: 1. AR mediates the differential splicing of GLS1. 2. GAC is capable of conferring therapy resistance to hormonal therapy. 3. Establish in vitro models for direct comparison of the enzymatic activity of the two isoforms.

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 (Time frame: Months 12-24).

The fact that GAC is largely expressed in the late-stage PCa, such as CRPC and SCNC, suggests that the emergence of GAC may mediate therapy resistance and disease progression. To test this hypothesis, we ectopically overexpressed GAC in hormone sensitive cell line, LNCaP (**Fig. 1A**) and subjected them to the ADT treatment. We found that overexpression of GAC was able to rescue the ADT-induced cell growth arrest (Fig. 1C). To further validate this observation in vivo, we subcutaneously injected the generated cell lines (LNCaP/vector and LNCaP/GAC) into mice. Immunohistochemical staining on the tumor xenografts confirmed the overexpression of GAC in the modified LNCaP cell line (**Fig. 1B**). Surgical castration or sham operation were performed in each group. Consistent with the findings in vitro, we found that ectopic expression of GAC promoted tumor growth and this effect was also evident under the castrated condition (**Fig. 1C and D**).

Additionally, supplementing extra glutamine was able to further enhance cellular viability in ADT treatment while altering glucose concentration did not influence cell growth (**Fig. 2A and B**), indicating that the robust function of GAC was achieved trough a glutamine-dependent mechanism.
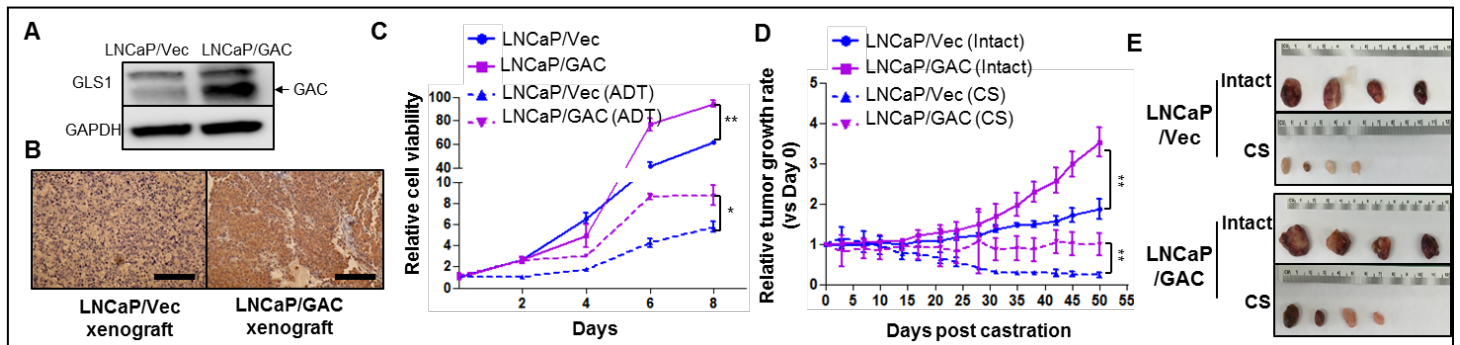
Fig. 1 GAC overexpression rescues the cell proliferation under the presence of ADT. **A and B.** Confirmation of GAC overexpression in LNCaP cells and the corresponding xenografts by western blot and IHC staining. **C.** Cell viability determining LNCaP/Vector and LNCaP/GAC cells in regular and ADT treatment. **D.** Tumor growth rate of LNCaP/Vector and LNCaP/GAC xenografts in control or castrated conditions. **E.** Representative images of tumor volume derived from D.
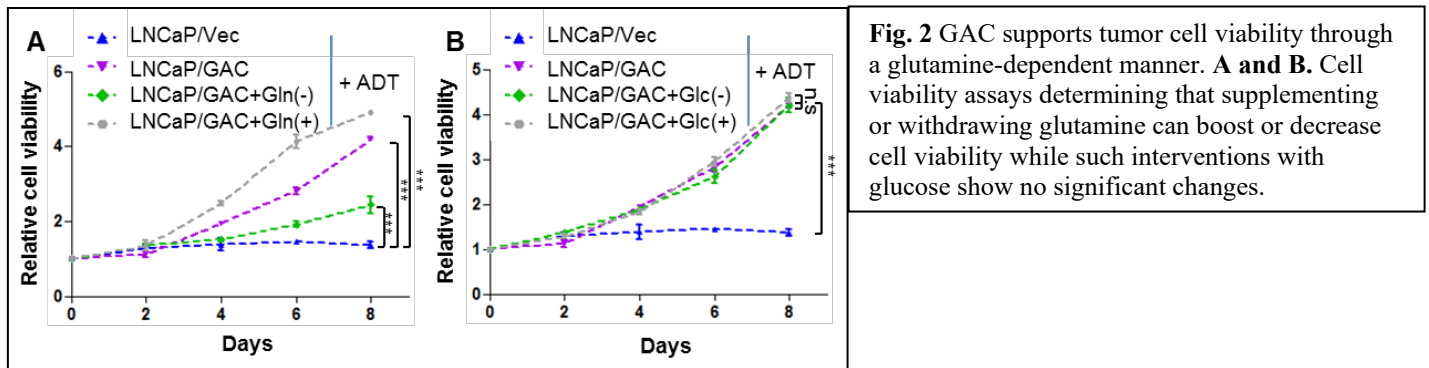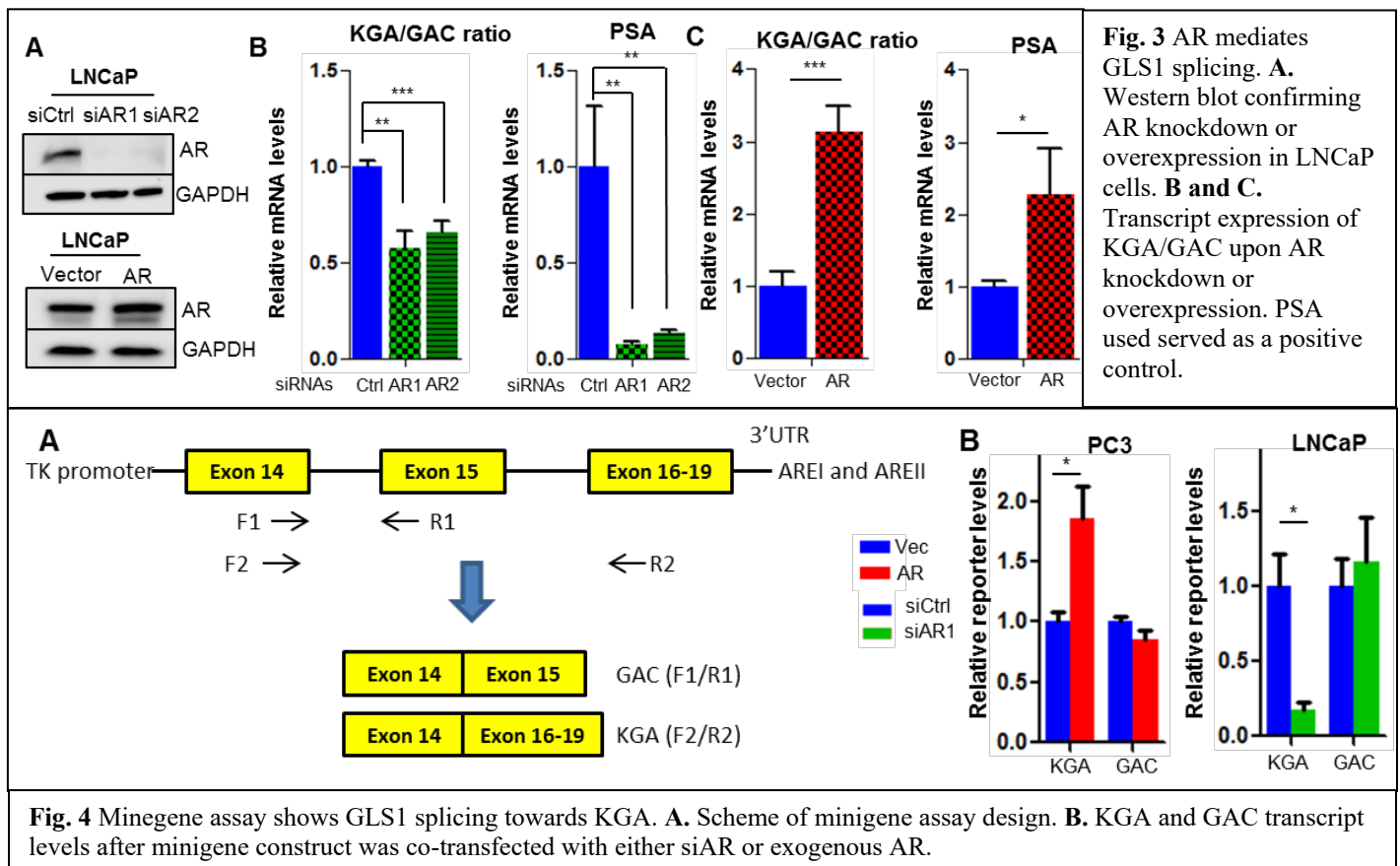

Fig. 2 GAC supports tumor cell viability through a glutamine-dependent manner. **A and B.** Cell viability assays determining that supplementing or withdrawing glutamine can boost or decrease cell viability while such interventions with glucose show no significant changes.

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

Our previous work has revealed that GLS1 is an AR-regulated gene as illustrated by ChIP-seq, ChIP-PCR and luciferase reporter assay (see the first-year annual report). Given that KGA shows positive association with AR level and activity, primarily in hormone-sensitive PCa, whereas GAC is associated with AR-independent characteristics, we hypothesized that AR may regulate GLS1 splicing. To answer this question, we knocked down AR expression by two sets of siRNA and specific primers for both isoforms were employed for the mRNA detection (KGA Forward: 5'-ACTGGAGATGTGTCTGCACTTCGAAG-3', Reverse: 5'-CCAAAGTGCAGTGCTTCATCCATGGGAGTG-3'. GAC Forward: 5'-TTGGACTATGAAAGTCTCCAACAAGAAC-3', Reverse: 5'-CCATTCTATATACTACAGTTGTAGAGATGTCCTC-3') (**Fig. 3A**). Although both KGA and GAC transcript levels were decreased upon loss of AR, we found a significant decrease of the ratio of KGA/GAC in both sets of siRNA targeting AR (**Fig. 3B**). Conversely, overexpression of AR dramatically elevated the ratio of KGA/GAC in LNCaP cells (**Fig. 3A and 3C**). These data suggest that AR promotes GLS1 splicing towards the KGA isoform.

To further determine if AR could affect GLS1 mRNA splicing, we performed a minigene assay using a GLS1 minigene reporter plasmid covering exon 14 to exon 19 of GLS1. Briefly, we obtained gBlocks custom-made by Integrated DNA Technologies (IDT), which contains exon 14-19 and their flanking intron regions of GLS1 gene and used it as the template for PCR amplification. The resulting DNA product was cloned into the plasmid vector pRL-TK (Promega) between NheI and XbaI sites. DNA sequencing confirmed the integrity of the final construct (**Fig. 4A**). The constructed plasmid was co-transfected with either a small-interfering RNA targeting AR into AR-positive LNCaP cells or AR into AR-negative PC3 cells. We showed that knocking down or overexpressing AR significantly upregulated or reduced KGA transcript levels but had little impact on GAC transcript levels (**Fig. 4B**). These results suggest that GLS1 RNA splicing is coupled to GLS1 gene transcription and is similarly governed by AR. Specifically, activated AR binds directly to GLS1 to upregulate its transcription and simultaneously regulate alternative splicing to favor the expression of the KGA isoform.

