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14. ABSTRACT

Advanced and aggressive prostate cancer (PCa) depend on glutamine for tumor survival and proliferation. We have previously shown that inhibition of glutaminase 1, which catalyzes the rate-limiting step of glutamine catabolism, achieves significant therapeutic effect; however, therapy resistance is inevitable. Here we report that while the glutamine carbon is critical to PCa survival, a parallel pathway of glutamine nitrogen catabolism that actively contributes to pyrimidine assembly is equally important for PCa cells. Importantly, we demonstrate a reciprocal feedback mechanism between glutamine carbon and nitrogen pathways which leads to therapy resistance when one of the two pathways is inhibited. Combination treatment to inhibit both pathways simultaneously yields better clinical outcome for advanced

15. SUBJECT TERMS Prostate cancer, glutamine metabolism, GLS1, CAD					
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TABLE OF CONTENTS

Introduction:

Prostate cancer (PCa) is the most common non-cutaneous cancer in men, leading to ~30,000 deaths annually in the US. Androgen deprivation therapy (ADT) remains the mainstay for patients with advanced and metastatic PCa. Although the treatment is efficacious initially, the disease will eventually recur as castration resistant PCa (CRPC). Second generation of hormonal therapy drugs, such as abiraterone and enzalutamide, are useful for CRPC but resistance eventually develops. Histologically, most cases relapse as adenocarcinoma, maintaining luminal differentiation including the expression of androgen receptor (AR) and prostate specific antigen (PSA). However, in up to 25% of the patients, the recurrent tumor after hormonal therapy shows neuroendocrine (NE) phenotype with suppressed AR signaling. Thus, developing therapeutic strategies independent of AR is a pressing unmet clinical need.

Rewired cellular metabolism is one of the most significant cancer hallmarks which is important to meet the needs of tumor cells' uncontrolled proliferation. In addition to the well-known Warburg effect which describes glucose flux primarily towards lactate, glutamine has also been implicated as a pleiotropic energy and building source in many solid cancer types. Our recent publication has discovered a unique propensity of therapy-resistant PCa being extremely addicted to glutamine9. Thus, targeting glutamine metabolism is an attractive therapeutic strategy for androgen/AR-independent PCa.

As an anaplerotic nutrient that fuels the tricarboxylic acid (TCA) cycle, glutamine's carbon backbone incorporates into α-ketoglutarate (α-KG) through glutaminase 1 (GLS1)-mediated catabolism. Although our study has demonstrated therapeutic potential of inhibiting GLS1 in therapy-resistant PCa, experience in other tumor types suggests that monotherapy targeting GLS1 produces limited efficacy. Importantly, the nitrogen released during the process of glutamine carbon catabolism is used for the synthesis of nucleotides and other nitrogen-containing molecules, which are also critical for the proliferating cancer cells. The coordinated roles of glutamine carbon and nitrogen in cancer cells' energy production and biosynthesis suggest that targeting both arms of glutamine metabolism (nitrogen and carbon) may result in better therapeutic outcomes than targeting either pathway alone in therapy-resistant PCa.

This research proposal has following specific aims: 1. To identify how glutamine carbon and nitrogen interact with one to the other and thereby overcome the inhibition of one of the glutamine metabolic branch; 2. To demonstrate whether inhibiting both glutamine carbon and nitrogen metabolism pathways would achieve better therapeutic outcomes than single treatment.

Keywords: prostate cancer, therapeutic resistance, glutamine metabolism, GLS1, CAD

Accomplishments:

1. Major Task 1: Training and educational development in prostate cancer research.

Subtask 1: Attend various scientific research workshops in Duke GCB department. The courses include genomic technologies, computational approaches, mass spectrometry analyses, etc (Time frame: Months 1-12).

Up to now, the PI has attended several GCB academic online courses (due to the COVID, most of the courses have been held online), including Fundamentals of Mass Spectrometry for Proteomics and Metabolomics and Introduction to DNA Sequencing Technology. With these coursed learned, the PI has obtained an overview and expanded the knowledge of the use of LC/MS/MS-based methods for proteomics and metabolomics and how recent new generation of high-throughput DNA sequencers has transformed biomedical and biotechnology research, and thus help the PI better understand how these technologies can help inform his research goals.

2. Research-Specific Tasks:.

Specific Aim 1: To dissect the underlying mechanisms of hyper-synthetic activity of pyrimidine in therapy-resistant PCa.

Major Task 1: To validate the hyper pyrimidine biosynthesis in therapy-resistant PCa.

