Title: Glutamate decarboxylase 65 signals through the androgen receptor to promote castration resistance in prostate cancer

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Running Title: GAD65 promotes CRPC development.

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Abstract
The transition from an androgen-dependent to a castration-resistant state is a critical event in the progression of prostate cancer. In this study, we compared metabolic pathways between isogenic human androgen-dependent and castration-resistant prostate cancer (CRPC) patient-derived xenograft (PDX) models, and found consistent activation of the γ-aminobutyric acid (GABA) shunt in CRPC. This difference was the result of phosphorylation and activation of glutamate decarboxylase 65 (GAD65), which synthesizes GABA from glutamate by decarboxylation. Mechanistic investigation showed that GABA binds to and retains the androgen receptor (AR) in the nucleus by facilitating AR association with the nuclear zinc finger protein ZNHIT3. GAD65 knockdown decreased the growth of multiple established CRPC xenografts and markedly delayed the time to emergence of castration resistance. These data encourage exploring GAD65 as a therapeutic target for CRPC.

Statement of Significance
This study reports metabolic alterations that could be responsible for the development of CRPC and identifies the GABA-producing enzyme GAD65 as a potential new therapeutic target.
Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer-related mortality in men (1). Androgen receptor (AR) signaling has been shown to promote prostate cancer progression. Accordingly, androgen-deprivation therapy (ADT) is a major component in the treatment of recurrent and metastatic prostate cancer. However, a considerable fraction of patients receiving ADT ultimately develop castration-resistance, mostly due to acquired mutations in the AR signaling pathway (2). These mutations typically occur after metastatic spread (3). As a result, the capability to survive ADT independent of AR pathway mutations has been hypothesized to be an early step in the evolution of some aggressive, AR-dependent castration-resistant prostate cancer (CRPC).

Metabolic reprogramming exemplified by the Warburg effect is a one of the hallmarks of cancer cells, and contributes to process of carcinogenesis and cancer progression. Identification of metabolic vulnerability of human cancers has led to the development of novel treatment strategies (4-5). For prostate cancer, metabolic change has been an area of active inquiry with potential application in metabolic imaging and biomarker diagnostics as well as for targeted therapies (6-7). Despite of significant genetic and clinical heterogeneity among prostate cancers, common metabolic reprogramming properties have been recognized. In comparison to normal prostate cells, citrate secretion is decreased and the Krebs cycle is re-activated in prostate cancer cells, with subsequent changes in cellular redox state and metabolic flux through interdigitated pathways (8). Another early and common metabolic alteration in prostate cancers is increased de novo lipogenesis (9-10). Increasing evidence suggests that metabolic reprogramming could promote tumor cell growth and survival in diverse microenvironmental conditions. For example, cancer cells adapt to hypoxia by utilizing physiological adaptation pathways that promote a switch from oxidative to glycolytic metabolism (11). Metabolic reprogramming is also critical for cancer cell survival upon distant metastasis in new environment. Specifically, upon metastasis into the liver, colorectal cancer cells undergo metabolic changes that allow using fructose (more abundant in the liver) as a major energy source (12). In the current study, we characterized metabolic reprogramming that allows prostate cancer cells to survive upon androgen deprivation.

Materials and Methods
**Cell culture.** The PC-AD and PC-CR xenografts were mechanically dissociated and cultured in RPMI-1640 (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS; for PC-AD cells) or 10% charcoal-stripped FBS (for PC-CR cells). After several serial passages, the serum was reduced to 3%, and 100 ng/ml IGF-1 (SAFC Biosciences) was added to enhance the growth of epithelial cells and suppress the proliferation of stromal cells. Prostate cancer cell lines LNCaP, abl, CWR22Rv1 and HEK293T cell line were obtained from American Type Culture Collection between 2010 and 2015 and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (for LNCaP cells and HEK293T cells) or 10% charcoal-stripped FBS (for abl and CWR22Rv1 cells). The identity of the cell lines was verified by short tandem repeat (STR) profiling (Shanghai Biowing Applied Biotechnology), absence of mycoplasma infection was verified by conventional PCR with the PromoKine PCR Mycoplasma Test Kit I/C. All stable cell lines were aliquoted and frozen after derivation and expansion, experiments were performed within the first 25 passages following thawing.

**Immunofluorescence, immunoprecipitation and Western blotting.** Immunofluorescence, immunoprecipitation and Western blotting were performed as previously described (13).

**FRAP analysis.** FRAP assay was performed in live cell chambers (Corning) at 37°C using a Zeiss LSM510 microscopy. The cells expressing GFP-AR were bleached with argon laser at 488nm wavelength. About 90% of the GFP signal was bleached in both the nuclei and cytoplasm. Images were then taken with 35 frames at 25s intervals. Average intensities in regions of interest were measured using MetaMorph software (Molecular Devices). The intensity in the pre-bleach image was set to 100%, and the first post-bleach image was set as time point 0. The recovery curves represent the average of at least six cells.

**Pull-down assays.** GST fusion proteins were expressed in BL21(DE3) bacteria, lysed by using a French press, isolated using glutathione beads, eluted in 50 mM Tris-Cl (pH 8.0) containing 10 mM glutathione, and dialyzed into phosphate-buffered saline (PBS). His-tagged proteins were expressed and purified using a Talon metal affinity resin (Clontech). Recombinant proteins were incubated in Tris buffered saline Nonidet P-40 buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na3VO4, 20 mM p-nitrophenyl phosphate) supplemented with a cocktail of protease inhibitors and 250 μM GABA at 4°C for 4h. Glutathione-agarose beads (GST pull-down) or Ni-NTA-agarose beads (His
pull-down) were then added in the incubation for additional 2 h. The bound proteins were resolved by SDS-PAGE and subjected to Western blotting.