Fig. 3 AR mediates GLS1 splicing. A. Western blot confirming AR knockdown or overexpression in LNCaP cells. B and C. Transcript expression of KGA/GAC upon AR knockdown or overexpression. PSA used served as a positive control.

Fig. 4 Minegene assay shows GLS1 splicing towards KGA. A. Scheme of minigene assay design. B. KGA and GAC transcript levels after minigene construct was co-transfected with either siAR or exogenous AR.

Although the above results reveal AR's metabolic function in PCa and how hormonal therapy inhibits KGA expression and glutamine catabolism to achieve therapeutic effect, it appears that the isoform switch can not be achieved by a single driver. Numerous studies have implicated that AR ligand-independent mechanisms dominate disease progression towards either CRPC or SCNC. Interestingly, MYC family proteins have been shown to regulate GLS1, particularly GAC, through either a direct or an indirect mechanism in other cancer types. MYC gene amplification has also been reported as one of the significant observation in recurrent PCa human tissues. Thus, these backgrounds impel us to further peruse the potential regulation of MYC on GAC,

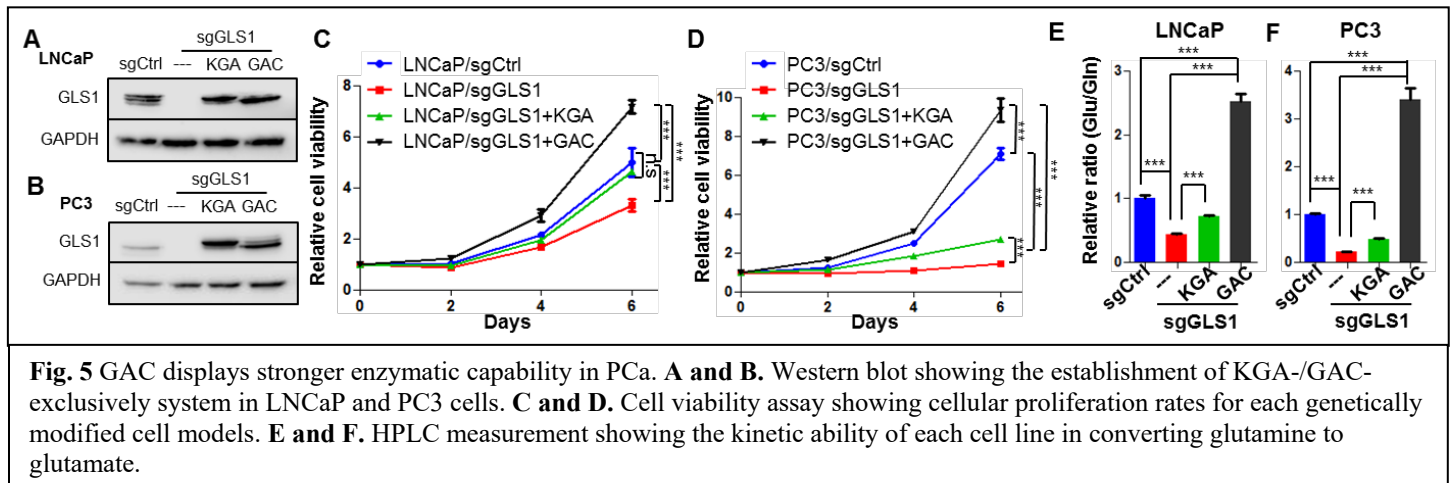which may reveal an important mechanism by which GAC becomes the predominant variant when disease recurs.

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).

To be performed.

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).

Our prior work has demonstrated that LNCaP cells, where KGA is dominant, are more sensitive to the reduction of KGA in terms of cell proliferation and glutamine utilization, whereas in PC3 cells, loss of GAC caused a significant decrease of glutamine utilization and a dramatic cell growth arrest. Given that PC3 is an AR-independent advanced PCa cell model, these results suggest that the strong enzymatic activity of GAC may be the main driver for tumor progression in an AR-independent manner.

Fig. 5 GAC displays stronger enzymatic capability in PCa. **A and B.** Western blot showing the establishment of KGA-/GAC-exclusively system in LNCaP and PC3 cells. **C and D.** Cell viability assay showing cellular proliferation rates for each genetically modified cell models. **E and F.** HPLC measurement showing the kinetic ability of each cell line in converting glutamine to glutamate.

To further validate that GAC is the enzymatically stronger isoform, we generated cell models that lack KGA or GAC, respectively. Basically, we used CRISPR/Cas9 technology to knock out GLS1 gene and employed lentiviral-mediated vectors to cDNA to introduce KGA or GAC, respectively, back to the GLS1-deficient cell lines. Using this strategy, we successfully established two sets of genetically modified cell models: LNCaP/Ctrl, LNCaP/sgGLS1, LNCaP/sgGLS1+KGA, LNCaP/sgGLS1+GAC, PC3/Ctrl, PC3/sgGLS1, PC3/sgGLS1+KGA and PC3/sgGLS1+GAC (Fig.5A-B). We then used MTS assay to determine the viability of each cell line. In both LNCaP and PC3 backgrounds, loss of GLS1 significantly reduced cell proliferation rates (Fig. 5C-D). Interestingly, we found that introducing back KGA only marginally rescued the cell growth arrest due to GLS1 depletion (Fig. 5C-D). In contrast, with the expression of GAC, cell proliferation was restored in both cell

models (Fig. 5C-D). Given that LNCaP and PC3 represent totally different genetic and phenotypic features, these results suggest that GAC is more potent regardless of disease stage. Additionally, to further show that GAC is enzymatically stronger, we determined intracellular glutamine and glutamate levels in these cell lines. The ratio of glutamate/glutamine can appropriately reflect the kinetics of conversion from glutamine to glutamate. Similarly, GLS1 deficiency led to significant decrease of glutamine utilization in both LNCaP and PC3 cells (Fig. 5E-F), while the ratio of glutamate/glutamine was much higher in GAC-expressing cell models (Fig. 5E-F), further supporting the notion that GAC possesses a much stronger enzymatic activity than KGA.

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

-   Nothing to Report.

How were the results disseminated to communities of interest?

Xu L, Yin Y, Li Y, Chen X, Chang Y, Zhang H, Liu J, Beasley J, McCaw P, Zhang H, Young S, Groth J, Wang Q, Locasale JW, Gao X, Tang DG, Dong X, He Y, George D, Hu H, Huang J. A glutaminase isoform switch drives therapeutic resistance and disease progression of prostate cancer. Proc Natl Acad Sci U S A. 2021 Mar 30;118(13):e2012748118. doi: 10.1073/pnas.2012748118.

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

-   We will finish the remaining work proposed in the Statement of Work.

4. **Impact:**

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

We have demonstrated the biological function of GAC in prostate cancer which contributes to therapeutic resistance and disease progression.

Another milestone we have achieved during the past year is that we have mechanistically revealed how AR regulates GLS1 transcription and splicing. AR is able to directly regulate GLS1 expression by binding to the 3'UTR of GLS1 gene. Furthermore, AR plays critical role in promoting GLS1 splicing towards the KGA isoform.

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

- **Nothing to report**

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:	*1*
Contribution to Project:	*Oversight of the entire project, Hypothesis, design, results interpretation*
Funding Support:	*N/A*

Name:	*Hong Zhang*
Project Role:	*Research specialist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*2*

Contribution to Project:	*Laboratory management including budgets and ordering supplies. Performance of some experiments as well as maintenance of supplies.*
Funding Support:	*N/A*
Name:	*William Butler*
Project Role:	*PhD Research Assistant*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*7*
Contribution to Project:	*Performance of experiments, results interpretation*
Funding Support:	*N/A*

Name:	*Qing Cheng*
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1*
Contribution to Project:	Data management. Bioinformatics analysis.
Funding Support:	*N/A*

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

- See attached.

What other organizations were involved as partners?

- Nothing to Report.

8. **Special Reporting Requirements**

Nothing to Report.

9. **Appendices**

1. Changes in Active Support

2. Award Chart

3. Publication: *A glutaminaseisoform switch drives therapeutic resistance and disease progression of prostate cancer*

Jiaoti Huang, MD, PhD

No Longer Active (Previous)
Title: Novel Tumor Suppressive Role of Phosphodiesterases in Prostate Cancer (PI: Huang, J.)
Time Commitment: 0%   0.0 calendar months*
Supporting Agency: DOD (W81XWH-16-1-0292)
Name and address of the Funding Agency's Procuring Contracting/Grants Officer: N/A
Performance Period: 08/01/2016 – 07/31/2021 (NCE)
Level of Funding:
Brief description of project's goals: The goal of this study is to investigate the roles of NF90 in prostate cancer, how it mediates the newly characterized function of EZH2 in transcriptional activation of the AR gene, and the efficacy of a new combinatorial therapeutic approach.
Specific Aims: Aim 1) Determine how NF90 cooperates with EZH2 to induce AR expression. Aim 2) Examine the molecular functions of NF90 in prostate cancer and determine NF90 expression in primary specimens and its association with EZH2. Aim 3) Characterize the role of NF90 in prostate cancer progression and test the efficacy of NF90/EZH2 inhibition in suppressing mCRPC and sensitizing tumors to enzalutamide.
Dr. Huang does not maintain effort on this project in its second NCE period

Title: Assessment of macrophage density in historical tNEPC tissue samples (PI: Huang, J.)
Time Commitment: 1%   0.12 calendar months
Supporting Agency: BioXcel Therapeutics
Name and address of the Funding Agency's Procuring Contracting/Grants Officer: N/A
Performance Period: 02/11/2019-02/10/2021
Level of Funding:
Brief description of project's goals: This study entails staining ~30 cases of neuroendocrine prostate cancer with 2 antibodies (anti-CD68 and anti-CD163).
Specific Aims: None specified

Title: Histologic and Immunohistochemical Biomarkers for Heavily Treated Metastatic Prostate Cancer (PI: Huang, J.)
Time Commitment: 30%   3.6 calendar months
Supporting Agency: NIH/NCI (5R01-CA205001-05)
Name and address of the Funding Agency's Procuring Contracting/Grants Officer: Jacquelyn Saval, (240) 276-6312, savalj@mail.nih.gov
Performance Period: 07/01/2016 – 06/30/2021
Level of Funding:
Brief description of project's goals: The major goals of the proposal are 1) Identify biomarkers to predict the outcome of patients with heavily treated metastatic adenocarcinoma of prostate origin and 2) Establish an immunohistochemical profile to aid in the diagnosis of the newly identified PCa histologic variant CYBAC.
Specific Aims: Aim 1) Identify biomarkers that predict the prognosis of patients with metastic AdenoCa. Aim 2) Establish diagnostic criteria for the newly identified Pca histologic Variant CYBAC.

