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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 PCa patients.

15. SUBJECT TERMS

Prostate cancer, glutamine metabolism, GLS1, CAD

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1. 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.

2. **Keywords:** prostate cancer, therapeutic resistance, glutamine metabolism, GLS1, CAD

3. Accomplishments:

Training-Specific Tasks:

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.

Subtask 2: Present research at weekly laboratory meetings and monthly GU joint meetings.

The PI has presented this research work at weekly laboratory meetings almost a year and half. And she will continue present this research until it done. All the lab members provide many constructive suggestions especially the mentor Dr.Huang. She also got many great suggestions and help from other collaborators.

Lab meeting and scientific communications give the PI many great ideas and suggestions which help her do a creative and meaningful research.

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 (Time frame: Months 1-2).

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.

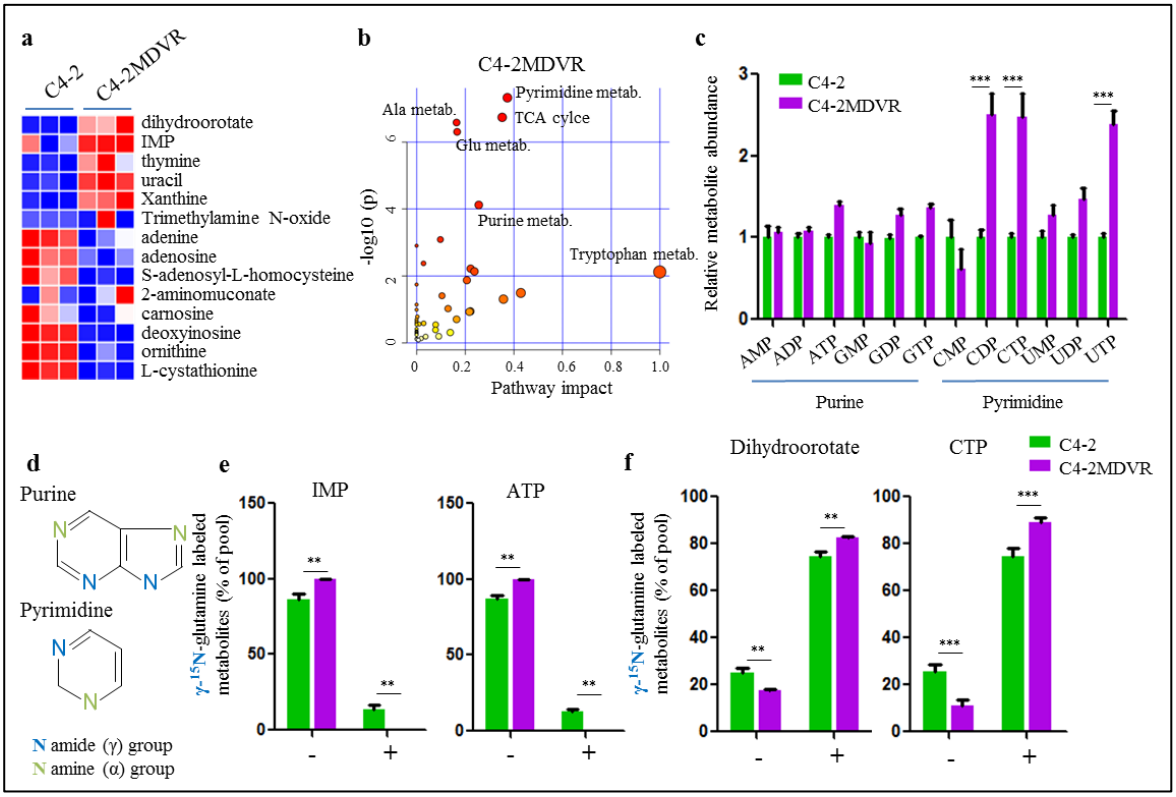


Fig. 1 Glutamine nitrogen is largely enriched in nucleotide biosynthesis in advanced PCa. **a.** Heat-map showing the comparison of nitrogen-contained metabolites in C4-2 and C4-2MDVR cells. **b.** Pathway impact analysis showing metabolic pathways that are significantly altered in C4-2MDVR cells. **c.** 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**). 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.