**Ethical issues.** Patients provided their written informed consent for the use of samples from their tumors for future investigations, and experiments were conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Second Military Medical University.

**Animals.** Four-week-old male NOG mice were used as transplant recipients. All animal procedures were performed in compliance with the institutional ethical requirements and approved by the Institutional Animal Care Use Committee of Second Military Medical University.

**Statistical analyses.** Statistical analysis was carried out with SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). Experimental data is expressed as means±SD and analyzed by two-tailed unpaired t-test. Association between Sirt5 expression and disease recurrence was analyzed by the Kaplan-Meier method, and curves were compared by the log-rank test. P<0.05 was considered to indicate statistical significance throughout the study.

**Results**

**GABA shunt is increased in CRPC.** Biopsy specimens were obtained from 7 patients with locally advanced or metastatic prostate cancer patients, and were immediately implanted subcutaneously into NOG mice in the presence of Matrigel. After a minimum of three generations of serial passaging, we were able to establish transplantable PDX lines in 5 of the 7 patients (PC-AD1, PC-AD2, PC-AD3, PC-AD4 and PC-AD7). The transplantable tumor lines retained major histopathologic characteristics of their matched patient tumor (Supplementary Table.1). Host castration resulted in a dramatic decrease in tumor volume in all established lines; however, a vestige of the regressed xenograft remained at the inoculation site. Within 7 months of continuing observation, 3 out of the 5 regressed xenografts relapsed (PC-AD1, PC-AD3 and PC-AD4; Supplementary Fig. S1A), indicating progression to an androgen-independent state. We successfully established the stable subcutaneous xenograft model from the first relapsed xenograft after five subcutaneous passages in castrated NOG mice (named PC-CR1, PC-CR3 and PC-CR4, respectively). We next compared the requirement for glucose or glutamine of hormone-dependent (AD) with paired castration-resistant (CR) lines. Similar to that in most proliferating cells, both PC-AD and PC-CR lines...
proliferated at a high rate when glucose and glutamine were abundant (Supplementary Fig. S1B), with high levels of glucose and glutamine consumption and lactate production (Supplementary Fig. S1C). Also, no apparent difference was observed in fatty acid uptake between the paired PC-AD and PC-CR xenograft cells (Supplementary Fig. S1D). TCA activity, as reflected by oxygen consumption rate (OCR), was similar between PC-AD and PC-CR cells (Supplementary Fig. S1E). Notably, steady state level of succinyl-CoA, but not other TCA cycle metabolites (αKG, succinate or malate), was reproducibly diminished in all PC-CR lines tested as compared with their androgen-dependent counterparts (Fig. 1A). In PC-AD cells, virtually all intracellular αKG, succinyl-CoA and succinate were rapidly labeled after addition of [U-13C]glucose or [U-13C]glutamine. In contrast, a substantial fraction of succinyl-CoA failed to become labeled with [U-13C]glucose or [U-13C]glutamine in PC-CR cells (Fig. 1B). Quantification of metabolite fluxes revealed significantly lower glucose and glutamine fluxes through succinyl-CoA in PC-CR cells (Fig. 1C) despite of similar flux of glucose- or glutamine-derived carbons through αKG in PC-CR versus PC-AD (Supplementary Fig. S1F).

The γ-aminobutyric acid (GABA) shunt bypasses the αKG dehydrogenase complex (KGDHC) and succinyl-coenzyme A ligase (SUCL) for the conversion of αKG into succinate, and thus decreases the formation of succinyl-CoA (14). PC-CR cells had substantial glutamine or glucose-dependent GABA and succinic semialdehyde (SSA) production (Fig. 1D). Also, these cells preserved larger intracellular pools of GABA and SSA than their parental hormone-naive cells (Fig. 1E). Glutamic acid decarboxylase (GAD), GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) are key enzymes in the GABA shunt. To assess the role of the GABA shunt in CRPC growth, we impaired the enzyme activity individually using RNA interference (Supplementary Fig. S1G). The growth of CRPC was markedly decreased by silencing the GAD65 isoform (Supplementary Fig. S1H-S1I), but not by knockdown of GAD67, GABA-T or SSADH (Supplementary Fig. S1J). Consistent with this finding, 13C-tracing experiments showed increased glutamate (and total glutamate) and decreased GABA (and total GABA) upon GAD65 knockdown in PC-CR4 cells (Fig. 1F), suggesting an increased GABA shunt activity in CRPC.