Title: Fortis Core Laboratory Agreement (PI: Huang, J.)
Time Commitment: 1%   0.12 calendar months
Supporting Agency: Fortis Therapeutics, Inc.
Name and address of the Funding Agency's Procuring Contracting/Grants Officer: N/A
Performance Period: 08/02/2018-08/01/2021
Level of Funding:
Brief description of project's goals: Duke shall make the Core Lab available to collaborate with Sponsor for the analysis of components of Sponsor's clinical study for evaluation of Sponsor's study drug.
Specific Aims: None specified

<u>New Active</u>
Title: Targeting NUAK2 to mitigate obesity-enhanced prostate cancer growth and metastasis (PI: Macias, E.)
Time Commitment: 3%   0.36 calendar months
Supporting Agency: DOD (W81XWH-21-1-0854)
Name and address of the Funding Agency's Procuring Contracting/Grants Officer: N/A
Performance Period: 09/1/2021-08/31/2024
Level of Funding:
Brief description of project's goals: The major goal of this proposal is to test if targeting NUAK2 genetically or with therapeutics will inhibit the effects of obesity on lethal prostate cancer.
Specific Aims: Aim 1) Evaluate the therapeutic potential of targeting NUAK2 in obesity-enhanced PC growth. Aim 2) Determine the therapeutic value targeting NUAK2 on obesity-enhanced PC metastasis. Aim 3) Evaluate the mechanisms by which NUAK2 promotes PC growth and progression under increased lipid and cholesterol rich host conditions.
Role: Co-Investigator

Qing Yang, PhD

<u>No Longer Active (Previous)</u>
Title: To Enhance Breast Cancer Survivorship of Asian Americans
Time Commitments: 0 calendar months
Supporting Agency: NIH/NCI 1R01-CA203719-01
6701 Democracy Blvd. Bethesda, MD 20892-4874
Contracting/Grants Officer: Rosemary Ward
Performance Period: 6/1/2016 - 5/31/2021
Level of Funding:
Project Goals: The goals of this study are to: (a) implement the program into various health care settings; (b) determine if the TICAA will lead to long-term improved health outcomes; and (c) fundamentally enhance the methodology/paradigm of culturally tailored technology-based interventions for ethnic minority groups of breast cancer survivors.
Specific Aims: Determine whether the intervention group (who uses the TICAA and the information on breast cancer by the American Cancer Society [ACS]) will show significantly greater improvements than the control group (who uses only the information on breast cancer by the ACS) in **primary outcomes** of the TICAA (needs for help, physical and psychological symptoms, and quality of life) from baseline (*pre-test*) to *Time Points 1 (post 1-month; primary) and 2 (post 3-months; secondary)*. **Aim 2.** Identify theory-based variables (attitudes, self-efficacy, perceived barriers, and social influences related to breast cancer survivorship) that **mediate** the intervention effects of the TICAA on the primary outcomes of the TICAA from baseline to Time Point 1.
Aim 3 (exploratory). Explore whether the effects of the TICAA on the primary outcomes are **moderated** by background factors and disease factors.
Overlap: None
Role: Co-Investigator

<u>New Active</u>
Title: EXpanding Technology-Enabled, Nurse-Delivered Chronic Disease Care (EXTEND)
Time Commitments: 1.43 calendar months
Supporting Agency: NIH/NINR 1R01-NR019594-01
National Institute of Nursing Research National Institutes of Health

31 Center Drive, Room 5B03
Bethesda, MD 20892-2178
Contracting/Grants Officer: Brian Albertini **Performance Period**: 3/15/2021 - 12/31/2025
Level of Funding:

Project Goals: The current proposal, EXpanding Technology-Enabled, Nurse-Delivered Chronic Disease Care (EXTEND) seeks to address current evidence gaps around real-world use of mobile technology-enabled telehealth for clinic-refractory chronic diseases, using high-risk patients with PPDM and hypertension.
Specific Aims: We will guide scaling and dissemination of the EXTEND interventions by: (A) stakeholders to clarify implementation barriers, facilitators and process requirements; and (B) comparing intervention costs against potential reimbursement. Finally we will explore the value of combining mobile monitoring and EHR data for predicting patient safety events in the study cohort over 24 months. Complex, clinic-refractory conditions like PPDM and hypertension demand innovative strategies such as the proposed intervention to improve outcomes and reduce complications and costs. Results will inform subsequent multi-site implementation and dissemination efforts.
Overlap: None
Role: Co-Investigator

Title: Integrating Risk Trajectories and Social Determinants to Enhance Cardiovascular Risk Assessment in Older Adults
Time Commitments: 0.43 calendar months
Supporting Agency: NIH/NIA 1R01-AG069938-01A1
National Institute on Aging Building 31, Room 5C27 31 Center Drive, MSC 2292. Bethesda, MD 20892
Contracting/Grants Officer: John Bladen
Performance Period: 8/15/2021 - 4/30/2026
Level of Funding:
Project Goals: We propose to address the limitations of the current CVD risk assessment tool by incorporating changes in risk factors over time and integrating social determinants of health to improve CVD risk-prediction and its translation to disease prevention in U.S. older adults.
Specific Aims: Aim 1: Classify trajectories of CVD risk factors among U.S. older adults. Aim 2: Predict CVD based on multi-trajectory profiles of risk factors. Aim 3: Identify factors associated with multi-trajectory risk profiles and CVD outcomes.
Overlap: None
Role: Co-Investigator

W81XWH1910411 / Targeting Glutaminase Isoforms for Therapy Resistant Prostate Cancer

PI: Jiaoti Huang, Duke University, North Carolina
Budget: $965,725

Topic Area: PCRP
Mechanism: IDA

Research Area(s): SCS Coding

Award Status: 09/30/2019 – 09/29/2022

Study Goals:

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.

Specific Aims:

- Aim 1: Functional studies of glutamine metabolism and GLS1 in prostate cancer and hormonal therapy.

- Aim 2: Functional studies of GLS1 isoform switching during therapy failure and disease progression of prostate cancer.

Key Accomplishments and Outcomes:

Publications:

1. Xu L, Yin Y, Li Y, Chen X, Chang Y, Zhang H, Liu J, Beasley J, McCaw P, Zhang H, Young S, Groth J, Wang Q, Locasale JW, Gao X, Tang DG, Dong X, He Y, George D, Hu H, Huang J. A glutaminase isoform switch drives therapeutic resistance and disease progression of prostate cancer. Proc Natl Acad Sci U S A. 2021 Mar 30;118(13):e2012748118. doi: 10.1073/pnas.2012748118.

Patents: none to date
Funding Obtained: none to date

A glutaminase isoform switch drives therapeutic resistance and disease progression of prostate cancer

Lingfan Xu[a], Yu Yin[a,1], Yanjing Li[a], Xufeng Chen[a], Yan Chang[a,2], Hong Zhang[a], Juan Liu[b], James Beasley[c], Patricia McCaw[c], Haoyue Zhang[c], Sarah Young[c,d], Jeff Groth[a], Qianben Wang[a], Jason W. Locasale[b], Xia Gao[b,e], Dean G. Tang[f], Xuesen Dong[g], Yiping He[a], Daniel George[h,i], Hailiang Hu[a,i,j,3], and Jiaoti Huang[a,b,i,3]

[a]Department of Pathology, Duke University School of Medicine, Durham, NC 27710; [b]Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC 27710; [c]Duke University Health System Biochemical Genetics Laboratory, Durham, NC 27713; [d]Department of Pediatrics, Duke University School of Medicine, Durham, NC 27710; [e]Duke Molecular Physiology Institute, Duke University School of Medicine, Durham, NC 27701; [f]Department of Pharmacology and Therapeutics, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263; [g]Department of Urologic Science, Vancouver Prostate Centre, University of British Columbia, Vancouver, BC, Canada BC V6H 3Z6; [h]Department of Medicine, Duke University School of Medicine, Durham, NC 27710; [i]Duke Cancer Institute, Duke University School of Medicine, Durham, NC 27710; and [j]School of Medicine, Southern University of Science and Technology, Shenzhen, China 510515

Cellular metabolism in cancer is significantly altered to support the uncontrolled tumor growth. How metabolic alterations contribute to hormonal therapy resistance and disease progression in prostate cancer (PCa) remains poorly understood. Here we report a glutaminase isoform switch mechanism that mediates the initial therapeutic effect but eventual failure of hormonal therapy of PCa. Androgen deprivation therapy inhibits the expression of kidney-type glutaminase (KGA), a splicing isoform of glutaminase 1 (GLS1) up-regulated by androgen receptor (AR), to achieve therapeutic effect by suppressing glutaminolysis. Eventually the tumor cells switch to the expression of glutaminase C (GAC), an androgen-independent GLS1 isoform with more potent enzymatic activity, under the androgen-deprived condition. This switch leads to increased glutamine utilization, hyperproliferation, and aggressive behavior of tumor cells. Pharmacological inhibition or RNA interference of GAC shows better treatment effect for castration-resistant PCa than for hormone-sensitive PCa in vitro and in vivo. In summary, we have identified a metabolic function of AR action in PCa and discovered that the GLS1 isoform switch is one of the key mechanisms in therapeutic resistance and disease progression.

prostate cancer | therapeutic resistance | glutaminase | GAC

Androgen receptor (AR) is required for the survival of prostate cancer (PCa) cells. Hormonal therapy, by suppressing androgen production and/or inhibiting AR function, is effective as a systemic treatment for PCa. Unfortunately, the therapy will eventually fail, and the development of castration-resistant PCa (CRPC) is inevitable (1–5). Newer drugs abiraterone and enzalutamide provide survival benefits for some CRPC patients, but treatment resistance and disease progression still occur. While most CRPCs still maintain adenocarcinoma histology (CRPC–adeno), some patients will develop an extremely aggressive PCa variant known as small-cell neuroendocrine carcinoma (SCNC) (4, 6).

AR is a physiological molecule and a key transcription factor in multiple organs which binds to numerous genes and activates many different downstream signaling pathways (4, 7, 8). However, which of the AR functions is required for PCa cell survival and the underlying molecular mechanisms of tumor cell inhibition after AR blockade remain unclear. We hypothesize that among many different functions of AR activation, only some are directly related to PCa cell survival. If we can identify the downstream effectors of AR responsible for PCa survival, future treatments targeting only the downstream effectors instead of AR can be more specific and effective as well as independent of tumor cells' AR status, resulting in better efficacy and fewer side effects.

Although genomic, epigenetic, transcriptional, translational, and posttranslational alterations all contribute to cancer initiation and progression, the final common pathway that regulates tumor cell proliferation is cellular metabolism. Abnormal metabolism is a

key cellular process that underlines cancer cells' malignant behavior (9–11). We hypothesize that AR regulates important metabolic pathways in PCa cells to support cell proliferation, and inhibition of AR by hormonal therapy disrupts the metabolic program of tumor cells, leading to tumor inhibition. We further hypothesize that tumor cells adapt to the androgen-deprived environment by restoring the metabolic function, resulting in tumor recurrence and disease progression. In this study, we show that AR up-regulates the expression of glutaminase 1 (GLS1), thus promoting glutamine utilization which is required for optimal growth of PCa cells. Hormonal therapy inhibits AR function, resulting in down-regulation of GLS1 which in turn suppresses glutamine utilization and tumor cell proliferation. Eventually the tumor cells

Significance

We report that androgen receptor (AR) promotes glutaminase 1 (GLS1) expression and glutamine utilization to support the survival of prostate cancer (PCa) cells. Hormonal therapy inhibits AR and decreases GLS1 expression and glutamine utilization to achieve therapeutic effect. Our results suggest that eventually the tumor cells switch GLS1 expression from the AR-dependent KGA isoform to the androgen-independent and enzymatically more potent GAC isoform, which increases glutamine utilization and can contribute to the development of castration-resistant PCa. Our work has discovered a previously unknown AR function, a metabolic mechanism of hormonal therapy and an important therapeutic target more specific than AR. Targeting GLS1 may achieve similar therapeutic efficacy but without the side effects resulting from inhibiting AR's other important physiologic functions.

express an androgen-independent GLS1 isoform resulting in tumor recurrence and disease progression.