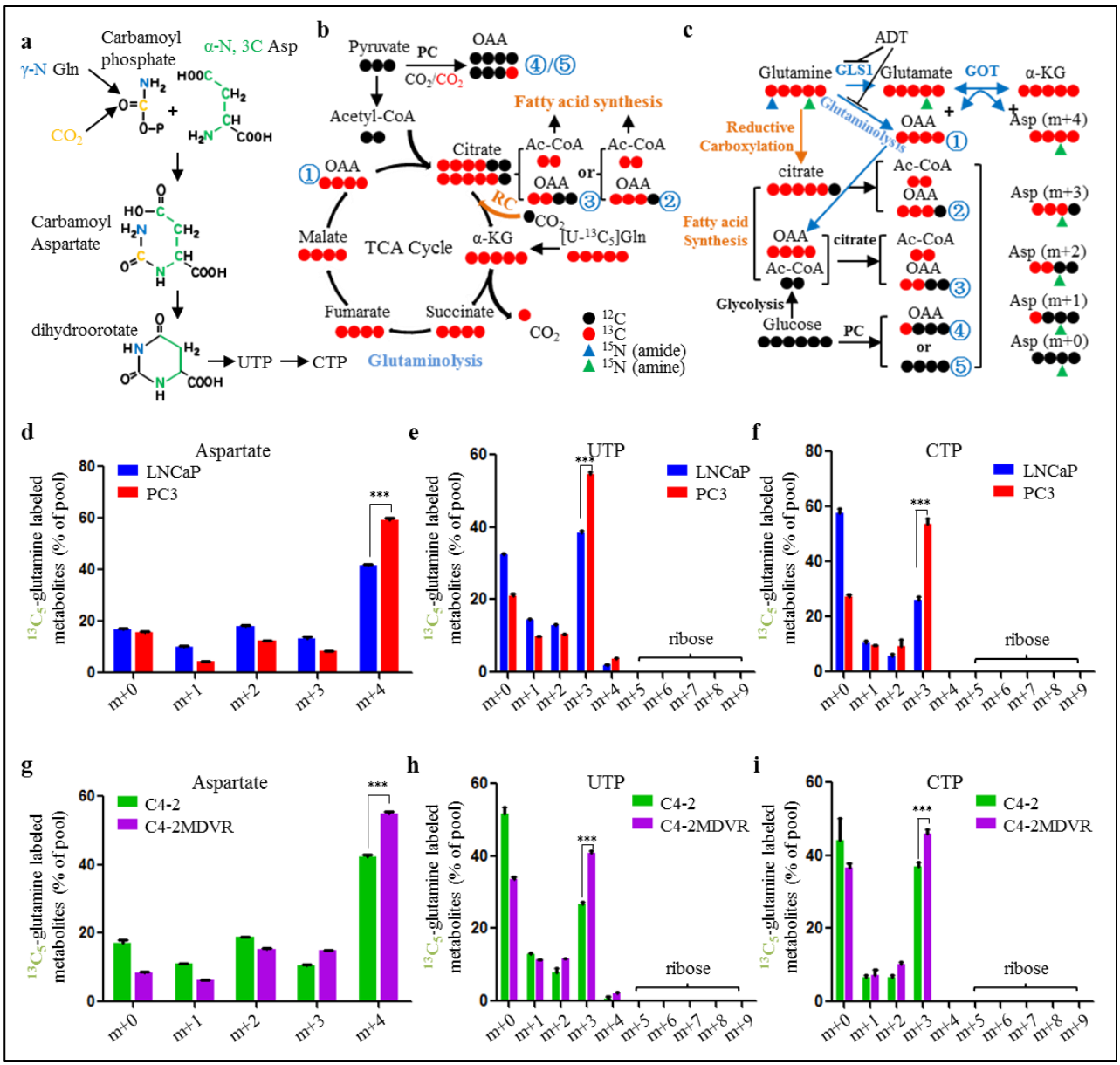


Fig. 2 Glutamine carbon is associated with glutamine nitrogen in building pyrimidine rings in PCa. **a, b and c.** Schematic overview of the synthesis of pyrimidine and glutamine carbon flux toward related metabolites. **d-i**, Fractions of $^{13}C_5$-glutamine derived isotopologues in aspartate, UTP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

Subtask 3: Measure extracellular metabolite concentration in cell culture medium (Time frame: Months 3-4).

To determine if primary PCa and therapy-resistant PCa have different degrees of dependency on glutamine, we compared paired PCa cell lines representing different disease stages, including LNCaP (AR-positive, hormonal therapy-sensitive) and PC3 (AR-negative, hormonal therapy-resistant), as well as C4-2 (enzalutamide-sensitive) and C4-2MDVR (enzalutamide-resistant) cells, respectively. We found that that both glutamine carbon and nitrogen are needed for optimal growth of advanced PCa. The high degree of addiction of therapy-resistant PCa to glutamine carbon, revealed by our work, can lead to potentially harmful levels of intracellular nitrogen. To further investigate how advanced PCa cells dispose of the accumulated nitrogen while utilizing glutamine carbon, we measure the extracellular metabolite concentration in cell culture medium, we used LNCaP, PC3, C4-2 and C4-2/MDVR cell lines.

The heatmap included metabolites involved in glycolysis, tricarboxylic acid (TCA) cycle, nonessential amino acids (NEAA) and nucleotides (**Fig. 3a, d**). It looks the level of extracellular metabolites has a big variation within group. and these is no difference between LNCaP and PC3, C4-2 and C4-2/MDVR group (**Fig. 3a, d**). We found that the extracellular Glutamine level slight decreased in PC3 cell culture medium and increased in C4-2/MDVR cell culture medium (**Fig. 3a, d**). Nitrogen-contained metabolites, nucleotides and derivatives metabolites in cells culture medium showed no difference between LNCaP and PC3 (**Fig. 3b, c**), C4-2 and C4-2/MDVR (**Fig. 3e, f**). We found that the results of extracellular metabolites are not consistent with the intracellular metabolites and have big variation.

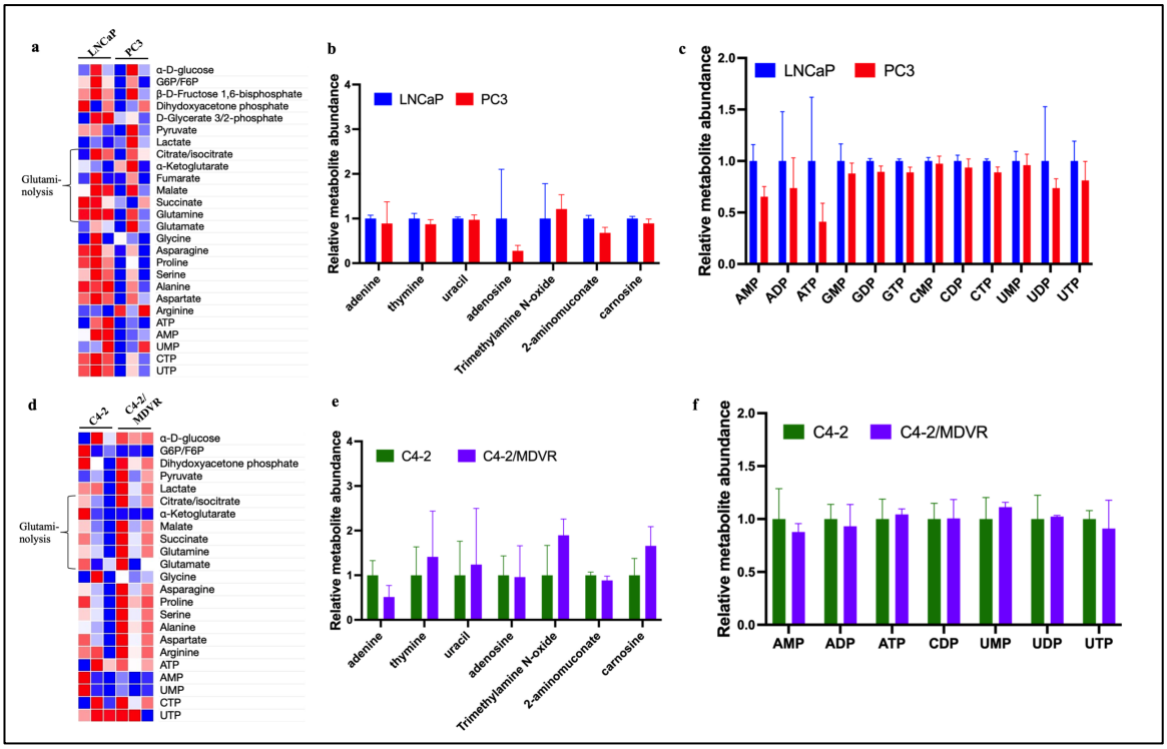


Fig. 3 Extra cellular metabolomics data analysis **a.d** Heat-map showing the comparison of important metabolism pathways. **a.** in LNCaP and PC3 cells culture medium. **d.** in C4-2 and C4-2MDVR cells culture medium. **b.e** Comparison of nitrogen-contained metabolites in cells culture medium. **b.** in LNCaP and PC3 cells culture medium. **e.** in C4-2 and C4-2/MDVR cells culture medium. **c.f** Metabolite abundance of nucleotides and derivatives in cells culture medium. **c.** in LNCaP and PC3 cells culture medium. **f.** in C4-2 and C4-2/MDVR cells culture medium.