**GAD65 is required for cell proliferation in CRPC.** Silencing GAD65 produced more profound inhibition
on the growth in PC-CR4 cells than in matched PC-AD4 cells (Fig. 2A, Supplementary Fig. S1H-S1I, Supplementary Fig. S1K). More robust inhibition by GAD65 silencing was also observed in the androgen-dependent prostate cancer cell line LNCaP than in its androgen-independent derivative LNCaP-abl (abl) (Fig. 2B and Supplementary Fig. S1L). In an in vivo mouse xenograft CRPC model employing CWR22Rv1 cells, tumors derived from GAD65 knockdown (KD) cells demonstrated decreased growth rate and masses with reduced cell proliferation rate assessed by immunohistochemistry (IHC) staining of Ki-67, compared to those derived from control cells (Fig. 2C, upper panels). However, the growth of xenografts derived from PC-AD4 and PC-AD4 GAD65 KD cells did not differ (Fig. 2C, lower panels). We generated “rescue” CRPC cells (PC-CR4 and abl) with stable knockdown of endogenous GAD65 and rescue expression of wild-type (WT) or an enzyme-dead K396R mutant (15) of a shRNA-resistant, flag-tagged human GAD65 form. Rescue expression of Flag-GAD65WT, but not the K396R mutant, prevented GAD65 shRNA from inhibiting the proliferation of PC-CR4 and abl cells (Fig. 2D). Moreover, rescue CWR22Rv1 cells expressing GAD65WT but not the K396R mutant demonstrated restored potential of tumor formation and growth (Fig. 2E). These data together suggested that GAD65 is necessary for CRPC proliferation.

**Phosphorylation at S6 activates GAD65.** To clarify whether activation of the GABA shunt in CRPC is due to increased GAD65 enzyme activity, we expressed flag-tagged GAD65 in PC-AD and PC-CR cells (Supplementary Fig. S2A). GDA65 activity after immunoprecipitation was significantly higher in PC-CR cells than in PC-AD cells (Fig. 3A). Phosphorylation status plays an important role in regulation of GAD activity (16). Four putative phosphorylation sites (serine 3, serine 6, threonine 95 and serine 417) have been identified in GAD65 by mass spectrometry (17). We mutated each of the 4 putative phosphorylation sites individually to alanine (A), and examined their phosphorylation. GAD65 phosphorylation was decreased by mutation at T95, and more so with S6 (by approximately 90%), but not at S3 and S417 (Fig. 3B), indicating that S6 is the major phosphorylation site in GAD65. To test the effect of S6 phosphorylation, we also generated the GAD65S6E mutant, in which S6 was substituted with glutamate to mimic constitutive phosphorylation, and over-expressed flag-tagged mutant or wild-type (WT) GAD65 in HEK293T cells (Supplementary Fig. S2B). GAD65 enzyme activity was significantly increased by substitution with
glutamate (GAD65\textsuperscript{S6E}) but not with alanine (GAD65\textsuperscript{S6A}) (Fig. 3C). Consistent with a stimulative effect of phosphorylation on GAD65 activity, inhibition of phosphatase by okadaic acid (OA) and vanadate (Vd) treatment significantly increased GAD65 activity in cells over-expressing wild-type GAD65 (Fig. 3D), but not in cells expressing either GAD65\textsuperscript{S6A} or GAD65\textsuperscript{S6E} mutant (Fig. 3D). Taken together, these data suggest that phosphorylation at S6 activates GAD65.

**GAD65\textsuperscript{S6} phosphorylation is elevated in CRPC.** Next, we generated an antibody specifically recognizing the S6-phosphorylated GAD65. The specificity of the anti-phospho-GAD65\textsuperscript{S6} antibody was verified by recognition of the S6-phosphorylated peptide but not the unphosphorylated control peptide (Supplementary Fig. S2C). Phosphorylation of GAD65 could readily be detected by this antibody; the signal was diminished by GAD65 knockdown (Supplementary Fig. S2D) and completely blocked by pre-incubation with the antigen peptide (Supplementary Fig. S2E), confirming the specificity of the anti-phospho-GAD65\textsuperscript{S6} antibody. GAD65\textsuperscript{S6} phosphorylation was negligible or absent in hormone-sensitive PC-AD and LNCaP cells but present in the paired castration-resistant PC-CR and abl cells (Fig. 3E). Consistent with the key role of PKCe in GAD65 phosphorylation (16), we found higher levels of the active PKCe in castration-resistant cells (Supplementary Fig. S2F). The activity of phosphoinositide 3-kinase (PI3K), an upstream activator of PKCe (18) and an essential regulator of CRPC (19), was also increased in castration-resistant cells (Supplementary Fig. S2G), suggesting major contribution of the PI3K/PKCe signaling to GAD65\textsuperscript{S6} phosphorylation in CRPC. Furthermore, we evaluated the kinetics of GAD65\textsuperscript{S6} phosphorylation in serial passages of PC-CR4 tumors in castrated mice. Percentage of the cells with GAD65\textsuperscript{S6} phosphorylation increased with passaging of the PC-CR4 line, from ~37% after the first passage to >80% by passage 5 (Fig. 3F). Such an increase was accompanied with slight increase in AR expression, moderate increases in PSA/TMPRSS2 expression (Supplementary Fig. S2H) and decreased tumor doubling time (9 days in P5 vs. 15 days in P1), suggesting a growth advantage of phosphorylated over unphosphorylated GAD65\textsuperscript{S6} cells in castrated mice.