Subtask 1: Measure intracellular nitrogen-contained metabolite levels and glutamine nitrogen incorporation.

Due to an isoform switch of GLS1, glutamine carbon is more efficiently oxidized via the TCA cycle in therapy-resistant PCa than primary hormone-sensitive PCa to help tumor cells escape the inhibitory effect of ADT (Xu, et al, PNAS, 2021). However, this process would generate excess glutamine nitrogen which is potentially toxic to tumor cells. To investigate how advanced PCa cells dispose of the accumulated nitrogen while utilizing glutamine carbon, we performed nitrogen-targeted metabolite profiling analyses by employing another paired cell lines, C4-2 and C4-2MDVR, to compare metabolic reprogramming in addition to the previously tested comparison between AR-positive, androgen-dependent LNCaP cells and androgen-independent, AR-null PC3 cells. Consistent with the resulted derived from LNCaP and PC3 cells, nitrogen was significantly enriched in metabolites involved in nucleotide biosynthesis pathways such as dihydroorotate and IMP, the precursors for pyrimidine and purine synthesis, respectively (**Fig. 1a**). Pathway impact and

enrichment analyses further demonstrated that pyrimidine and purine synthesis were among the top affected metabolic pathways in the advanced C4-2MDVR cells (**Fig. 1b**). Additionally, cellular nucleotides and their derivatives were increased in C4-2MDVR cells compared to C4-2 cells (**Fig. 1c**). These results suggest that advanced PCa preferentially uses nitrogen to synthesize nucleotides.

To determine how glutamine nitrogen contributes to nucleotide biosynthesis in advanced PCa, we performed ^{15}N-glutamine isotopomer tracing studies in PCa cell lines. Surprisingly, the amide nitrogen group, which directly participates in purine and pyrimidine synthesis (**Fig. 1d**), was minimally assimilated into the purine synthetic pathway in the more advanced and C4-2MDVR cells, as there was minimal labeling of IMP and ATP by glutamine (**Fig. 1e**). In contrast, the labeled fractions of dihydroorotate and CTP were high in C4-2MDVR cells (**Fig. 1f**), suggesting that advanced, therapy-resistant PCa preferentially utilizes glutamine amide-nitrogen for pyrimidine over purine production. In support of this conclusion, although we found that C4-2MDVR cells had higher levels of purine and pyrimidine concentrations than C4-2 cells, the increases in the levels of pyrimidine nucleotides (CDP, CTP and UTP) were much more pronounced (**Fig. 1c**). Taken together, these findings demonstrate that advanced PCa cells utilize the released glutamine nitrogen for nucleotide, particularly pyrimidine, biosynthesis to keep pace with the hyper-activity of glutamine carbon oxidation towards the TCA cycle.