Major Task 2: To indentify CAD as the key factor enhancing pyrimidine synthetic activity in therapy-resistant PCa.

Subtask 1: Assess CAD protein expression in human prostate tissue (TMA) (Time frame: Months 4-5).

The above findings clearly demonstrate that the addiction of advanced PCa to glutamine leads to not only hyper activity of the TCA cycle, but also enhanced pyrimidine biosynthesis in tumor cells. Bioinformatics analyses of public datasets of human PCa specimens confirmed that genes belonging to pyrimidine synthesis pathway were significantly enriched in small cell neuroendocrine prostate cancer (SCNC), the most aggressive histologic variant of PCa often seen in terminal stages of the disease after hormonal therapy **(Fig. 4a).** There are three dominant enzymes in this anabolic pathway, carbamoyl-phosphate synthetase 2, aspartate transcarbamylase and dihydroorotase (CAD), dihydroorotate dehydrogenase (DHODH) and glutamic oxaloacetic transaminase (GOT). CAD is responsible for the first three steps of pyrimidine synthesis while DHODH catalyzes the subsequent reaction converting dihydroorotate to orotate **(Fig. 4b)**. GOT is the key enzyme for aspartate generation catalyzing the transfer of an amine-group nitrogen between glutamate and aspartate **(Fig. 4b**). To determine if some or all of the pyrimidine biosynthesis enzymes play a role in advanced PCa, we queried publicly available datasets to compare gene expression between primary adenocarcinoma and therapy-resistant tumors. In all the cohorts analyzed, CAD was universally upregulated in advanced PCa, including SCNC and metastatic PCa, in comparison to primary adenocarcinoma samples **(Fig. 4c).** To confirm these results, we examined CAD expression by immunohistochemical (IHC) staining using tissue microarrays (TMAs) including benign prostate, primary PCa, castration-resistant PCa (CRPC) and SCNC. We found that CAD positive cells were rarely present in benign tissues **(Fig. 4d**), which was consistent with the findings from the Cancer Genome Atlas (TCGA) dataset showing an overall lower expression of CAD in benign tissues **(Fig. 4e**). In contrast, CAD expression was slightly elevated in adenocarcinoma, and significantly enriched in CRPC and SCNC **(Fig. 4d**). These results strongly support those of bioinformatics analyses and together, suggest that CAD is associated with disease progression of human PCa **(Fig. 4b).**