To determine whether GAD65\textsuperscript{S6} phosphorylation can be detected in clinical CRPC, the anti-phospho-GAD65\textsuperscript{S6} antibody was characterized by its suitability for immunohistochemistry. We found that this antibody could detect strong signals that were specifically blocked by the phospho-S6 antigen
peptide in paraffin-embedded normal colon tissues (Supplementary Fig. S2I). In soft-tissue and bone metastases obtained from 16 men who died from CRPC, majority (12/16) exhibited high level of GAD65<sup>S6</sup> phosphorylation (>30% phosphorylated S6-positive cells) (Fig. 3G and 3H). We also stained tissue microarrays containing samples from individuals with benign prostatic hyperplasia (BPH), hormone-naive prostate cancer, prostate cancer treated with neoadjuvant hormone ablation and CRPC. We detected high GAD65<sup>S6</sup> phosphorylation in 1.5%, 12.3%, 56.7% and 83.1% of these samples, respectively. The mean percentage of cells positive for GAD65<sup>S6</sup> phosphorylation among all samples increased from 2.7% in benign prostatic hyperplasia to 5.3% in hormone-naive disease, 40.5% in men treated with neoadjuvant ADT and 76.2% in CRPC (P<0.01) (Fig. 3I and 3J). These data demonstrate that GAD65<sup>S6</sup> phosphorylation is rare in untreated androgen-dependent prostate cancer, increases with androgen deprivation and is highest in CRPC.

**GAD65 maintains AR nuclear accumulation in CRPC via GABA.** To decipher the role of GAD65 in bioenergetics and anabolic biosynthesis of CRPC, we performed a set of metabolic assays using CRPC cells with GAD65 knockdown. In comparison with the empty vector control, GAD65 knockdown did not affect intracellular ATP level, glucose uptake, glycolytic rate, lactate production, oxygen consumption rate, glutamine uptake and glutaminolysis rate (Supplementary Fig. S3A-S3G). GAD65 knockdown also did not affect oxidative pentose phosphate pathway (PPP) flux (Supplementary Fig. S3H) and the overall lipid and RNA biosynthesis (Supplementary Fig. S3I-S3J).

Since GAD65 knockdown reduced cell proliferation and tumor growth, we hypothesized that other mechanisms beyond metabolic defects might be responsible for the proliferative disadvantage in cells conferred by GAD65 knockdown. Further analyses showed that knockdown of GAD65 selectively inhibited transcriptional activity of AR and reduced the mRNA levels of AR target genes (PSA and TMPRSS2) in CRPC cells (Fig. 4A-4B). Such effects were reversed by ectopic expression of shRNA-resistant GAD65<sup>WT</sup> or the GAD65<sup>S6E</sup> mutant, but not the GAD65<sup>S6A</sup> mutant (Fig. 4C-4D). GAD65 knockdown did not significantly change the levels of AR (Supplementary Fig. S4A), but substantially reduced the levels of nuclear AR in abl and PC-CR3 cells (Fig. 4E), which was confirmed by immunofluorescence (Fig. 4F). No similar phenomena were detected in the matched androgen-sensitive...
cells (Fig. 4E-4F).

We further explored the molecular mechanism by which GAD65 regulates AR nuclear localization. GAD65 did not form a protein complex with AR. We speculated that GAD65 may indirectly regulate AR nuclear localization by controlling intracellular levels of GABA shunt intermediates. Addition of GABA up to 250μM in the culture media restored intracellular GABA levels in GAD65 knockdown cells (Supplementary Fig. S4B). Interestingly, the decrease in AR nuclear localization caused by GAD65 knockdown in CRPC cells was also reversed by GABA treatment, but not by succinate or SSA treatment (Fig. 4G-4H). We also demonstrated the ability of GABA to restore AR transcriptional activity in GAD65 knockdown cells by quantifying ARE reporter activity (Fig. 4I), as well as the expression of AR target genes (Fig. 4J). Consequently, addition of GABA, but not succinate or SSA, significantly reversed the reduced cell proliferation due to GAD65 knockdown (Fig. 4K). Based on these results, we hypothesize that GAD65-mediated conversion of glutamate into GABA functions in a pathway that is used to promote AR nuclear accumulation in CRPC.

GABA enhances AR binding with ZNHIT3. To distinguish whether GAD65 facilitates AR nuclear accumulation through either nuclear import or nuclear retention, we analyzed the nuclear localization of AR in GAD65 knockdown abl cells after treatment with leptomycin B (LMB), an inhibitor of protein nuclear export by CRM1/exportin 1 (20). As shown in Fig. 5A, AR was primarily localized in the nucleus in the presence of LMB, strongly suggesting that the cytoplasmic localization of AR after GAD65 knockdown is not due to defects in nuclear import but due to a combination of deficient nuclear retention and effective nuclear export. Co-immunoprecipitation (co-IP) of AR followed by a mass spectrometry (MS) analysis suggested binding of AR to zinc finger HIT domain-containing protein 3 (ZNHIT3) in PC-CR4 cells (Supplementary Table. S2), as well as in PC-CR3 and abl cells (Fig. 5B). Such an interaction was significantly disrupted by treating cells with a GAD65 shRNA (Fig. 5C). Next, we performed cell-free pull-down assays using purified recombinant AR incubated with purified ZNHIT3, in the presence of increasing amounts of GABA. Although ZNHIT3 displayed negligible interaction with AR, GABA (50-250 μM) increased AR-bound ZNHIT3 in a concentration-dependent manner (Fig. 5D). More importantly, we found that purified AR pre-treated with increasing concentrations of GABA displayed increasing binding
ability to ZNHIT3, whereas GABA pre-treated ZNHIT3 had no effect (Fig. 5E), suggesting that GABA directly affects the AR-ZNHIT3 complex. In line with this, a radiometric metabolite-protein interaction analysis using $^{14}$C-labeled metabolites revealed that labeled GABA but not succinate, or SSA, was retained on purified recombinant AR (Supplementary Fig. S4C). To determine the selectivity of GABA binding to AR, we generated an AR mutant with substitutions at L704, Q711, M745, R752 and F764. These residues were predicted to be critical for GABA binding to AR by molecular docking (Fig. 5F). The results demonstrated that the L704A/Q711A/M745A/R752A/F764A (5A) mutant was resistant to GABA binding (Supplementary Fig. S4D); the association between the 5A mutant and ZNHIT3 was no longer induced by GABA (Fig. 5G). To clarify whether ZNHIT3 facilitates AR nuclear retention, we performed fluorescence recovery after photobleaching (FRAP) GFP-AR in abl cells expressing scramble shRNA or ZNHIT3 shRNA (Fig. 5H). After photobleaching of GFP-AR in the nucleus, no significant differences were observed in the recovery of nuclear GFP-AR among control and ZNHIT3 knockdown cells. In contrast, after photobleaching of cytoplasmic AR, reduction of nuclear GFP-AR was significantly less in control cells compared with ZNHIT3 shRNA-expressing cells, thus indicating an important role of ZNHIT3 in AR nuclear retention.