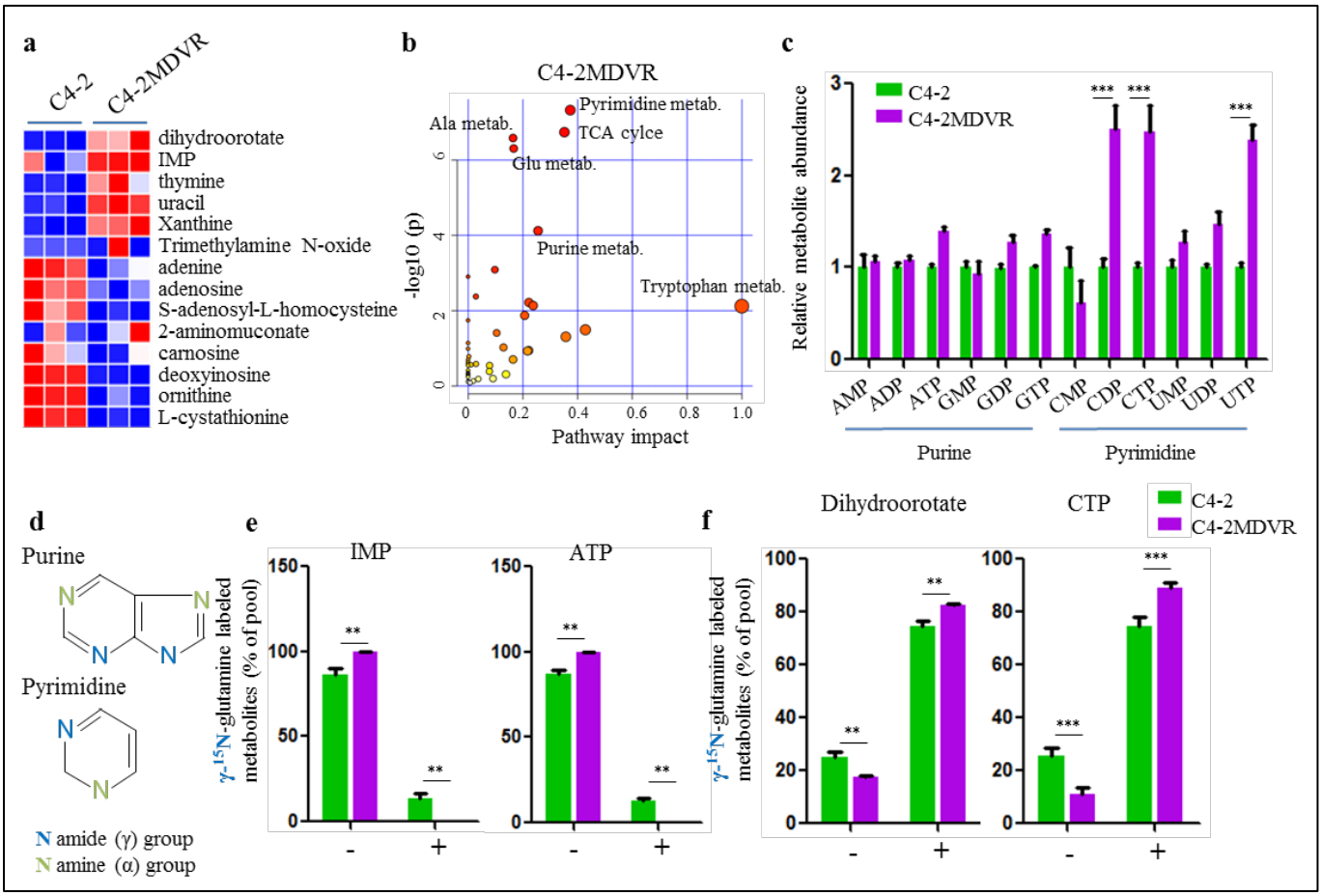

Fig. 1 Glutamine nitrogen is largely enriched in nucleotide biosynthesis in advanced PCa. **A.** Heat-map showing the comparison of nitrogen-contained metabolites in C4-2 and C4-2MDVR cells. **B.** Pathway impact analysis showing metabolic pathways that are significantly altered in C4-2MDVR cells. **C.** Metabolite abundance of nucleotides and derivatives in C4-2MDVR cells. **D.** Chemical structure of purine and pyrimidine bases. **E and F.** Amide (γ)-^{15}N-glutamine tracing analysis showing the incorporation of glutamine into IMP, ATP, dihydroorotate and CTP in C4-2 and C4-2MDVR cells.

Subtask 2: Measure intracellular glutamine carbon incorporation in nucleotides. (Time frame: Months 2-3).

In addition to the requirement of glutamine nitrogen, glutamine carbon is also an important contributor for nucleotide biosynthesis. Generally, glutamine passages its carbon to aspartate which is the direct precursor of dihydroorotate (m+3) (**Fig. 2a**). This carbon transfer is usually mediated by the generation of oxaloacetic acid (OAA). Isotopomer tracing flow shows that glutaminolysis is the pathway for m+4 OAA generation while the reductive carboxylation pathway is able to produce m+3 and m+2 OAA as the by-products split from citrate (**Fig. 2b**). Moreover, m+1 and m+0 OAA can be traced from glycolysis depending on whether the assimilated carbon dioxide (CO2) is derived from glutamine or not (**Fig. 2b**).