Results

Androgen Deprivation Therapy Suppresses Proliferation and Metabolic Activities of PCa Cells while Inducing Glutamine Accumulation. Since AR is critical for PCa survival, we hypothesized that AR function likely supports certain metabolic activities required by the tumor cells. We took an unbiased approach to test this hypothesis by conducting a metabolite-profiling study of two hormone-sensitive PCa cell lines, LNCaP and LAPC4, cultured either with (normal fetal bovine serum, FBS) or without androgen (charcoal-stripped FBS which mimics the clinically used androgen deprivation therapy or ADT), respectively. Androgen withdrawal inhibited the proliferation of the tumor cells (*SI Appendix*, Fig. S1*A*) and resulted in remarkable changes in the levels of many metabolites

and metabolic pathways in both cell lines at different time points, as illustrated by principal component analysis, heat map alignment, and pathway enrichment analysis (*SI Appendix*, Fig. S1 *B–F*). They included metabolites involved in glycolysis, tricarboxylic acid (TCA) cycle, nonessential amino acids (NEAA), nucleotides, and pentose phosphate pathway (PPP).

The decrease in metabolite levels after androgen withdrawal was consistent with the observation that tumor cells' proliferation was significantly inhibited by ADT. Surprisingly, we observed a significant increase in glutamine levels in the two cell lines after ADT (Fig. 1*A* and *SI Appendix*, Fig. S2 *A–D*). The increased intracellular glutamine could be caused by either enhanced glutamine synthesis or decreased glutamine catabolism by the tumor cells (*SI Appendix*, Fig. S2*E*). By examining the expression of glutamine transporter ASCT2 (encoded by *SLC1A5* gene) in LNCaP cells cultured with or without androgen, we found that
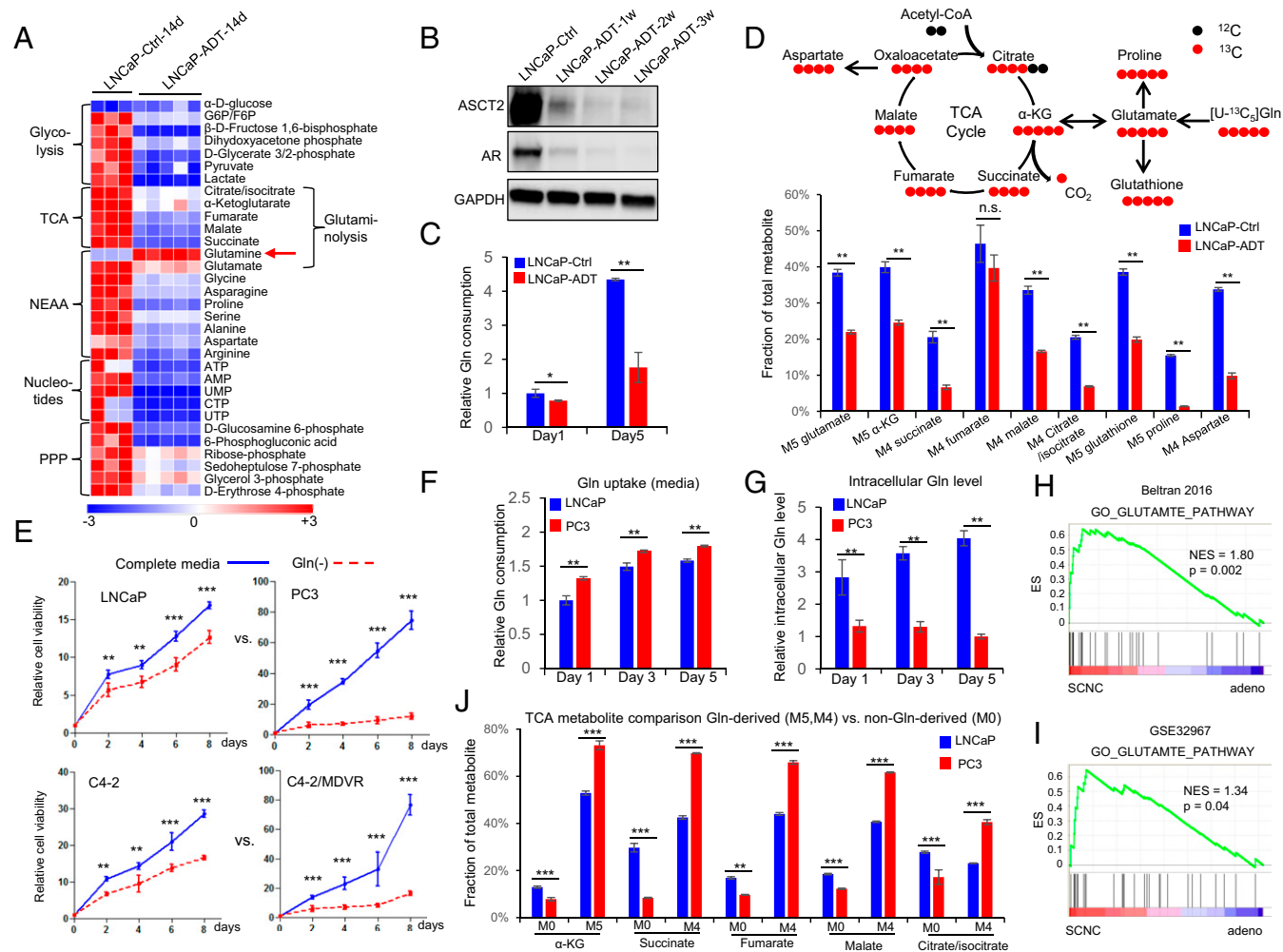
Fig. 1. Androgen deprivation inhibits glutamine catabolism, and therapy-resistant PCa cells are more addicted to glutamine. (*A*) Heat map shows that ADT decreases levels of metabolites involved in important metabolism pathways (*n* = 3 and 5 replicates for the control and ADT groups, respectively). (*B*) Western blot shows that glutamine transporter ASCT2, similar to AR, is decreased after ADT. (*C*) UPLC-MS analysis of cell culture medium to compare glutamine consumption with or without ADT (*n* = 3 cultures per group). (*D*) Tracing of ¹³C-labeled glutamine influx and mass isotopomer analysis of [U–¹³C₅] glutamine-derived metabolites in LNCaP cells with or without ADT. α-KG, α-ketoglutarate (*n* = 3 cultures per group). (*E*) Relative cell viability of LNCaP, PC3, C4-2, and C4-2MDVR cells cultured with or without glutamine (*n* = 3 replicates for two independent experiments). (*F* and *G*) Ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis of glutamine uptake and intracellular glutamine levels in LNCaP and PC3 cells. Metabolites were extracted from culture medium and cell pellets at the indicated time points (*n* = 3 cultures per group). (*H* and *I*) GSEA of "GO_GLUTAMATE_PATHWAY" gene sets to compare SCNC and adenocarcinoma in Beltran 2016 (12) and GSE32967. ES, enrichment score; NES, normalized enrichment score. (*J*) Mass isotopomer analysis of TCA cycle metabolite abundance in LNCaP and PC3 cells (*n* = 3 cultures per group). M0 represents the non-Gln–derived (mainly glucose-derived) metabolite pool, whereas M5 and M4 represent the ¹³C-labeled Gln-derived metabolite pool. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 by two-tailed Student's *t* test. n.s., not significant.

ADT significantly decreased ASCT2 message RNA (mRNA) and protein expression (Fig. 1B and *SI Appendix*, Fig. S2F) and inhibited glutamine uptake from the medium (Fig. 1C), suggesting that increased intracellular glutamine after ADT is not a result of increased glutamine uptake. This conclusion was further supported by the fact that varying extracellular glutamine concentration had little impact on ADT-induced growth arrest of tumor cells (*SI Appendix*, Fig. S2G).

Glutamine is an NEAA which can be synthesized from other nutrients by the enzyme glutamine synthetase (GS), in addition to direct uptake from the extracellular space. The $^{13}C_5$-labeled glutamine-tracing study showed a reduction of intracellular M5–glutamine fraction after ADT, consistent with attenuated glutamine uptake. However, we noticed an increase in M0–glutamine fraction after ADT (*SI Appendix*, Fig. S2H), indicating increased glutamine synthesis inside the tumor cells. Indeed, we observed a stepwise elevation of GS expression after androgen withdrawal (*SI Appendix*, Fig. S2I). The $^{13}C_6$-labeled glucose-tracing analysis also showed that ADT resulted in increased glutamine production from glucose (*SI Appendix*, Fig. S2J). These findings suggest that in PCa cells, ADT inhibits glutamine uptake from the extracellular space but increases glutamine production intracellularly.

The intracellular level of glutamine can also be influenced by its catabolism. The $^{13}C_5$-labeled glutamine-tracing analysis revealed that ADT significantly reduced the levels of almost all the metabolites downstream of glutamine catabolism, including glutamate (M5), glutathione (M5), proline (M5), aspartate (M4), α-ketoglutarate (α-KG) (M5), and other TCA cycle intermediates (M4) (Fig. 1D), suggesting that ADT suppresses glutamine utilization by the tumor cells. To determine if this may be responsible for the observed growth inhibition after ADT, we supplemented the culture media with the downstream metabolites NEAA (excluding glutamine) and α-KG and observed a substantial rescue of ADT-induced growth inhibition (*SI Appendix*, Fig. S2 K and L). Together, these studies demonstrate that a mechanism by which ADT inhibits tumor cell viability is in part through suppression of glutamine catabolism.

Advanced and Therapy-Resistant PCa Cells Are More Addicted to Glutamine. The above results suggest that AR promotes glutamine utilization which may be essential for PCa cell survival. Hormonal therapy, through inhibition of AR function, inhibits glutamine utilization, leading to inhibition of cellular proliferation. Since hormonally treated PCa always recurs as CRPC–adeno or SCNC (3, 4), we hypothesized that the recurrent tumor cells may have regained the ability to utilize glutamine despite the absence of androgen. To test this hypothesis, we examined the degree of glutamine dependency across several PCa cell lines that represent different stages of PCa: LNCaP (AR-positive, androgen-sensitive) versus PC3 [AR-negative, castration-resistant, with features of SCNC (13, 14)] and C4-2 (castration-resistant but enzalutamide-sensitive) versus C4-2MDVR (C4-2 cells that are enzalutamide-resistant after long-term culture with the drug). Glutamine was required for optimal growth of all examined PCa cells (Fig. 1E). However, PC3 and C4-2MDVR cells, representing very advanced stages of the disease, showed higher degrees of dependency on glutamine than LNCaP and C4-2 cells (Fig. 1E). In contrast, glucose deprivation had much smaller effect on these PCa cells compared to glutamine deprivation (*SI Appendix*, Fig. S3A), suggesting that PCa cells become increasingly addicted to glutamine as the disease progresses. This conclusion was further supported by glutamine consumption study showing that PC3 and C4-2MDVR cells utilized more glutamine than LNCaP and C4-2 cells (Fig. 1 F and G and *SI Appendix*, Fig. S3 B and C), respectively. Glutamine addiction of advanced PCa was also observed in gene set enrichment analysis (GSEA), showing that the expression levels of genes involved in glutamate metabolism pathway were correlated with those genes associated with SCNC, the most