ZHIT3 binding in the hinge region facilitates AR nuclear retention. In the next experiment, GST-fusion constructs of different AR truncation mutants lacking the NTD, hinge or DBD domains (Fig. 6A) were immobilised on glutathione resin; in vitro binding assays were performed to examine their ability of binding to ZNHIT3 in the presence of GABA. As illustrated in Fig. 6B, GABA induced ZNHIT3 binding only to AR constructs that contain the hinge region, and not any other AR domains. To locate the segment critical for ZNHIT3 association, we generated and characterized several mutants bearing smaller deletions within the AR hinge region (Fig. 6C). The AR (∆625-634) and AR (∆653-660) mutants had full binding affinity for ZNHIT3 upon GABA treatment, whereas AR (∆625-660) completely lost binding affinity for ZNHIT3 (Fig. 6D), suggesting amino acids 635-652 is absolutely required for GABA-induced AR binding to ZNHIT3. Next, we transfected HEK293 cells with the GFP-AR or GFP-ARΔ635-652 construct. Transfected AR constructs underwent nuclear import within 4 hours after DHT induction. In the absence of DHT, GFP-AR, but not GFP-ARΔ635-652, exhibited prolonged nuclear retention in the presence of GABA.
(Fig. 6E). These results indicate that GABA-induced ZNHIT3 binding in the hinge region retains AR in the nucleus to promote its nuclear accumulation. To further explore how GABA promotes ZNHIT3 binding, we generated an antibody to a specified hinge segment (amino acids 633-653) including amino acids 635 to 652 for the purpose of testing whether the GABA binding alters hinge accessibility. Immunoblotting showed that this antibody binds to hinge, hinge lacking the amino acids 655 to 668, but does not recognize NTD, LBD and DBD (Fig. 6F). In the next experiment, this anti-hinge antibody was used to probe for hinge accessibility in the GST-hinge-LBD (amino acids 624 to 920) by ELISA. The GST-hinge-LBD protein was adsorbed to microtiter wells, based on detection with an anti-GST antibody. The addition of GABA increased the anti-hinge antibody binding to GST-hinge-LBD protein by approximately 3 folds (Fig. 6G), indicating that GABA binding promotes AR hinge accessibility to ZNHIT3.

**Inhibition of the GABA shunt by GAD65 knockdown impedes the development and growth of CRPC.**

A retroviral vector, in which GAD65 shRNA was expressed under the control of the Dox-inducible activator rtTA (LV-tet-on-shGAD65), was constructed and used to infect PC-CR3, abl and CWR22Rv1 cells. Dox treatment induced knockdown of endogenous GAD65 in these cells (Supplementary Fig. S5A). After palpable tumor formation, castrated mice bearing infected PC-CR3, abl or CWR22Rv1 xenografts were treated twice weekly with PBS or Dox for 5 weeks. Dox administration significantly slowed the growth of all three tumor models, with the most prominent effect in CWR22Rv1 xenograft (Fig. 7A). Dox-treated tumors were pale, non-adherent to underlying muscle, and noninvasive histologically, whereas control tumors grossly invaded underlying muscle (Fig. 7B). Metastasis to lymph nodes was less frequent in Dox-treated mice (1 out of 5 mice) versus the control (5 out of 5) (Fig. 7C). Prolonged administration of Dox led to long-term growth suppression (Supplementary Fig. S5B) and increased the duration of survival by >100% in mice bearing abl tumors. Dox-treated tumors had large areas of cell loss, reduced proliferation (Ki-67 staining) and fewer blood vessels (CD31 staining) compared to the PBS control (Fig. 7D).

In the last set of experiments, androgen-sensitive PC-AD3 cells were transfected with the Dox-inducible vector for GAD65 shRNA expression, and then implanted subcutaneously into castrated mice. Treatment of the mice with Dox significantly delayed time to castration resistance (Fig. 7E). At week 6 after implantation, progression to castration-resistance (defined as serum PSA at >8 ng/ml) was apparent in 8 out...
of 10 mice and none of the 10 Dox-treated mice (Fig. 7F). Of note, AR immunostaining in xenografts treated with Dox was predominantly localized to the cytoplasm of tumor cells, whereas the PBS control tend to have higher level of AR in the nucleus (Supplementary Fig. S5C). These findings suggest that GAD65 inhibition maintain the sensitivity of AR in androgen-sensitive tumors in castrated hosts and inhibit the development of castration-resistance.

