

Supplementary Materials and Methods

Chemicals. Methyltrienolone (R1881) was purchased from PerkinElmer (Waltham, MA) and dissolved in ethanol. Bicalutamide (Casodex) was provided as a gift from P. Turnbull (GlaxoSmithKline, Research Triangle Park, NC) and resuspended in a 1:1 mixture of ethanol and dimethylsulfoxide (DMSO). Cycloheximide was obtained from Sigma (St Louis, MO) and dissolved in DMSO. Compound C (in DMSO) was from Calbiochem (San Diego, CA). STO-609 was purchased from Tocris (Ellisville, MO) and resuspended in 100 mM NaOH. 5-aminoimidazole-4-carboxamide 1- β -D-ribo-furanoside (AICAR) was from Enzo Life Sciences (Plymouth Meeting, PA) and dissolved in water.

Antibodies. The CaMKK antibody used, unless otherwise specified, was from BD Biosciences (Palo Alto, CA). The CaMKK β (clone 1A11) antibody was from Abnova (Walnut, CA). The v5 antibody was purchased from Invitrogen (Carlsbad, CA). The GAPDH and AR antibodies have previously been described (1). Phospho-CaMKI (T177), CaMKI and Lamin A antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-AMPK α (T172), AMPK α , phospho-acetyl-CoA carboxylase (S79), and acetyl-CoA carboxylase antibodies were from Cell Signaling Technology (Danvers, MA).

Plasmids. The CMV- β gal and PSA-Luc plasmids were previously described (2). The pGL4.14 (lacks both basal promoter and enhancers) and pGL4.26 (lacks enhancer, but contains basal promoter) vectors were obtained from Promega (Madison, WI). MSCV-GWb-GAL4(DNA-binding domain (DBD))-IRES-EGFP, MSCV-GWb-CaMKK β -IRES-EGFP, MSCV-GWb-v5-ARwt-IRES-EGFP and MSCV-GWb-v5-AR(C562S)-IRES-EGFP were created using the Invitrogen Gateway recombinase subcloning system according to the manufacturer's instructions. To do this, GAL4(DBD), CaMKK β ,

v5-ARwt or v5-AR(C562S) were shuttled from pENTR-GAL4(DBD), pENTR-v5-ARwt, pENTR-v5-AR(C562) or pOTB7-CaMKK β (prostate splice variant) (American Type Culture Collection (ATCC), Manassas, VA) to MSCV-IRES-EGFP that was converted to a Gateway destination vector. The pGL4.14-CaMKK β promoter construct was created by PCR amplifying a 2.1 kb genomic sequence that encompassed the *CaMKK β* transcriptional start site through the potential AR binding site identified using ChIP on Chip (previously described (3)). This fragment was then cloned into the pGL4.14 vector using NheI and HindIII restriction sites. Subsequent deletion constructs were created by PCR amplifying smaller fragments that were cloned into pGL4.26 using NheI and HindIII restriction sites. Finally, the pGL4.14-CaMKK β promoter-ARE deletion construct was created from the original pGL4.14-CaMKK β promoter construct using the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All primers used for the creation of constructs are listed in Supplementary Table 1. All sequences were confirmed using restriction digests and sequencing.

Creation of stable cell lines. To create LNCaP-GAL4, LNCaP-CAMKK β , LNCaP-v5-ARwt and LNCaP-v5-AR(C562S) cells, parental cells were infected with retrovirus expressing MSCV-GWb-GAL4(DBD)-IRES-EGFP (negative control), MSCV-GWb-CAMKK β -IRES-EGFP, MSCV-GWb-v5-ARwt-IRES-EGFP or MSCV-GWb-v5-AR(C562S)-IRES-EGFP. EGFP positive cells were then selected through two rounds of cells sorting using flow cytometry and expression levels were confirmed by western blot and/or qPCR.

Supplementary References

1. Frigo DE, McDonnell DP. Differential effects of prostate cancer therapeutics on neuroendocrine transdifferentiation. *Mol Cancer Ther* 2008; 7: 659-69.
2. Frigo DE, Sherk AB, Wittmann BM, et al. Induction of Kruppel-like factor 5 expression by androgens results in increased CXCR4-dependent migration of prostate cancer cells in vitro. *Mol Endocrinol* 2009.
3. Wang Q, Li W, Liu XS, et al. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol Cell* 2007; 27: 380-92.