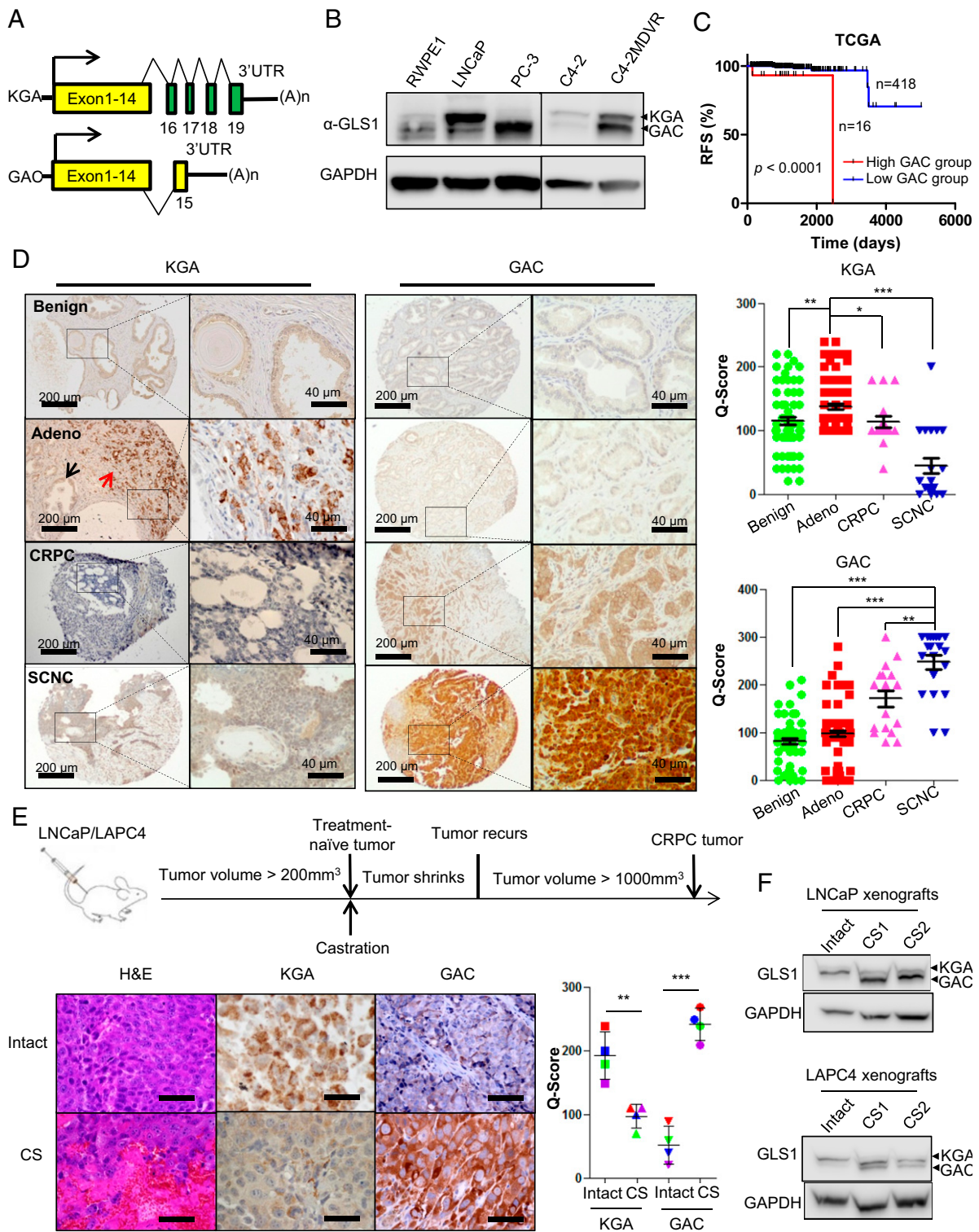advanced, therapy-resistant and lethal histologic variant of PCa (Fig. 1 H and I).

Our group has reported that in advanced PCa, glucose is shunted toward the glycolytic pathway instead of entering the more energy-efficient TCA cycle (15, 16). However, more advanced PCa cells have higher levels of TCA cycle activity than hormone-sensitive PCa (17), suggesting that they are able to efficiently use nutrient sources other than glucose to maintain the TCA cycle. We performed a ^{13}C-labeled glutamine-tracing experiment which showed that the AR-negative PC3 cells and the enzalutamide-resistant C4-2MDVR cells had more α-KG (M5) and other TCA cycle intermediates (M4) derived from glutamine, whereas LNCaP and C4-2 cells had more TCA cycle intermediates derived from nonglutamine sources (M0) (Fig. 1J and *SI Appendix*, Fig. S3 D and E). These data suggest that instead of glucose, glutamine becomes the major carbon source for the maintenance of TCA cycle in advanced and therapy-resistant PCa (*SI Appendix*, Fig. S3F).

The critical role of glutamine in advanced PCa was also demonstrated by the metabolite-profiling study showing that glutamine and TCA cycle-related metabolic pathways were enriched in tumor cells that are AR-negative or show features of prostate SCNC such as PC3 and C4-2/N-Myc cells [C4-2 cells overexpressing N-Myc (18–20)] (*SI Appendix*, Fig. S4A). We also observed that glutamine deprivation led to distinct metabolic changes in AR-negative PC3 cells and AR-positive LNCaP cells (*SI Appendix*, Fig. S4 B–F), further supporting the notion that primary and advanced tumors respond differently to glutamine withdrawal.

Differential Expression of Glutaminase Isoforms in PCa of Different Stages. The results above suggest that hormonal therapy inhibits PCa cell survival in part through inhibition of glutamine utilization, but advanced and therapy-resistant PCa cells regain the ability to use glutamine which may contribute to therapy resistance and disease progression. To investigate the underlying mechanism, we examined the expression of glutaminase (GLS, including GLS1 and GLS2), the first rate-limiting enzyme in glutaminolysis (21–23), in a panel of PCa cell lines. GLS2 can be a tumor suppressor (24, 25), and our study showed that it was not expressed in PCa tissues or a panel of PCa cell lines examined (*SI Appendix*, Fig. S5 A and B). The other glutaminase, GLS1, has two isoforms, kidney-type glutaminase (KGA) and glutaminase C (GAC), due to differential RNA splicing (26) (Fig. 2A). GAC is much more potent than KGA in catalyzing the conversion of glutamine to glutamate in vitro and in vivo (27). We found that KGA was highly expressed in AR-positive PCa cells, such as LNCaP, VCaP, LAPC4, and C4-2, whereas GAC expression predominated in the more advanced and therapy-resistant C4-2MDVR and LNCaP95 cells (both resistant to enzalutamide) as well as PCa cells that are AR-negative and/or with neuroendocrine/SCNC features such as PC3, NCI-H660, and DU145. (Fig. 2B and *SI Appendix*, Fig. S5 C and D). We also observed a correlation of the enzymatically strong GAC isoform with increasing Gleason scores in the TCGA database. The mRNA level of GAC, but not KGA, showed a stepwise increase with increasing Gleason scores (*SI Appendix*, Fig. S5E). Additionally, patients whose tumors harbored high levels of GAC had much shorter survival than those whose tumors had low GAC (Fig. 2C). In contrast, KGA levels did not show such clinical relevance (*SI Appendix*, Fig. S5F).

Adenocarcinoma of the prostate consists of bulk luminal-type tumor cells that are AR-positive and a minor population of AR-negative neuroendocrine (NE) tumor cells. Our group has recently demonstrated that CXCR2, an interleukin-8 receptor, is specifically expressed in NE tumor cells of human PCa (28). In the same study, we showed that NE tumor cells possess molecular features associated with aggressive cancer phenotypes such as stemness, epithelial-mesenchymal transition, and angiogenesis. We purified NE tumor cells from fresh primary human PCa tissue (prostatectomy specimens) by fluorescence-activated cell

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Fig. 2. Isoform switch of GLS1 during disease progression of PCa. (*A*) Gene structures of *KGA* and *GAC* isoforms of *GLS1*. (*B*) Western blot to determine KGA and GAC protein levels in RWPE1, LNCaP, PC3, C4-2, and C4-2MDVR cells with an anti-GLS1 antibody that recognizes both isoforms. (*C*) Recurrence-free survival of patients stratified by GAC mRNA expression in the TCGA dataset. Log-rank (Mantel–Cox) test. (*D*) Representative images of KGA and GAC IHC staining of tissue microarrays containing benign prostate tissue (Benign, *n* = 67), primary prostate adenocarcinoma (Adeno, *n* = 76), castration-resistant PCa (CRPC, *n* = 17), and small-cell neuroendocrine PCa (SCNC, *n* = 20). Black and red arrows denote benign and tumor compartments (KGA/Adeno). The *Right* panels are scores for KGA and GAC staining (staining intensity × the percentage of positive cells) by using the Quick-Score (Q-Score) system. *P* values were calculated using unpaired *t* test. Scale bars are shown as indicated. (*E*) Hematoxylin and eosin (H&E) staining and IHC analysis of KGA and GAC expression in mouse LNCaP xenograft tumors before castration (Intact) and in recurrent tumors after castration (CS) (*n* = 4). *Upper* panel shows the experimental procedure. (Scale bar, 40 μm.) *Bottom Right* panel shows a plot of Q-Score for KGA and GAC staining in each xenograft tumor group. (*F*) Western blot analysis of KGA and GAC protein levels in hormone-sensitive xenograft tumors (Intact) and castration-resistant xenograft tumor (CS) with GLS1 antibody. *P* values were calculated using two-tailed Student's *t* test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

sorting using an anti-CXCR2 antibody and determined the ratios of KGA and GAC mRNA levels using isotype-specific primers (29). Luminal-type tumor cells were purified and used as controls. In four of five samples, the ratio of GAC/KGA was dramatically increased in the CXCR2-positive NE cells compared to that in the CXCR2-negative luminal cells (*SI Appendix*, Fig. S5G), further supporting the notion that GAC expression is associated with a more aggressive phenotype.

The above results were confirmed in human PCa tissue by immunohistochemical (IHC) staining of tissue microarrays (TMAs) with KGA- and GAC-specific antibodies, respectively. Consistent with the in vitro data, KGA expression was the highest in untreated (hormone-naive) prostate adenocarcinoma and decreased in the more advanced and aggressive CRPC and SCNC, whereas GAC expression was lower in hormone-naive adenocarcinomas and higher in CRPC and SCNC (Fig. 2D). Notably, KGA expression was inversely correlated with GAC expression in PCa across different clinical stages (hormone-sensitive adenocarcinoma, CRPC and SCNC) (*SI Appendix*, Fig. S5H). These results demonstrate that the two isoforms of GLS1 display differential expression patterns in different stages of PCa. Specifically, the enzymatically weaker KGA isoform is expressed early in the disease process, and as the disease progresses to advanced and therapy-resistant stages, the enzymatically stronger GAC becomes the predominant isoform in tumor cells.

To determine whether the GLS1 isoform switch from KGA to GAC during disease progression can be observed in vivo longitudinally, we employed hormone-sensitive xenograft tumor models wherein established LNCaP and LAPC4 tumors in mice regressed upon castration but eventually recurred as CRPC (30) (*SI Appendix*, Fig. S5 I and J). IHC as well as Western blot analyses of the xenograft tumors showed that in comparison to tumors from intact mice (hormone-sensitive), the recurrent tumors after castration (CRPC) expressed lower levels of KGA but higher levels of GAC (Fig. 2 E and F), again demonstrating a GLS1 isoform switch during the process of therapy resistance and disease progression.