Discussion

In efforts to develop more effective therapies, it is critical to improve the understanding of molecular mechanisms underlying CRPC development. In the current study, we approached this question by identifying “metabolic vulnerabilities” specifically required for CRPC development. Distinct metabolic states are required for tumor growth under different environments (11-12, 21). We found increased GABA shunt activity due to GAD65 activation in CRPC. The GAD65 activation was due to S6 phosphorylation, and mediated by the PI3K-PKCε signaling axis. This finding is consistent with a previous study showing a critical role of the PI3K-PKCε signaling axis in the development and maintenance of CRPC (19). Specifically, we detected GAD65 S6 phosphorylation in multiple CRPC cell lines and in a majority of metastatic and castration-resistant prostate cancer tissues. Interestingly, GAD65 S6 phosphorylation increased with passaging of castration-resistant tumors in the xenograft models, suggesting that phospho-S6-positive cells have a growth advantage over phospho-S6-negative cells. We also provided convincing evidence showing that activated GAD65 promotes CRPC proliferation via facilitating AR nuclear accumulation. It is well recognized that AR is activated by androgen binding and translocates to nucleus to activate transcription of AR-target genes. However, in CRPC cells, AR remains in the nucleus even in the absence of androgens, transactivating androgen-responsive genes and leading to uncontrolled growth (22). Blockade of AR nuclear localization is a putative treatment strategy for CRPC. The results from the current study showed that GAD65 selectively promotes AR nuclear retention in CRPC by controlling intracellular levels of its product GABA. GABA is the major inhibitory neurotransmitter in the mammalian brain but recent studies have suggested that GABA is also implicated in many other biological functions (23-24), as well as in the development of a variety of cancers (25). Uptake of GABA and its subsequent catabolism via the GABA shunt increase NADH levels in tumor microenvironment, which in
turn confer a proliferative advantage to tumor cells (26). Also, GABA acting via its receptors ensures a beneficial and important function on tumor cell proliferation and a “GABAergic Mr. Hyde” has been described in different types of tumors where components of the GABAergic signaling are overexpressed (25). Our findings link the GABA shunt to AR nuclear accumulation in CRPC and clarify a signaling function of GABA that is independent of its role in cell metabolism or GABA receptor-mediated effects.

GABA serves as a proto-oncometabolite by binding to AR in its ligand binding domain. GABA induces a conformational switch in AR that increases hinge accessibility to ZNHIT3. ZNHIT3 is a nuclear zinc finger protein previously implicated in transcriptional regulation and in small nucleolar ribonucleoprotein particle assembly and thus possibly to pre-ribosomal RNA processing (27-28). ZNHIT3 inhibits nuclear export and promotes nuclear retention of AR. These results suggest that metabolic intermediates may function as signaling molecules to allow crosstalk between metabolic and cell signaling pathways. For example, we previously reported that TCA cycle intermediate succinate binds to and activates the ROS scavenging enzyme TrxR2 to maintain redox homeostasis (29), whereas others have found that acetoacetate specifically enhances binding of BRAF V600E to MEK1 in V600E-positive cancer cells to promote activation of MEK-ERK signaling (30). On the other hand, increasing evidence suggests that post-translational modifications, including tyrosine phosphorylation (31-32) and lysine acylation (33-34) of metabolic enzymes, are common and important to link cell signaling pathways to metabolic pathways in cancer cells. These findings together represent a realm of crosstalk with “back and forth” signal flows between metabolic and cell signaling networks that “acutely” regulate cell metabolism and proliferation, which, unfortunately, are “hijacked” by cancer cells.

Lastly, the findings from the current study encourage exploring the GABA shunt, GAD65 in particular, as a molecular target in the treatment of CRPC. Future studies, however, must address the specificity issue due to the critical functional role of GABA in the central nervous system (14).

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Figure Legends

Figure 1. CRPC cells exhibit increased GABA shunt activity. A. Intracellular metabolite levels of the paired PC-AD and PC-CR cells. a.u., arbitrary units. B. Fraction of each metabolite labeled by $^{13}$C derived from [U-$^{13}$C]glucose (left panels) or [U-$^{13}$C]glutamine (right panels) over time (0-12 h) in the paired PC-AD3 and PC-CR3 cells. C. [U-$^{13}$C]glucose (left panel) and [U-$^{13}$C]glutamine (right panel) flux through the succinyl-CoA pool in the paired PC-AD and PC-CR cells. D. The paired PC-AD and PC-CR cells were incubated with medium containing [U-$^{13}$C]glucose (left panels) or [U-$^{13}$C]glutamine (right panels) for 4 h and the fraction of GABA and SSA containing glucose- or glutamine-derived carbons was determined. E. Intracellular GABA (left panel) and SSA (right panel) levels of the paired PC-AD and PC-CR cells. a.u., arbitrary units. F. Relative metabolite abundance in PC-CR4 cells grown in [U-$^{13}$C]glucose (left panel) or [U-$^{13}$C]glutamine (right panel) upon GAD65 knockdown. Data are presented as the total metabolite pool and the $^{13}$C-labeled metabolite pool. All experiments were independently repeated three times in triplicate. Values shown are mean ± S.D. A two-tailed unpaired t-test was used to compare experimental groups. **, p<0.05.