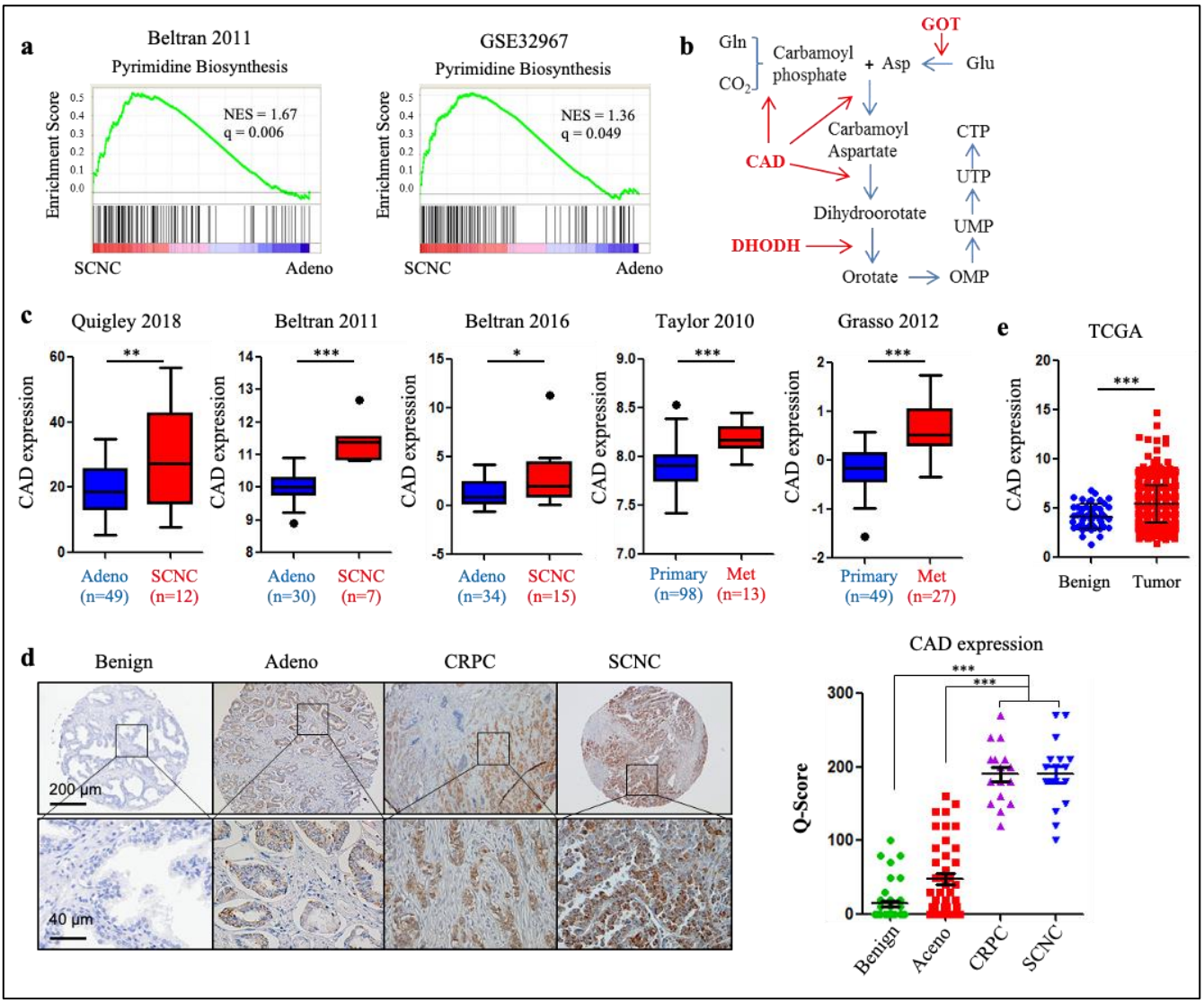


Fig. 4 CAD plays a predominant role in advanced PCa. **a.** GSEA of pyrimidine biosynthesis signatures in SCNS and adenocarcinoma samples from Beltran 2011 and GSE32967 datasets. **b.** Schematic overview showing the process and the key enzymes of pyrimidine synthesis. **c.** Bioinformatics analysis showing CAD gene expression in a variety of study cohorts. **d**. Representative images of CAD IHC staining on the TMAs including benign prostate cancer tissue (n=40), primary prostate adenocarcinoma (n=40), CRPC (n=16) and SCNC (n=17). A plot of Quick-score quantifying CAD expression in each group. **e.** Bioinformatics analysis showing CAD gene expression in benign samples and tumor samples from the TCGA dataset.

Subtask 2: Assess CAD protein level in different PCa cell lines (Time frame: Months 5-6).

In all the cohorts analyzed, CAD was universally upregulated in advanced PCa, including SCNC and metastatic PCa, in comparison to primary adenocarcinoma samples **(Fig. 4c).** IHC results shows that CAD expression was slightly elevated in adenocarcinoma, and significantly enriched in CRPC and SCNC **(Fig. 4d**). Based on these results, we wonder the CAD expression level in different PCa cell lines. We used LNCaP (AR-positive, hormonal therapy-sensitive), PC3 (AR-negative, hormonal therapy-resistant), C4-2 (enzalutamide-sensitive), C4-2MDVR (enzalutamide-resistant), DU145 (AR-negative, hormonal

therapy-resistant), 22RV1(AR-positive, hormonal therapy-sensitive), NCI-H660 (SCNC), LAPC4 (AR-positive, hormonal therapy-sensitive), LNCaP95 (AR-positive, hormonal therapy-resistant), totally nine different PCa cell lines.

These qPCR and WB results showed that CAD was highly expressed in 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. 5a, b)**. In AR-positive PCa cells, such as LNCaP, VCaP, LAPC4, and C4-2, CAD has a lower mRNA and protein expression level **(Fig. 5a, b)**. These results were consistent with the IHC results and the bioinformatics analysis.

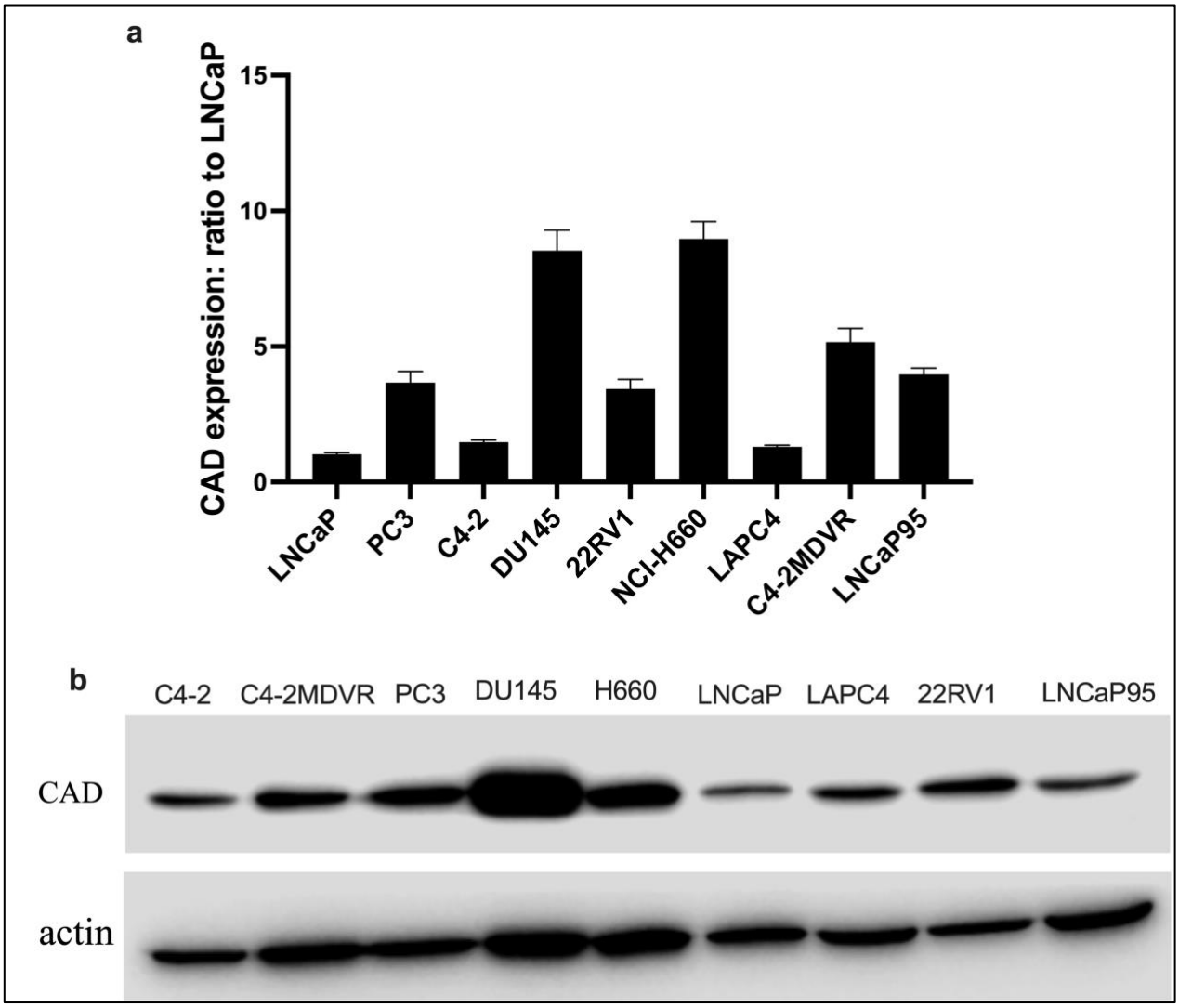
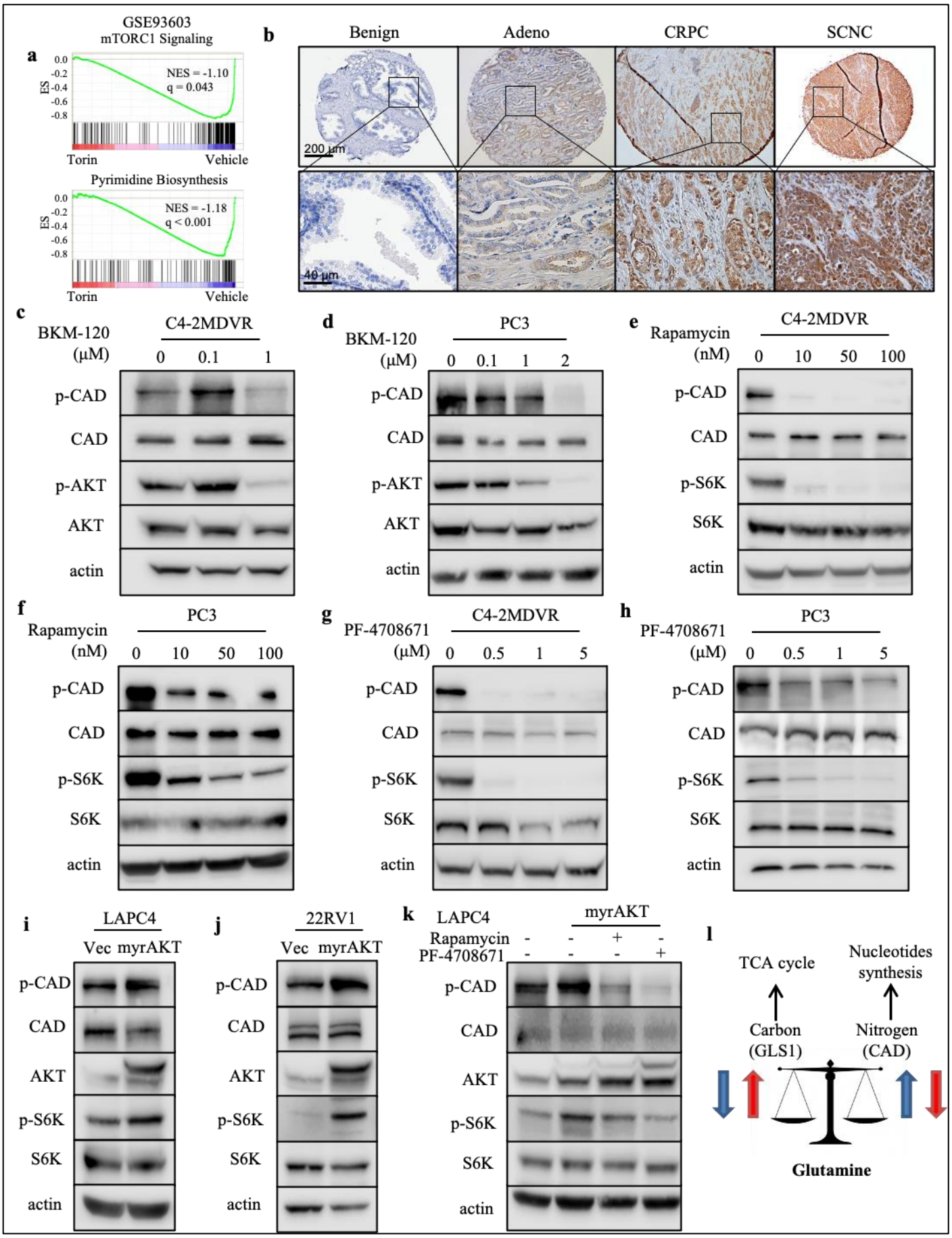