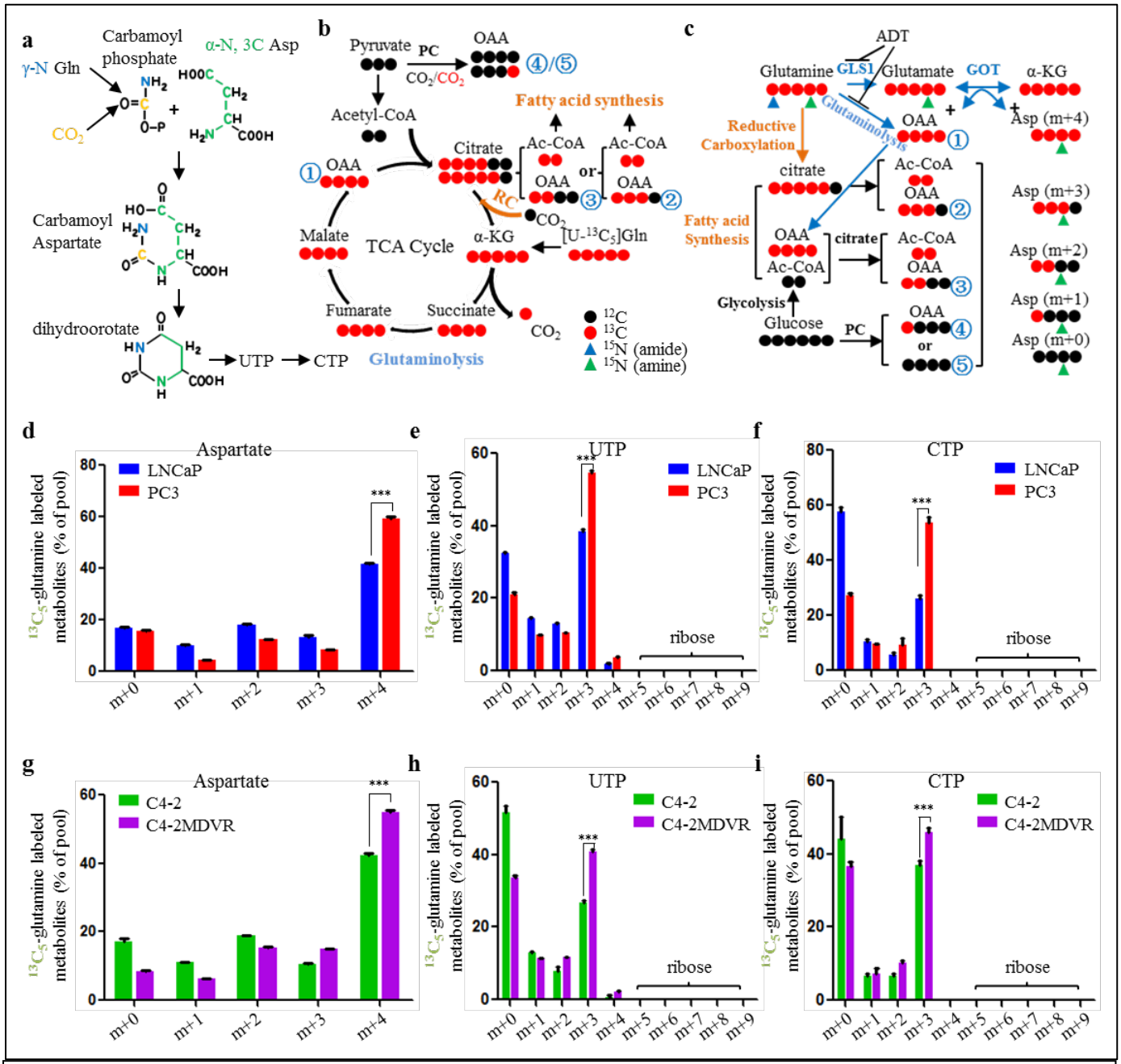

Fig. 2 Glutamine carbon is associated with glutamine nitrogen in building pyrimidine rings in PCa. **A, B and C.** Schematic overview of the synthesis of pyrimidine and glutamine carbon flux toward related metabolites. **D-I**, Fractions of $^{13}C_5$-glutamine derived isotopologues in aspartate, UTP and CTP in the indicated PCa cell lines.

Accordingly, m+4, m+3, m+2, m+1 and m+0 aspartate are formed through transamination by which OAA accepts amine-nitrogen from glutamate while retaining the carbon backbone from glutamine/glutamate (**Fig. 2c**). To determine whether glutamine carbon catabolism is associated with its nitrogen catabolic rates when advanced PCa cells actively synthesize pyrimidine, we traced the $^{13}C_5$-glutamine isotope-labeled intermediates in pyrimidine biosynthesis. Across all the tested cell line models, the proportion of m+4 aspartate was the largest among the aspartate pool, suggesting that glutaminolysis is the dominant pathway for aspartate synthesis in PCa (**Fig. 2d,g**). In comparing the metabolic differences between cell lines representing different

stages of the disease, we found that the more advanced PC3 and C4-2MDVR cells had more m+4 aspartate proportions than LNCaP and C4-2 cells (**Fig. 2d,g**). Accordingly, we observed significantly increased levels of glutamine-aspartate-derived pyrimidine nucleotides (m+3 UTP and m+3 CTP) in advanced PCa cells (**Fig. 2e,f,h,i**). These findings, together with our previously published data, indicate that while advanced PCa avidly consumes glutamine carbon, the intermediates (such as OAA and aspartate) can coordinate with the released glutamine nitrogen to participate in pyrimidine biosynthesis.