The Isoform Switch from KGA to GAC Drives Therapy Resistance. We next determined whether the isoform switch of GLS1 from KGA to GAC increases glutamine utilization in tumor cells and leads to castration resistance. We specifically knocked down KGA and GAC, respectively, using short-hairpin RNAs (shRNAs) in LNCaP and PC3 cells (Fig. 3 A and B). LNCaP cells were more sensitive to the reduction of KGA in terms of cell proliferation and glutamine utilization (Fig. 3 A and C), consistent with our observation that KGA is the dominant isoform of GLS1 in androgen-dependent cells, including LNCaP cells. In PC3 cells, in contrast, loss of GAC caused a significant decrease of glutamine utilization and a dramatic cell growth arrest, while knocking down KGA had little effect (Fig. 3 B and D). To further validate the function of GAC in tumor cells, we generated CRISPR/Cas9-mediated *GLS1*-deficient cells and introduced back KGA and GAC, respectively (Fig. 3 E and F and *SI Appendix*, Fig. S5K). PC3 cells exhibited a greater degree of dependency on *GLS1* gene compared to LNCaP cells (Fig. 3 E and F). Reexpression of KGA partially rescued cell proliferation in both LNCaP and PC3 cells (Fig. 3 E and F). In contrast, cell proliferation was remarkably increased after reintroducing GAC in the two cell lines (Fig. 3 E and F). These results suggest that the stronger enzymatic activity of GAC confers the rapid proliferation of advanced PCa through enhanced glutamine catabolism. Additionally, in both cell models, the ratio of glutamate/glutamine (reflecting the kinetics of conversion from glutamine to glutamate) was higher in GAC-expressing cell models (Fig. 3 G and H), supporting previous reports that GAC possesses a much stronger enzymatic activity than KGA.
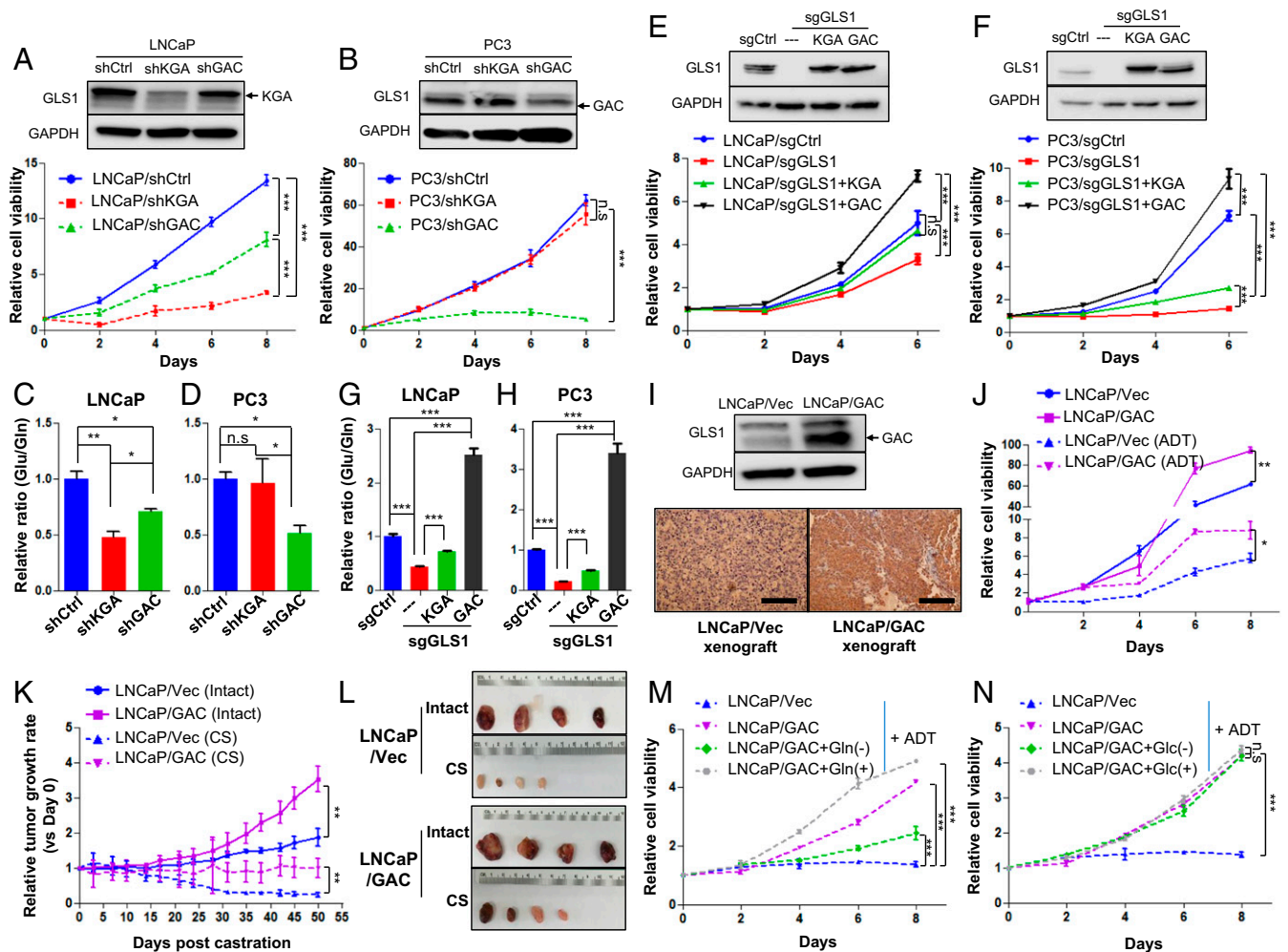
To determine whether the switch from KGA to GAC drives castration resistance, we generated LNCaP cells stably over-expressing the GAC isoform and subjected the cells to androgen deprivation (Fig. 3I). We observed that GAC overexpression in LNCaP cells partially rescued the proliferation of LNCaP cells under androgen-deprived conditions (Fig. 3J). This observation was confirmed in vivo with the xenograft tumor model (Fig. 3I). Overexpression of GAC increased the sizes of LNCaP xenograft tumors in intact animals, and such tumors were more resistant to castration than control tumors (Fig. 3 K and L). Furthermore, increased proliferation of LNCaP cells overexpressing GAC was abrogated by depleting glutamine and enhanced by increasing glutamine concentration in the androgen-deprived medium (Fig. 3M). In contrast, changing glucose concentration had little effect under the same experimental conditions (Fig. 3N), suggesting that overexpression of GAC leads to increased utilization of glutamine and confers the tumor cells resistance to ADT.

GLS1 Inhibitor CB-839 Preferentially Suppresses Advanced and Therapy-Resistant PCa. Having documented that advanced and therapy-resistant PCa overexpress GAC and are more addicted to glutamine, we next studied if therapy-resistant PCa is more sensitive to GLS1 inhibition than primary PCa. We tested a specific GLS1 inhibitor CB-839 (i.e., Telaglenastat, Calithera Biosciences) (31, 32), which is in clinical trials for multiple solid tumors (#NCT02071862), including renal cell carcinoma, for its potential in inhibiting PCa. CB-839 had a more potent inhibitory effect on PC3 cells (IC_{50} = 481 nM) than on RWPE1 (a benign prostate epithelial cell line) and LNCaP cells ($IC_{50} > 1,000$ nM) (Fig. 4A). Furthermore, CB-839 more potently inhibited cell growth, colony formation, and invasion of advanced PCa cells than those of hormone-dependent cells (Fig. 4 B–D and *SI Appendix*, Fig. S6 A and B), supporting our finding that advanced cancer cells are more addicted to glutamine and more sensitive to GLS1 inhibition. In vivo xenograft models further confirmed the more potent inhibitory effect of CB-839 on PC3 xenografts than LNCaP tumors (Fig. 4 E and F) as determined by tumor sizes and the proliferation marker Ki67 (*SI Appendix*, Fig. S6C). In addition, $^{13}C_5$-labeled glutamine-tracing experiments showed that CB-839 inhibited glutamine utilization in both LNCaP and PC3 cells, but the inhibitory effect was more pronounced in the AR-negative, androgen-independent PC3 cells (Fig. 4G and *SI Appendix*, Fig. S6 D and E). These results suggest that inhibition of glutamine catabolism is more efficacious in advanced and hormonal therapy-resistant PCa than in primary PCa.

Next, we determined if CB-839 has different specificity for the two isoforms of GLS1. We employed the previously generated cell lines specifically expressing KGA or GAC (Fig. 3 E and F and *SI Appendix*, Fig. S5K) and subjected them to CB-839 treatment. CB-839 showed a greater inhibitory effect on GAC-expressing cell lines (Fig. 4H).

AR and MYC Cooperatively Contribute to the GLS1 Isoform Switch. A unique feature of PCa is that the tumor cells require androgen for their survival (17, 33). Given that the expression of KGA is associated with a hormone-sensitive state while that of GAC is associated with castration resistance after AR-targeted therapy, we studied if AR regulates *GLS1* alternative splicing during disease progression. We first analyzed the AR ChIP-sequencing datasets (34, 35) and found a strong AR-binding peak in the 3′ untranslated region (UTR) of *GLS1* in dihydrotestosterone (DHT)-stimulated LNCaP cells (Fig. 5A). Detailed DNA sequence analysis revealed two putative androgen response elements (AREs) in this region (*SI Appendix*, Fig. S7A), and ChIP–qPCR verified the binding of AR to this region (Fig. 5B). We then cloned 3′UTR of *GLS1* containing the two AREs into a cytomegalovirus (CMV)-driven *Renilla* luciferase reporter construct (pRL) and measured the luciferase activity in the presence or absence of

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Fig. 3. The GLS1 isoform switch confers castration resistance to PCa cells. (*A* and *B*) Western blots show KGA and GAC expression of LNCaP and PC3 cells transduced with KGA-specific, GAC-specific, or control shRNAs and the corresponding cell viability for each cell line (*n* = 3 replicates for two independent experiments). (*C* and *D*) Glutamine utilization indicated by the ratio of intracellular glutamate/glutamine of cells in *A* and *B* (*n* = 3 cultures per group). (*E* and *F*) Cell viability after knocking out *GLS1* and introducing *KGA* and *GAC* back, respectively (*n* = 3 replicates for two independent experiments). (*G* and *H*) Enzymatic activity of KGA and GAC as measured by the ratio of intracellular glutamate/glutamine in cells described in *E* and *F* (*n* = 3 cultures per group). (*I*) Western blot and IHC staining of GAC overexpression in LNCaP cells and the corresponding xenograft tumor. (*J*) Cell viability of LNCaP cells overexpressing GAC and LNCaP/Vec control in regular medium or charcoal-stripped medium (ADT) (*n* = 3 replicates for two independent experiments). (*K* and *L*) Xenograft tumor growth curves with or without surgical castration (CS) and representative images of tumor size (*n* = 4 in each group). (*M* and *N*) Cell viability of LNCaP/ Vec and LNCaP/GAC treated with glutamine (Gln)/glucose (Glc) depletion or supplemented glutamine/glucose in the charcoal-stripped medium (ADT) (*n* = 3 replicates for two independent experiments). Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by two-tailed Student's *t* test. n.s., not significant.