Fig. 5 CAD RNA and protein level in different PCa cell lines. **a.** mRNA level of CAD was detected by qPCR in C4-2, C4-2MDVR, PC3, DU145, NCI-H660, LNCaP, LAPC4, 22RV1 and LNCaP95 cell lines. **b.** Western blot assessment of total CAD level in C4-2, C4-2MDVR, PC3, DU145, NCI-H660, LNCaP, LAPC4, 22RV1 and LNCaP95 cell lines.

Subtask 3: Determine CAD phosphorylation regulated by mTOR pathway (Time frame: Months 6-8).

CAD can be phosphorylated by mammalian target of rapamycin(mTOR)-S6K axis on Ser^{1859}. Given that alterations of the PI3K-phosphatase and tensin homolog (PTEN)-mTOR pathway are extremely frequent in PCa (including both CRPC and SCNC), we hypothesized that activation of PI3K-AKT-mTOR pathway may phosphorylate and activate CAD. Furthermore, inhibition of mTOR with Torin1 significantly suppressed the expression of genes involved in pyrimidine synthesis (**Fig. 6a**), suggesting that pyrimidine synthesis is dependent on mTOR signaling. Indeed, IHC staining of human PCa TMAs also displayed pronounced expression of CAD^{S1859} in CRPC and SCNC where mTOR pathway is more frequently activated in comparison to benign prostate tissue and primary adenocarcinoma (**Fig. 6b**). In PCa, activation of PI3K-AKT-mTOR pathway is often trigged by PTEN deletion. Treatment with the PI3K inhibitor BKM-120 reduced CAD^{S1859} phosphorylation without changing total CAD levels in PC3 and C4-2MDVR cells (Fig. 6c, d). Specific inhibitors for downstream mediators of the PI3K pathway, rapamycin (an inhibitor of mTOR) and PF-4708671 (an inhibitor of S6K1), also significantly inhibited CAD^{S1859} phosphorylation (**Fig. 6e, h**). We overexpressed myristoylated AKT in PTEN WT cell lines LAPC4 and 22RV1 where the endogenous phospho-AKT levels are low and observed increased CAD phosphorylation along with activation of S6K (**Fig. 6i, j**). Targeting the cells with rapamycin or PF-4708671 abrogated the myristoylated AKT-induced CAD phosphorylation (**Fig. 6k**). Together, these findings suggest that loss of PTEN stimulates CAD phosphorylation mediated by PI3K-AKT-mTOR-S6K signaling axis which contributes to increased pyrimidine synthesis in advanced PCa.