Altogether, the accomplishments so far can be summarized below:

1. We have verified a glutamine nitrogen metabolic feature in advanced PCa that glutamine nitrogen preferentially fluxes to participate in pyrimidine synthesis.
2. With powerful metabolome data delivered from accomplished tasks, we have also identified a cooperation between glutamine carbon and nitrogen metabolism in advanced PCa. We have demonstrated how these two metabolic pathways coordinate with one to the other to confer the maximal utilization of glutamine for the tumor cells.

3. Opportunities for training and professional development.

Please see **Major Task 1**.

4. Dissemination of results to communities of interest.

Nothing to report.

5. Plans for next reporting period.

Due to the unexpected COVID-19 situation, the PI, Dr. Lingfan Xu can not be able to fulfill the rest of the reward and it will be transferred to Dr. Xue Jiang, who has the equivalent education and research background of Dr. Xu. Dr. Jiang will continue to finish unaccomplished work as stated in the SOW. Specifically, Dr. Jiang will identify CAD as the key factor enhancing pyrimidine synthetic activity in therapy-resistant PCa and evaluate the synergistic effect of targeting both carbon and nitrogen metabolism of glutamine in therapy-resistant PCa.

Impact:

1. Impact on the development of the principal disciplines.

Currently, there is no effective treatment for CRPC. In addition, some particular subtypes, including AR

indifferent PCa and small cell neuroendocrine carcinoma, do not respond to AR targeted therapies. We believe that a lack of understanding of exactly how metabolic reprogramming in PCa development, treatment and progression is largely responsible for the slow progress we have made in combating advanced and aggressive PCa. Our recent study showed that advanced PCa is addicted to glutamine, a process that is regulated by AR. Targeting GLS1, the rate-limiting enzyme for glutamine catabolism, is likely a more specific and efficacious approach than AR-directed therapy for advanced PCa. However, acquired treatment resistance is still inevitable. With the proposed study, we aim to identify activation of nitrogen metabolism following GLS1 inhibition as an important mechanism of therapy resistance and reveal a reciprocal regulation of the glutamine carbon and nitrogen pathways governed by the balance of GLS1 and CAD, key enzymes for the two pathways, respectively. We also expect to show that glutamine carbon and nitrogen pathways coordinate with each other in advanced PCa to fuel tumor cell proliferation. Inhibition of one pathway will eventually activate the other through a mechanism of reciprocal regulation, leading to therapy failure. A combinatorial therapeutic strategy by targeting both pathways of glutamine catabolism would be better to improve therapeutic efficacy.

2. **Impact on other disciplines.**

   Nothing to report.

3. **Impact on technology transfer.**

   Nothing to report.

4. **Impact on society beyond science and technology.**

   Nothing to report.

Changes/Problems:

Change of PI.

Products:

Manuscripts:

1. Xu L, Zhao B, Butler W, Xu H, Song N, Chen X, Hauck S, 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, accepted

Participants and other collaborating organizations

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Nearest person month worked	4
Contribution to Project:	Study design, research performance
Funding Support	W81XWH2110034

Name:	Jiaoti Huang
Project Role:	Mentor
Researcher Identifier	HUANG88
Nearest person month worked	0
Contribution to Project:	For this award, Dr. Huang has mentored Dr. Xu through multiple mechanisms including frequent meetings and emails to discuss her project. In addition to frequent meetings and emails with Dr. Jiang, Dr. Huang also runs a weekly lab meeting where Dr. Xu presented his data to the entire group and received constructive feedbacks.
Funding Support	N/A

Name:	Daniel George
Project Role:	Co-Mentor
Researcher Identifier	DAN.GEORGE
Nearest person month worked	0
Contribution to Project:	Dr. George's clinical expertise and insights into the medical oncology aspects of prostate cancer has been a great resource for Dr. Xu's success so far. Dr. George has attended meetings where Dr. Xu presented his results and data interpretation and provided highly valuable feedbacks, particularly related to the translational and clinical aspects of prostate cancer.
Funding Support	N/A

Special reporting requirements:

Nothing to report.