DHT. DHT up-regulated luciferase activity of the construct containing the *GLS1*-wide-type–3′UTR in comparison to the vector control (*SI Appendix*, Fig. S7B). Three other constructs containing the *GLS1*–3′UTR with deletions of each ARE individually or in combination were generated, and luciferase reporter assays showed that each ARE mutation diminished the luciferase activity and the mutation combination had the strongest effect (*SI Appendix*, Fig. S7B), suggesting that AR binds to the AREs of *GLS1* and up-regulates its expression. Additionally, we found that ADT of LNCaP cells repressed GLS1 expression (both KGA and GAC), and adding back DHT restored the protein levels of GLS1 (Fig. 5C), further supporting the conclusion that *GLS1* is an AR-regulated gene. These results were also consistent with our previous finding that ADT leads to glutamine accumulation by inhibiting glutamine catabolism (Fig. 1*A*).
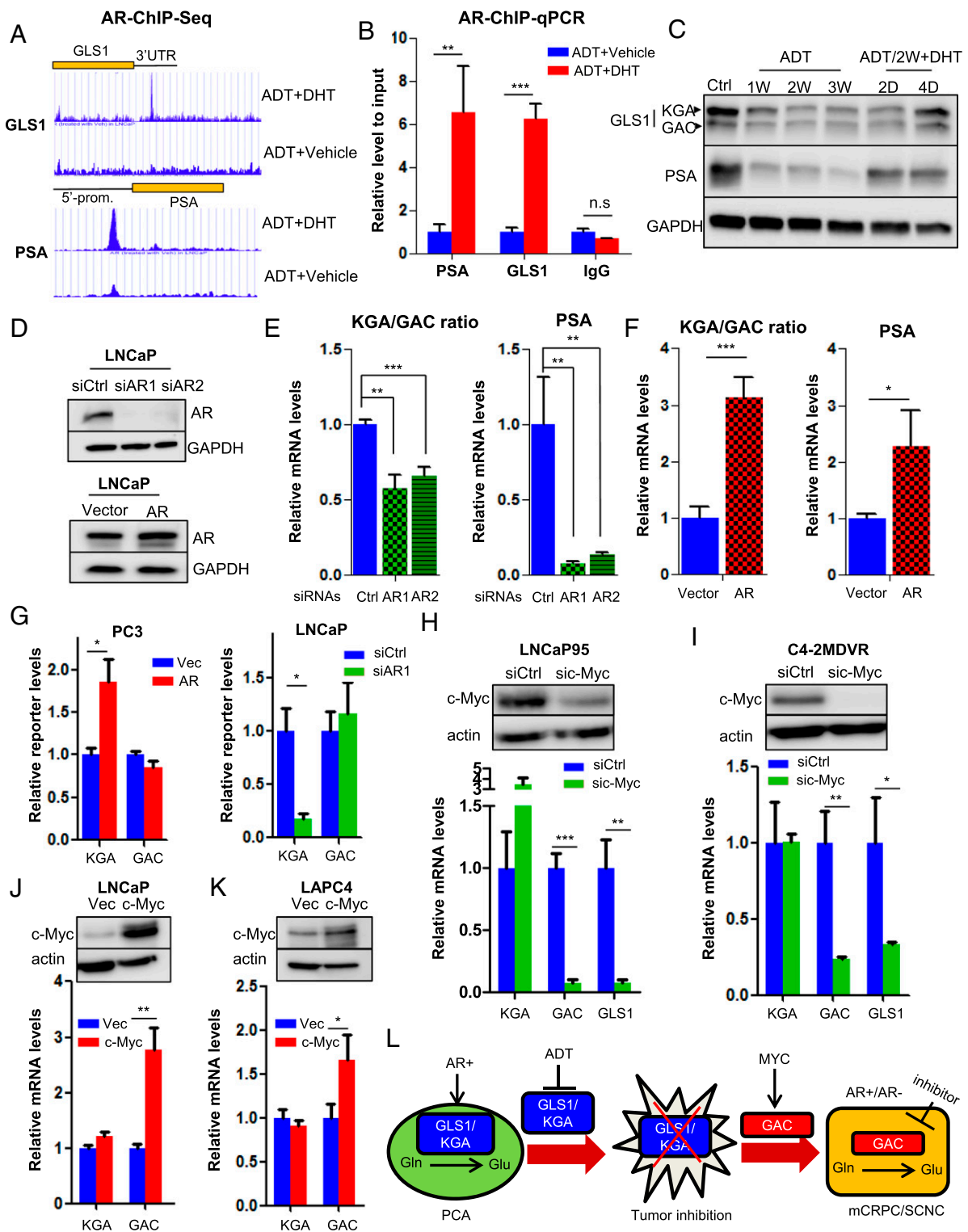
To investigate if AR activity regulates the expression of the two isoforms differentially, we knocked down or overexpressed AR, respectively, in LNCaP cells. Although both KGA and GAC

levels were affected, AR knockdown or overexpression decreased and increased the ratio of KGA/GAC (Fig. 5 *D–F*), respectively. To further determine if AR could affect *GLS1* mRNA splicing, we performed a minigene assay using a *GLS1* minigene reporter plasmid covering exon 14 to exon 19 of *GLS1* (*SI Appendix*, Fig. S7C). The plasmid was cotransfected with either a small-interfering RNA targeting *AR* (siAR) into AR-positive LNCaP cells or exogenous *AR* into AR-negative PC3 cells. We showed that knocking down or overexpressing AR significantly up-regulated or reduced *KGA* transcript levels but had little impact on *GAC* transcript levels (Fig. 5G). These results suggest that *GLS1* RNA splicing is coupled to *GLS1* gene transcription governed by AR. Specifically, activated AR binds directly to *GLS1* to up-regulate its transcription and simultaneously regulate alternative splicing to favor the expression of the KGA isoform (*SI Appendix*, Fig. S7D).

The above results reveal AR's metabolic function in PCa and how hormonal therapy inhibits KGA expression and glutamine catabolism to achieve therapeutic effect. We then sought to
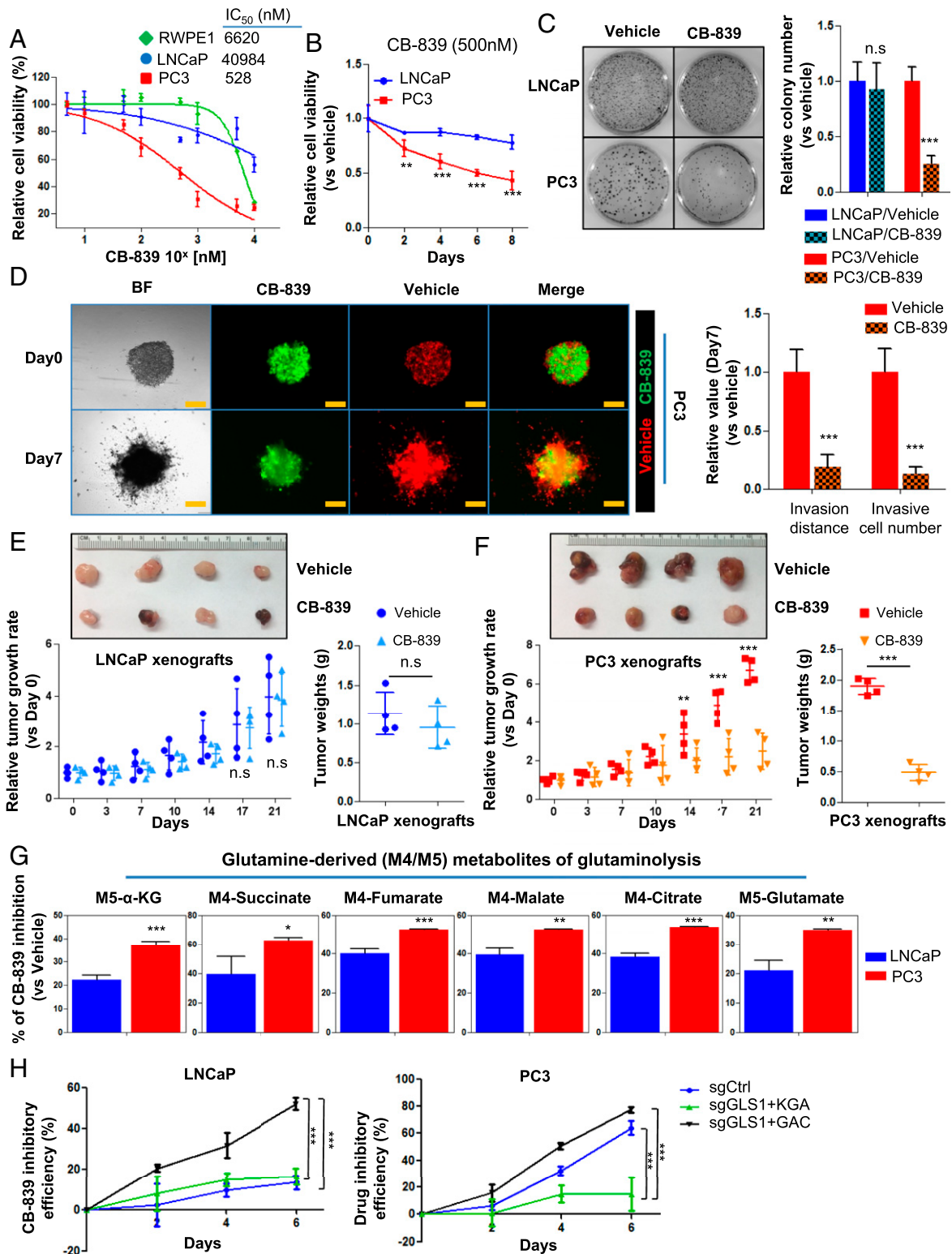
Fig. 4. GLS1 inhibitor CB-839 preferentially inhibits therapy-resistant PCa. (*A*) IC$_{50}$ of CB-839 in RWPE1, LNCaP, and PC3 cells after 72 h of drug treatment (*n* = 3 replicates for two independent experiments). (*B*) Cell viability of LNCaP and PC3 cells treated with 500 nM CB-839 (*n* = 3 replicates for two independent experiments). (*C*) Colony formation of LNCaP and PC3 cells treated with CB-839 (500 nM) for 14 d (*n* = 3 replicates for two independent experiments). (*D*) Spheroid invasion assay showing the inhibitory effect of CB-839 on the migration of PC3 cells (*n* = 3 replicates for two independent experiments). Representative images (*Left*) and quantifications of relative cell invasion (*Right*) are shown. Cells labeled with RFP or GFP denote those cultured with vehicle or CB-839, respectively. (Scale bar, 200 μm.) (*E* and *F*) Representative images of tumor sizes (*Top*) and xenograft tumor growth curves (*Bottom Left*) and tumor weights (*Bottom Right*) with placebo or CB-839 oral administration (*n* = 4 in each group). (*G*) Mass isotopomer of ^{13}C-glutanime–tracing analysis showing the inhibitory efficacy of CB-839 on glutaminolysis in LNCaP and PC3 cells (*n* = 3 cultures per group). (*H*) CB-839 inhibitory efficacy in LNCaP and PC3 cells expressing KGA only or GAC only in comparison to control single guide RNA (sgCtrl) (*n* = 3 replicates for two independent experiments). Data are depicted as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by two-tailed Student's *t* test. n.s., not significant.

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Fig. 5. AR and MYC cooperatively regulate GLS1 isoform switch. (*A*) Identification of AR-binding sites in GLS1–3′UTR by ChIP sequencing. Prostate-Specific Antigen (PSA) is used as a positive control. (*B*) ChIP–qPCR showing AR binding to GLS1–3′UTR upon DHT stimulation. (*C*) Western blot showing KGA and GAC expression in LNCaP cells treated with ADT and followed by 10 nM DHT at the indicated time points. (*D*) Western blot showing AR expression in LNCaP cells transduced with siRNAs or complementary DNA (cDNA) plasmid expressing AR. (*E* and *F*) Transcript ratio of KGA/GAC in LNCaP cells after knockdown or overexpression of AR. (*G*) GLS1 minigene reporter was cotransfected with either AR cDNA plasmid in PC3 cells or AR-targeting siRNA in LNCaP cells. Transcript levels of isoforms were detected by qPCR by using *KGA*- and *GAC*-specific primer pairs. (*H* and *I*) Western blot showing knockdown of c-Myc by specific siRNA pool in LNCaP95 and C4-2MDVR cells (*Top*) and transcript levels of *KGA*, *GAC*, and *GLS1* after c-Myc reduction (*Bottom*). (*J* and *K*) Western blot showing ectopic expression of c-Myc in LNCaP and LAPC4 cells (*Top*) and transcript levels of KGA, GAC, and GLS1 after c-Myc overexpression (*Bottom*). (*L*) A model depicting how GLS1 isoform switch drives castration resistance and disease progression in PCa. PCA, prostate adenocarcinoma; Glu, glutamate; Gln, gluta-mine; mCRPC, metastatic castration-resistant PCa; SCNC, small-cell neuroendocrine PCa. For each assay, $n = 3$ replicates were performed. Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by two-tailed Student's *t* test. n.s., not significant.