Fig. 6 PI3K-AKT signaling phosphorylates CAD in advanced PCa. **a.** GSEA of "mTORC1 Signaling" and "Pyrimidine Biosynthesis" gene sets in the compassion of cells treated with torin or vehicle. **b.** Representative images of phosphorylated CAD^{S1859} IHC staining on the TMAs including benign prostate tissue (n = 40), primary prostate adenocarcinoma (n = 40), CRPC (n = 16) and SCNC (n = 17). Adeno, adenocarcinoma. CRPC, castration-resistant prostate cancer. SCNC, small neuroendocrine prostate cancer. **c, d.** Western blot assessment of phosphorylated CAD^{S1859}, total CAD, phosphorylated AKT^{S473} and total AKT protein levels after treatment with the PI3K inhibitor BKM-120 for 48 hours in C4-2MDVR (c) and PC3 (d) cells. **e, f.** Western blot assessment of phosphorylated CAD^{S1859}, total CAD, phosphorylated $S6K^{T389}$ and total S6 protein levels after treatment with the mTOR inhibitor rapamycin for 48 hours in C4-2MDVR (e) and PC3 (f) cells. **g, h.** Western blot assessment of phosphorylated CAD^{S1859}, total CAD, phosphorylated $S6K^{T389}$ and total S6 protein levels after treatment with the S6K inhibitor PF-4708671 for 48 hours in C4-2MDVR (g) and PC3 (h) cells**. i, j,** Western blot assessment of phosphorylated CAD^{S1859}, total CAD, total AKT, phosphorylated $S6K^{T389}$ and total S6 protein levels after infection of myristoylated AKT in LAPC4 (i) and 22RV1 (j) cells. **k,** Western blot assessment of phosphorylated CAD^{S1859}, total CAD, total AKT, phosphorylated $S6K^{T389}$ and total S6 protein levels after infection of myristoylated AKT together with the PI3K inhibitor (BKM-120) or the mTOR inhibitor (rapamycin) treatment in LAPC4 cells. **l,** Diagram of proposed mechanism for the crosstalk within glutamine metabolic network.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q = 0.043
ES
Torin
Vehicle
Pyrimidine Biosynthesis
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q < 0.001
b
Benign
Adeno
CRPC
SCNC
200 μm
40 μm
c
BKM-120 (μM)
C4-2MDVR
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p-CAD
CAD
p-AKT
AKT
actin
d
BKM-120 (μM)
PC3
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e
Rapamycin (nM)
C4-2MDVR
0 10 50 100
p-S6K
S6K
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PC3
g
PF-4708671 (μM)
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k
LAPC4
myrAKT
Rapamycin
PF-4708671
l
TCA cycle
Nucleotides synthesis
Carbon (GLS1)
Nitrogen (CAD)
Glutamine

Specific Aim 2: To evaluate the synergistic effect of targeting both carbon and nitrogen metabolism of glutamine in therapy-resistant PCa.

Major Task 3: To test the combinatorial effect of targeting glutamine carbon and nitrogen *in vitro*.

Subtask 1: Generate doxycycline-inducible shRNA system specifically targeting CAD expression (Time frame: Months 8-10).

We next performed experiments to study the function of CAD in PCa. Ectopic expression of short-hairpin RNAs (shRNAs) targeting CAD reduced cellular proliferation of PC3 and C4-2MDVR cells (**Fig. 7a, d**). Similar results were obtained by colony formation assays (**Fig. 7b, e**). To confirm the results obtained through constitutive expression of shRNAs, we employed a doxycycline-inducible shRNA knockdown model to study loss-of-function phenotypes. Similarly, cellular proliferation decreased dramatically upon doxycycline treatment targeting CAD (**Fig. 7c, f**). These results suggest that CAD is required in advanced PCa.

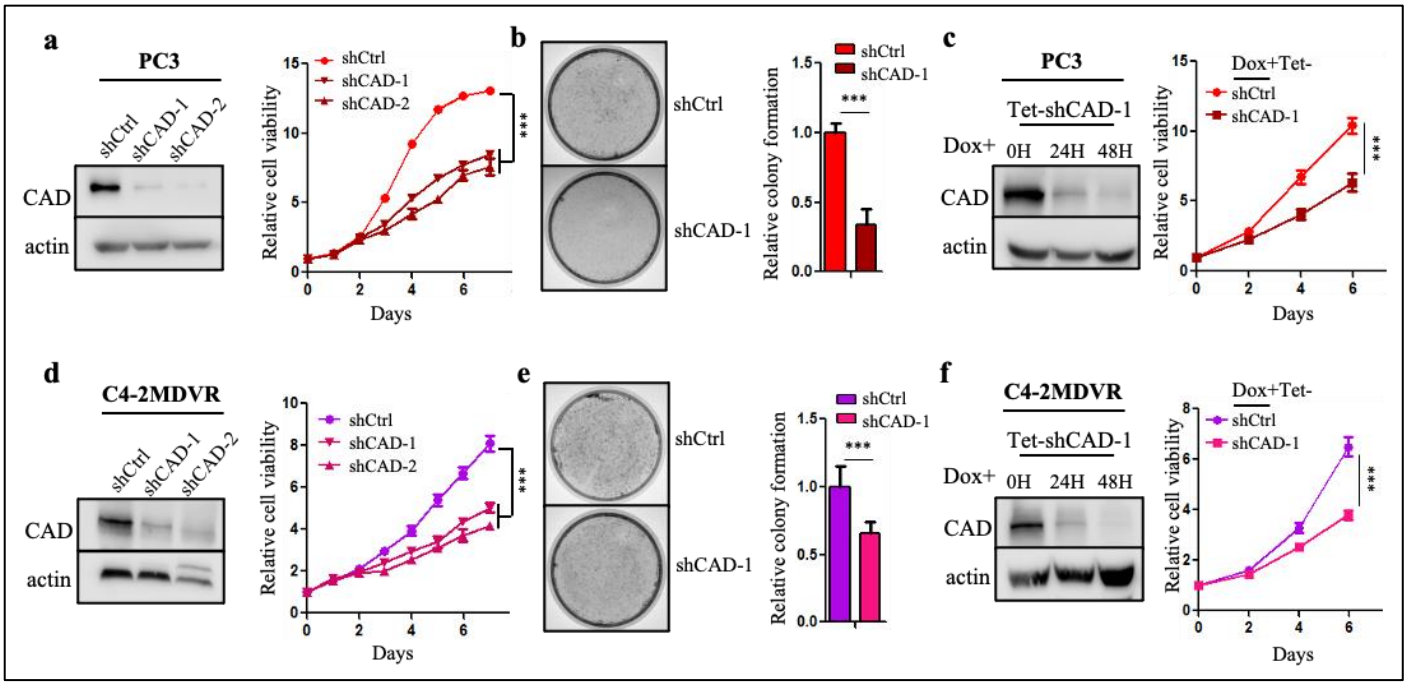

Fig. 7. **a, d.** Western blot showing two sets of shRNAs targeting CAD in PC3 (**a**) and C4-2MDVR (**d**) cells. Cellular viability was measured in the corresponding cell lines. **b, e.** Colony formation assay determining tumorigenic capability in the indicated cell lines in PC3 (**b**) and C4-2MDVR (**e**) cells. **c, f.** Western blot showing CAD protein levels in PC3 (**c**) and C4-2MDVR (**f**) cells transduced with shRNAs against CAD in an inducible vector system at various time points. Cellular viability was measured in the corresponding cell lines.