Figure 2. GAD65 is required for androgen-independent proliferation of CRPC. A-B. Colony formation of the paired androgen-dependent and castration-resistant PC cells (A: PC-AD4 vs. PC-CR4; B: LNCaP vs. abl) with GAD65 knockdown. C. Left two panels: tumor growth and size of xenograft mice injected with parental or GAD65 knockdown CWR22Rv1 or PC-AD4 cells. Middle panels show the dissected tumors in representative mice. Right two panels show representative images and quantification of IHC staining of Ki-67 of tumors. D. Effect of GAD65 knockdown on cell proliferation rates of PC-CR4 or abl cells expressing GAD65 WT or K396R. Right panels: the presence of ectopically expressed and endogenous GAD65 was verified by Western blotting. E. Left two panels: tumor growth and size of xenograft mice injected with parental or GAD65 knockdown CWR22Rv1 cells with rescue expression of Flag-GAD65 WT or enzyme deficient K396R mutant. Right panel shows the dissected tumors in representative mice. All experiments were independently repeated three times in triplicate. Values shown are mean ± S.D. A two-tailed unpaired t-test was used to compare experimental groups. **, p<0.05. Bars: 50 μm.

Figure 3. GAD65 enzymatic activity in CRPC is enhanced by phosphorylation at residue serine 6. A.
Flag-tagged GAD65 was ectopically expressed in the paired PC-AD and PC-CR cells, after which enzyme activity of exogenous GAD65 was determined. B. Analysis of phosphorylation of individual GAD65 mutants. The indicated plasmids were transfected into PC-CR3 cells and proteins were immunoprecipitated for phosphorylation analysis. C. The indicated Flag-tagged GAD65 proteins were overexpressed in HEK293T cells. Wild-type and mutant GAD65 proteins were purified by Flag beads and eluted with Flag peptide, followed by determination of their enzyme activity. D. The indicated Flag-tagged GAD65 proteins were overexpressed in HEK293T cells, with or without combined Okadaic acid and Vanadate treatment for 48 h. Wild-type and mutant GAD65 proteins were purified by Flag beads and eluted with Flag peptide, followed by determination of their enzyme activity (upper panel) and their serine phosphorylation (lower panels). E. GAD65S6 phosphorylation in the paired androgen-dependent vs. castration-resistant PC cells. F. FACS analysis of phosphorylated GAD65S6 in serial passages (P) of PC4 from AD (PC-AD4) to CR (PC-CR4). G. Immunohistochemical analysis of GAD65S6 phosphorylation in multiple prostate cancer metastases. Tumor tissues from LNCaP, PC-AD3 and PC-AD4 xenografts were also included for comparison. H. Phosphorylated GAD65S6 immunohistochemistry of high-expression prostate cancer metastases (M), showing clear staining in M13, M12 and M10 and negligible staining in PC-AD3. I. Immunohistochemical evaluation of GAD65S6 phosphorylation in 136 prostate cancer patients with localized, hormonally treated, and castration resistant prostate cancer. BPH: benign prostate hyperplasia; NHT: neoadjuvant hormone therapy; CRPC: castrate resistant prostate cancer. J. BPH were negative, while untreated tumors show rare immunoreactivity for phosphorylated GAD65S6. GAD65S6 phosphorylation was significantly upregulated in NHT samples, and also in CRPC. The data in C and E show mean percentage of cells staining positive for phosphorylated GAD65S6 among all patients. All experiments were independently repeated three times in triplicate. Values shown are mean ± S.D. A two-tailed unpaired t-test was used to compare experimental groups. **, p<0.05. Bars: 50 μm.

**Figure 4. GAD65 contributes to AR nuclear accumulation by controlling intracellular GABA level.**

A-B. AR transcriptional activity (A) and mRNA levels of PSA/TMPRSS2 (B) in the paired androgen-dependent vs. castration-resistant PC cells with GAD65 knockdown. C-D. PC-CR3 and abl cells with stable knockdown of endogenous GAD65 were transfected with wild-type GAD65 or the S6A/E
mutants, after which transcriptional activity (C) and mRNA levels of PSA/TMPRSS2 (D) were determined.

E. Western analysis of endogenous AR from cytosolic and nuclear fractions of the paired androgen-dependent vs. castration-resistant PC cells with GAD65 knockdown. Lamin B and tubulin were used as loading controls for nuclear and cytoplasmic fractions, respectively. F. Subcellular localization of AR in the paired androgen-dependent vs. castration-resistant PC cells with GAD65 knockdown. G-K. PC-CR3 and PC-CR4 cells with stable knockdown of GAD65 was treated with or without cell-permeable GABA, succinate or SSA, after which cytosolic and nuclear AR expression (G), subcellular localization of AR (H), AR transcriptional activity (I), mRNA levels of PSA/TMPRSS2 (J) and proliferation rates (K) were determined. All experiments were independently repeated three times in triplicate. Values shown are mean ± S.D. A two-tailed unpaired t-test was used to compare experimental groups. **, p<0.05. Bars: 10 μm.