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A glutaminase isoform switch drives therapeutic resistance and disease progression of prostate cancer

understand the molecular mechanisms that up-regulate GAC expression after hormonal therapy and focused on c-Myc which mediates AR's ligand-independent function and drives disease progression toward CRPC (36). Relevant to our study, it has been reported that c-Myc up-regulates GAC expression (37–40). Bioinformatic analyses revealed that c-Myc was up-regulated in PCa, particularly in metastatic PCa (*SI Appendix*, Fig. S8*A*). IHC staining of TMA containing human PCa tissues and Western blot analysis of PCa cell lines showed that CRPC tissues and the castration-resistant CWRR1 and C4-2MDVR cells had the highest levels of c-Myc (*SI Appendix*, Fig. S8 *B and C*). In addition, in the longitudinal xenograft tumor model (Fig. 2*E*), c-Myc expression was elevated in the recurrent castration-resistant tumors in comparison to primary tumors (*SI Appendix*, Fig. S8*D*). These findings demonstrate strong association of c-Myc expression with castration resistance. Interestingly, we also discovered a strong positive correlation of c-Myc expression with GAC but not with KGA by studying the TCGA dataset (*SI Appendix*, Fig. S8*E*). Knocking down c-Myc in advanced PCa cells (LNCaP95 and C4-2MDVR) reduced the expression of GAC (Fig. 5 *H* and *I*). Conversely, overexpressing c-Myc in hormone-sensitive PCa cells (LNCaP and LAPC4) increased GAC levels (Fig. 5 *J* and *K*). In contrast, manipulation of c-Myc expression did not change KGA expression (Fig. 5 *H–K*). These results suggest that c-Myc promotes the expression of the enzymatically potent GAC which may contribute to therapy resistance and disease progression.

Recurrent PCa after hormonal therapy usually maintains adenocarcinoma histology (CRPC–adeno), but a subset of recurrent tumors belongs to a variant histologic type known as SCNC which is composed of pure NE tumor cells with extremely aggressive behavior (41). Interestingly, MYC family members have been implicated in governing the histological differentiation of PCa in the advanced stages. c-Myc amplification was frequently detected in the CRPC tumors with adenocarcinoma histology but not in SCNC, both in human tissues and in an NE cell model (NCI-H660) (*SI Appendix*, Fig. S8 *B and C*). In contrast, *N*-Myc was amplified and overexpressed in SCNC (*SI Appendix*, Fig. S8*G*) (42, 43), while there was no significant up-regulation of c-Myc in SCNC (*SI Appendix*, Fig. S8*F*). Although *N*-Myc and c-Myc are expressed in advanced PCa of different histologies (CRPC–adeno vs. SCNC, respectively), *N*-Myc similarly promoted the expression of GAC, not KGA, in LNCaP and C4-2 cells (*SI Appendix*, Fig. S8 *H* and *I*).

AR inhibition promotes the emergence of SCNC through lineage plasticity (44, 45). 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. 5*C*). However, when *N*-Myc is overexpressed, ADT led to an eightfold increase of GAC (*SI Appendix*, Fig. S8*I*), suggesting that ADT and *N*-Myc cooperate to induce the expression of GAC, resulting in increased glutamine utilization and aggressive behavior of SCNC.

The above results firmly establish 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. However, the isoform switch from KGA to GAC could also be due to differences in RNA stabilities. To rule out this possibility, we treated LNCaP and PC3 cells, which express predominantly KGA and GAC, respectively, with actinomycin D (ActD) to inhibit *GLS1* gene transcription. We observed no significant differences of RNA decay rates between *KGA* and *GAC* (*SI Appendix*, Fig. S8 *J* and *K*), indicating that the isoform switch is not due to different mRNA stability.

Discussion

Despite decades of research, how AR activation promotes PCa cell proliferation remains largely unknown. Consequently, the molecular mechanisms of hormonal therapy and the subsequent

development of therapy resistance are poorly understood. Our study has uncovered previously unrecognized mechanisms of action for AR and hormonal therapy in PCa: 1) Glutamine is an important nutrient for PCa, particularly in advanced, therapy-resistant, and aggressive PCa. 2) AR binds to the AREs within the 3′UTR of the *GLS1* gene, promotes *GLS1* expression, and regulates transcription-coupled RNA splicing to favor the production of the KGA isoform. As a result, KGA is the predominant isoform of GLS1 in untreated, hormone-sensitive PCa, and AR is required for PCa cell survival. 3) Hormonal therapy suppresses AR function and GLS1 (predominantly the KGA isoform) expression, thus inhibiting glutamine catabolism and tumor cell survival. 4) Eventually, PCa cells regain the ability to utilize glutamine and resume proliferation, leading to therapy resistance and disease progression to the castration-resistant stage clinically. 5) The MYC family proteins c-Myc and *N*-Myc play important roles in this process along with AR. For many tumors at this stage, AR expression is maintained, and an important ligand-independent function of AR is transcriptional activation of c-Myc (36) which in turn induces GAC expression. Due to GAC's extremely potent enzymatic activity, the tumor cells can utilize glutamine more efficiently, resulting in aggressive behavior. This is characteristic of CRPC–adeno with expression of AR and c-Myc. 6) A significant number of tumors at this stage are histologically classified as SCNC in which GAC expression is equally important for the tumor cells' rapid proliferation and aggressive behavior. In SCNC, the increased expression of GAC is not mediated by c-Myc but by a combination of loss of AR expression (through lineage plasticity) (45, 46) and *N*-Myc overexpression (43). These important findings are depicted in Fig. 5*L*.

Our study has important clinical implications: 1) Currently used hormonal therapy targeting AR has significant side effects including sexual dysfunction and disorders of the cardiovascular and metabolic systems (47) because AR has diverse physiological functions in many organs and cells. A therapeutic strategy directly targeting GLS1 isoforms but not interfering with AR's physiological function in normal tissue can potentially have a therapeutic efficacy similar to or better than hormonal therapy but with much fewer side effects. 2) Due to the complexity of the intracellular signaling networks and their extensive cross-talks, PCa cells can quickly evolve to adapt to an androgen-deprived environment by a variety of mechanisms, such as activation of glucocorticoid receptor signaling (48), production of constitutively active AR splicing variants lacking the ligand-binding domain (1), and transdifferentiation toward an NE phenotype (45), resulting in tumor recurrence (CRPC–adeno or SCNC). A therapeutic strategy targeting GLS1 will bypass the complex upstream signaling pathways and directly suppress the production of energy and building blocks required by PCa cells, starving tumor cells to death. Since metabolic activity directly controls cellular proliferation (49, 50), it may be more difficult for the tumor cells to overcome a metabolic inhibition to develop resistance. 3) AR-targeted treatment approaches will not work for PCa cells in which AR activity is not required such as in AR indifferent tumors (30) and SCNC (12). Our study indicates that glutamine addiction is common across the entire spectrum of PCa including tumors lacking the expression of AR. In fact, advanced tumors and tumors independent of AR activity are more addicted to glutamine. As a result, many patients, particularly those who have exhausted currently available treatment options, will benefit from a treatment strategy targeting glutamine catabolism. CB-839, a specific small-molecule inhibitor of GLS1, has been evaluated in multiple clinical trials for solid and hematological malignancies as a single agent and in combination with an immune checkpoint inhibitor (38). Our data demonstrate that advanced and therapy-resistant PCa, where GAC is overexpressed, are highly sensitive to CB-839 inhibition. GLS1 inhibitor may thus provide clinical benefits.

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There are still important yet unanswered questions related to the development of advanced PCa. An important factor in SCNC is hypoxia mediated by the master NE-differentiation regulator ONECUT2 (51). Future studies are needed to address if hypoxia affects the GLS1 isoforms and glutamine utilization in SCNC. Additionally, the molecular and cellular events described above are consistent with an "adaptation" model which states that PCa cells switch isoform expression from the AR-dependent KGA isoform to the androgen-independent GAC isoform in order to survive the androgen-deprived environment, resulting in more efficient glutamine utilization and more aggressive behavior seen in recurrent tumors (CRPC–adeno or SCNC). However, the findings may also be explained by the "clonal selection" model. This alternative model states that in untreated and hormone-sensitive PCa, the tumor cells are heterogeneous with KGA being the predominant isoform in the majority of the tumor cells, while a minority of tumor cells express GAC predominantly. Hormonal therapy creates an androgen-deprived environment leading to the inhibition of KGA-expressing tumor cells and the selection of GAC-expressing tumor cells, and the latter eventually populate the recurrent tumors. Further studies are needed to distinguish these two interesting possibilities.

Materials and Methods

See *SI Appendix* for further information.

Cell Culture, Proliferation Assay, Colony Formation Assay, Gene Silence, and Overexpression. Cells were maintained under standard or specific conditions (for details, see *SI Appendix*). Cell viability assay and colony formation assay are described in *SI Appendix*. Cells used in each experiment were performed under identical and well controlled conditions to test a specific hypothesis. However, conditions such as the starting cell numbers and the sizes of the tissue culture plates were not identical among different experiments described in different figures. Therefore, the results of different experiments may not be directly comparable. shRNA lentiviral plasmids, Crispr single guide RNA (sgRNA) plasmids, specific siRNA pool, and ORF plasmids were either commercially purchased or kindly given from collaborators. Detailed protocols are described in *SI Appendix*.

Tissue Microarrays and Immunohistochemistry. Tissue microarrays, including treatment-naive, adjacent benign, CRPC and SCNC samples, were constructed and have been reported previously (13, 52). Immunohistochemistry was performed following standard procedures, and the Quick-score system was applied to determine the intensity of staining (13).

Metabolite Extraction, Isotope Tracing, and Mass Spectrometry. As described previously (53), cells were harvested from 6-well plate after corresponding treatment. For isotope-tracing analysis, medium was replaced by fresh glutamine/glucose-deprived medium supplemented with 2 mM ^{13}C–glutamine or 4.5 g/L ^{13}C–glucose isotope. Metabolites were extracted in 80% cold methanol followed by speed vacuum drying. The dried pellets were sent for further LC/MS analyses.

Minigene Assay. The gBlock, which contains exon 14–19 and their flanking intron regions of GLS1 gene, was synthesized by Integrated DNA Technologies and used as the template for PCR amplification. The DNA fragment product was cloned into the plasmid vector pRL-TK (Promega) between NheI and XbaI sites. DNA sequencing confirmed the integrity of the final construct, which was subsequently transfected into desired cells. KGA- and GAC-specific primers were used to determine the transcript levels by qPCR.

Animal Experiments. For drug efficacy experiments, LNCaP and PC3 cells were injected subcutaneously in mice. For each tumor type, mice were randomly distributed into two groups receiving either vehicle or CB-839 (200 mg/kg oral administration) (32). For longitudinal observation of the isoform switch, surgical castration was performed when subcutaneously implemented tumors reached ~200 mm^3. Animals were killed after tumor recurred significantly or tumor volumes exceeded 1,000 mm^3. Harvested tumors were embedded into paraffin followed by hematoxylin and eosin staining and immunohistochemistry staining.

Data availability. All study data are included in the article and/or supporting information.

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