Subtask 2: Measure downstream metabolite levels of CAD and GLS1 (Time frame: Months 10-11).

Although acute inhibition of either CAD or GLS1, key enzymes for glutamine nitrogen and carbon catabolism, respectively, can achieve significant tumor suppression in advanced PCa, the inhibitory effect gradually diminished over time (**Fig. 8d**). This observation raised the possibility that tumor cells may acquire resistance through compensatory mechanisms after either pathway is inhibited. Given that glutamine carbon and nitrogen pathways both promote tumor proliferation and are tightly connected, we hypothesized that there may be a reciprocal regulation of the two pathways in PCa cells so that inhibition of one pathway will eventually lead to the activation of the other, resulting in therapeutic resistance. To determine whether the cellular metabolism underwent reprogramming in those genetically modified cells that are resistant to either GLS1 or CAD inhibition, we performed metabolite profiling experiments in cells with single knockdown of GLS1 or CAD, respectively, and their combination knockdown in PC3 and C4-2MDVR cells (**Fig. 8a and Fig. 8e**). As expected, the downstream products of CAD (dihydroorotate, orotate, UMP and CTP) and GLS1 (glutamate, α-KG and fumarate), respectively, were markedly reduced when CAD and GLS1 were knocked down (**Fig. 8b and Fig. 8h**). In the meantime, many other metabolic pathways and metabolites were upregulated. For instance, loss of CAD activated ammonia assimilation pathways, such as urea cycle and ammonia recycling (**Fig. 8c**), largely because pyrimidine synthesis pathway can also function to scavenge the accumulated ammonia. Surprisingly, loss of CAD also led to significantly enhanced glutaminolysis-related pathways including glutamate metabolism, aspartate metabolism and the TCA cycle (**Fig. 8c**), as well as increased levels of specific intermediate (glutamate, α-KG and fumarate) (**Fig. 8b and Fig. 8h**). Conversely, suppressing GLS1 elevated pyrimidine synthesis activity (**Fig. 8b, c and Fig. 8h**). The reciprocal regulation of glutamine carbon and nitrogen catabolism was abolished when both enzymes were repressed (**Fig. 8b and Fig. 8h**). Interestingly, while there was a moderate elevation of intracellular glutamine concentration in cells deficient in either GLS1 or CAD, the increase was much more dramatic in cells in which both enzymes had been knocked down (**Fig. 8f, g**), indicating that inhibition of both pathways maximally prevents glutamine from being utilized by the tumor cells.