Figure 5. GABA binding to AR facilitates its association with ZNHIT3 and promotes AR nuclear retention. A. Subcellular localization of AR in abl cells with stable knockdown of GAD65 was determined in the presence or absence of LMB (25 μM, 4h). B. Co-IP of AR and ZNHIT3 in PC-CR3 and abl cells. C. Co-IP of AR and ZNHIT3 in PC-CR3 and abl cells with stable knockdown of GAD65. D. Effect of GABA on AR-ZNHIT3 binding in cell-free, in vitro assays using purified recombinant AR and ZNHIT3. E. Effect of pre-treatment of AR (left) or ZNHIT3 (right) with increasing concentrations of GABA on AR-ZNHIT3 binding in cell-free, in vitro assays. F. Schematic representation of molecular docking study of GABA based on the crystal structure of AR. GABA is docked in a pocket surrounded by residues, L704, Q711, M745, R752 and F764. G. Effect of GABA on the interaction of Flag-AR WT and L704A/Q711A/M745A/R752A/F764A (5A) mutant with ZNHIT3 in cell-free, in vitro assays. H. Abl cells with ZNHIT3 knockdown were transfected with GFP-AR and were subjected to FRAP analysis. The intensity of nuclear fluorescence was monitored for ~750 s and plotted against time. All experiments were independently repeated three times in triplicate. Values shown are mean ± S.D. A two-tailed unpaired t-test was used to compare experimental groups. **, p<0.05. Bars: 10 μm.

Figure 6. GABA increases the accessibility of AR hinge region for ZNHIT3. A. Plasmid construction of full length AR and AR deletion mutant fusion proteins. AR contains four domains: NTD, DBD, H, and
LDB. The domain deletion mutants ∆hinge (∆H), ∆DBD and ∆NTD were created. B. Purified recombinant His-ZNHIT3 protein (1μg) was incubated with glutathione-Sepharose beads coupled with wild-type or domain deletion mutant GST·AR proteins (10μg) in the presence of GABA (250μM). Subsequently, proteins bound to the beads were analyzed by PAGE-Western blotting analysis using an anti-His antibody (upper panel). Total input wild-type GST·AR and mutant GST·AR detected by anti-GST antibodies are shown in the lower panel. C. Schematic presentation of AR deletion mutants used for identification of amino acid sequence required for ZNHIT3 association. D. Purified wild-type or deletion mutant GST·AR proteins (10μg) were immobilized on glutathione-Sepharose beads. The beads were then incubated with purified recombinant His-ZNHIT3 protein (1μg) in the presence of 250μM GABA. Proteins bound to the beads were detected by Western blotting with an anti-His antibody (upper panel). Total input wild-type GST·AR and mutant GST·AR detected by anti-GST antibodies are shown in the lower panel. E. HEK293 cells transfected with GFP-AR or GFP-ARΔ635-652 were treated with 10nM DHT for 4 h, washed to remove DHT, and then treated with GABA (250μM) for the indicated times. Nuclear fractions from the cells and whole cell lysates were subjected to Western blotting analysis. F. Specificity of antibody against amino acid 633-653 of AR was determined by probing the indicated Flag-tagged proteins (upper panel). Total input of the indicated Flag-tagged AR proteins was detected by an anti-Flag antibody (lower panel). G. The GST-hinge-LBD fusion protein was adsorbed to microtiter wells and incubated with a dilution series of anti-AR (633-653) antibody in the presence or absence of 250μM GABA. The binding of the anti-AR (633-653) antibody was measured by ELISA. All experiments were independently repeated three times in triplicate. Values shown are mean ± S.D. A two-tailed unpaired t-test was used to compare experimental groups.

Figure 7. Knockdown of GAD65 inhibits growth of established tumor and block progression to castration resistance in vivo. PC-CR3, abl and CWR22Rv1 cells infected with LV-tet-on-shGAD65 were transplanted subcutaneously into castrated NOG mice. A. In vivo castration-resistant growth inhibition of PC-CR3, abl and CWR22Rv1 tumors upon treatment with PBS or Dox (8 mg/kg, twice weekly), beginning when subcutaneous tumors were palpable in castrated mice. Right panel shows the dissected tumors in representative mice. B-C. Histological analyses of PC-CR3 tumors treated with PBS or Dox, showing
decrease in tumor-muscle invasion (B) and metastases to axillary lymph nodes (C). D. Quantification of changes between PBS- and Dox-treated PC-CR3 tumors in the following parameters: hypocellular regions by H&E staining (upper panels); CD31 staining (middle panels) and Ki-67 staining (lower panels). Quantification was determined by counting five different fields per tumor, followed by averaging the values for the three tumors. E-F. PC-AD3 cells infected with LV-tet-on-shGAD65 were transplanted subcutaneously into castrated SCID mice. PC-AD3 tumor emergence in castrated mice was compared between PBS and Dox (8 mg/kg, twice weekly) treatment group (E). Intact: mice bearing PC-AD3 tumors without castration. Serum PSA was measure to determine relapse of PC-AD3 tumors 6 weeks after implantation (F). A PSA value of 8 ng/ml, marked by the dotted line, is considered indicative of tumor relapse. Values shown are mean ± S.D. A two-tailed unpaired t-test was used to compare experimental groups. **, p<0.05. Bars: 50 μm.
Figure 7  

A.  

B.  

C.  

D.  

E.  

F.
Glutamate decarboxylase 65 signals through the androgen receptor to promote castration resistance in prostate cancer


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