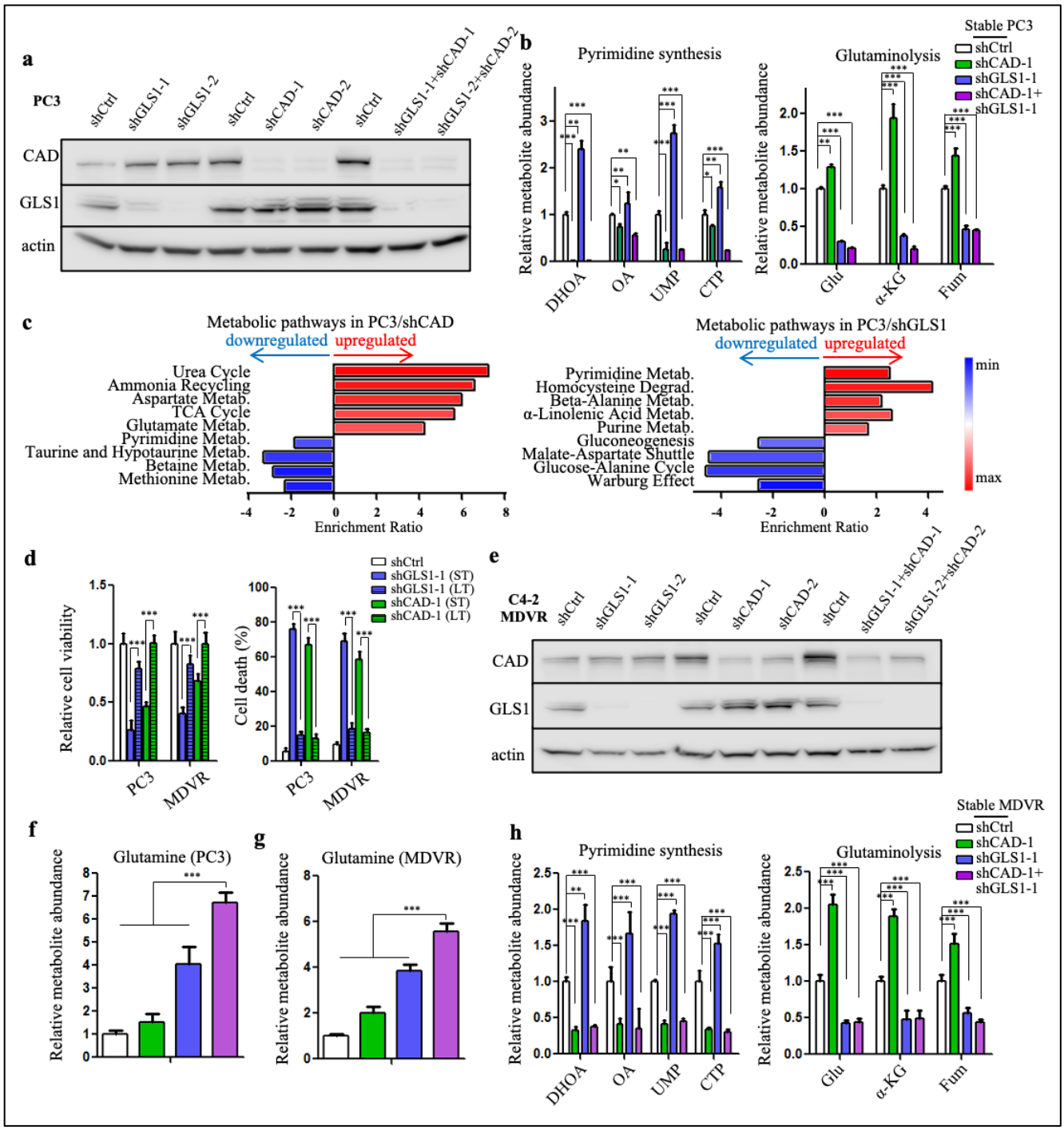

Fig. 8. **a.** Western blot showing knockdown of either GLS1 or CAD or in combination mediated by shRNAs in PC3 cells. **b.** Metabolite profiling results showing levels of representative metabolites in pyrimidine synthesis and glutaminolysis upon indicated treatment in PC3 cells. **c.** Pathway enrichment analysis showing upregulated metabolic pathways in CAD-deficient (left panel) and GLS1-deficient (right panel) PC3 cells. **d.** Cellular viability and apoptosis assays in PC3 and C4-2MDVR cells treated with either short-term (ST) or long-term (LS) knockdown mediated by shRNAs targeting GLS1 or CAD. **e.** Western blot showing knockdown of either GLS1 or CAD or in combination mediated by shRNAs in C4-2MDVR cells. **h.** Metabolite profiling results showing levels of representative metabolites in pyrimidine synthesis and glutaminolysis upon indicated treatment in C4-2MDVR cells. **f, g.** Relative intracellular glutamine levels in PC3 (**f**) and C4-2MDVR (**g**) cells

Subtask 3: Determine cell viability after treatment of either CAD knockdown of CB-839 alone or combination (Time frame: Months 11-12).

PCa cells Since the reciprocal regulation of the two branches of glutamine catabolism network is an important resistance mechanism after glutamine carbon- or nitrogen-targeted monotherapy, we tested whether simultaneously targeting both pathways in advanced PCa could achieve a synergistic effect. Doxycycline-induced reduction of GLS1 or CAD displayed modest activity in inhibiting PC3 and C4-2MDVR cells (**Fig. 9a, b**). However, their combined knockdown led to a more profound inhibition of cell viability (**Fig. 9a, b**). The combination of CAD knockdown together with CB-839, a selective GLS1 inhibitor, also achieved significant inhibitory effects in advanced PCa cells (**Fig. 9c, d**).

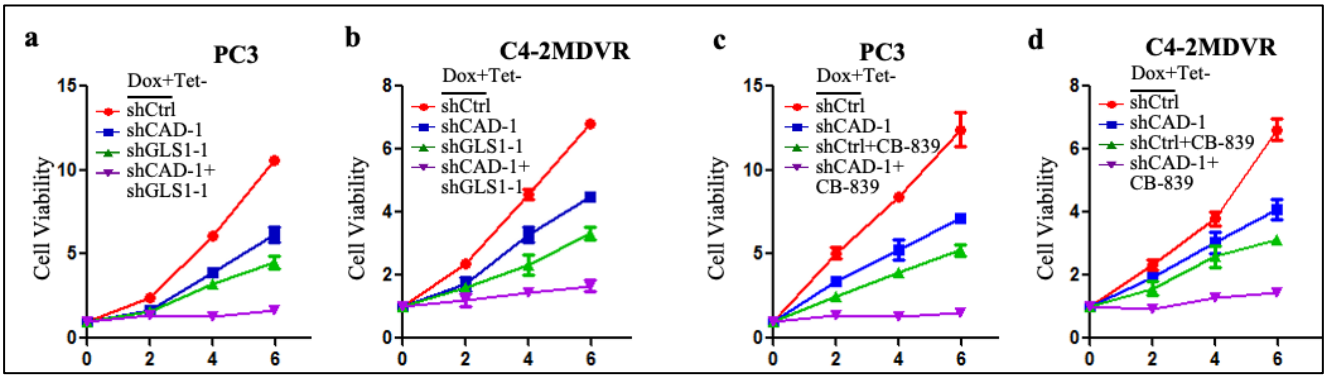

Fig. 9. **a, b.** Cellular viability of scramble control, CAD/GLS1 knockdown alone or in combination upon doxycycline induction (100 ng/ml) in PC3 (**a**) and C4-2MDVR (**b**) cells. **c, d.** Cellular viability of scramble control, CAD knockdown/CB-839 (500 nM) alone, or in combination upon doxycycline induction (100 ng/ml) in PC3 **(c)** and C4-2MDVR **(d)** cells.

Altogether, the accomplishments so far can be summarized below:

We have verified a glutamine nitrogen metabolic feature in advanced PCa that glutamine nitrogen preferentially fluxes to participate in pyrimidine synthesis.

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.

We 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. Our study

shows 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.

Opportunities for training and professional development.

Please see **Training-Specific Tasks.**

Dissemination of results to communities of interest.

Nothing to report.

Plans for next reporting period.

During the next reporting period, we plan to finish the in vivo experiments. We plan to use PC3, PC3/shCAD, PC3/shGLS-1, C4-2MDVR, C4-2MDVR/shCAD and C4-2MDVR/shGLS1 cell lines

4. **Impact:**

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.

Impact on other disciplines.

Nothing to report.

Impact on technology transfer.

Nothing to report.

Impact on society beyond science and technology.

Nothing to report.

5. Changes/Problems:

Due to the unexpected Pandemic, the previous PI, Dr. Lingfan Xu cannot be able to fulfill the rest of the reward and it transferred to Dr. Xue Jiang. The PI change approval was very delayed. It took almost 10 months. We cannot move on this project smoothly during this pending state. For this reason, many experiments are delayed, especially the mouse experiments.

Dr. Jiang will continue to finish unaccomplished work as stated in the SOW (major task 3 and 4). Specifically, Dr. Jiang will identify CAD as the key factor enhancing pyrimidine synthetic activity in vivo and evaluate the synergistic effect of targeting both carbon and nitrogen metabolism of glutamine in vivo.

And the NCE was approved on 18-Jan-2023, so the period of performance is: 1 March 2021-28 February 2024.

6. Products:

Publication:

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.

7. Participants and other collaborating organizations

Name:	Xue Jiang
Project Role:	PI
Researcher Identifier	JIANG_XUE
Nearest person month worked	12
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. Jiang 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. Jiang 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. Jiang's success so far. Dr. George has attended meeting where Dr. Jiang presented her results and provided highly valuable feedbacks, especially related to the translational and clinical aspects of prostate cancer.
Funding Support	N/A

8. **Special reporting requirements:**

Nothing to report.