Supplementary Figure Legends

Supplementary Figure S1. Validation of CaMKK β protein bands. For CaMKK β western blot analysis, a different monoclonal CaMKK β antibody (clone 1A11) was used than in Fig. 1. CaMKK β protein levels were normalized to GAPDH loading control. Results are expressed as fold induction over vehicle-treated cells + SE (n = 2). *, $P < 0.05$ indicates significant changes from mock-transfected cells.

Supplementary Figure S2. CaMKK β levels are increased in prostate cancer samples. Independent microarrays were analyzed using the OncoPrint resource. A, four separate studies determined that CaMKK β levels were elevated in prostate cancer samples (red) compared to normal prostate controls (blue). B, CaMKK β levels correlated with disease progression. C, CaMKK β levels are significantly higher in prostate cancer samples compared to other cancers (a-bladder, b-kidney, c-colon, d-breast, e-esophageal, f-liver, g-lung, h-ovarian, i-pancreatic, j-squamous cell lung). All changes in expression were at the $P < 0.001$ level.

Supplementary Figure S3. The prostate expresses different splice variants of CaMKK β compared to brain (expanded Figs. 2A and B) A, schematic of CaMKK β splice variants. B, RT-PCR using primers spanning specific exon-exon boundaries (indicated in right schematic) was performed on cDNA generated from various tissues and cell lines.

Supplementary Figure S4. CaMKK β is required for androgen-mediated cell migration in prostate cancer cells. A, LNCaP cells were pretreated for 1 h with vehicle, 10 or 30 nM STO-609 prior to

overnight treatment with vehicle, 100 pM or 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (right). Phospho-CaMKI (p-CaMKI) levels were normalized to total CaMKI. Results are expressed as fold induction/phosphorylation over double vehicle-treated cells + SE (n = 2). *, $P < 0.05$ indicates significant changes from vehicle-treated cells. #, $P < 0.05$ indicates significant changes from vehicle (no STO-609)-treated cells. B, VCaP cells were plated in 96-well plates and grown for 3 d. Cells were treated +/- 1 nM R1881 and +/- 30 nM STO-609 on d 3, d 5, and d 7. On d 10, cells were lysed and the relative number of cells was measured with the fluorescent DNA binding dye FluoReporter Blue. Each sample was performed in triplicate, and results from a representative experiment are shown. Results are expressed as relative cell number \pm SE (n = 2). *, $P < 0.05$ indicates significant changes from vehicle (no R1881)-treated cells. C, VCaP cells were pretreated for 1 h +/- 30 nM STO-609 prior to overnight treatment +/- 10 nM R1881. Cells were then dissociated and reseeded into the top chamber for a Boyden dual chamber migration assay. Fresh medium with the corresponding treatments was added to the top and bottom chambers while either no chemoattractant or 5% FBS (serum) was added to the bottom chamber. After 16 h, migrated cells were fixed, stained with crystal violet and counted in three different microscopic fields and added together. The results are expressed as mean \pm SE (n = 2). *, $P < 0.05$ indicates significant changes from vehicle (no R1881)-treated cells. D and E, densitometry results for western blots in Figs. 3C and D respectively. *, $P < 0.05$ indicates significant changes from vehicle-treated (D) or GAL4 control (E) cells. #, $P < 0.05$ indicates significant changes from control (siLacZ)-transfected cells (D).

Supplementary Figure S5. Androgen mediated prostate cancer cell migration requires functional AR-mediated transcription. A and B, an example of the AR replacement strategy is shown. This method has the advantage of using cells with endogenous androgen signaling as opposed to the common

reintroducing of AR into AR-negative cells, which often has artificial biological consequences. Here, cells that express endogenous AR, in this case LNCaPs, were retrovirally infected to create stable cell lines expressing a control (GAL4) or a v5-tagged version of AR (wild type or a DNA-binding domain mutant (C562S)) linked to an IRES-EGFP. Cells were then selected using 2 rounds of flow cytometry. Subsequently, EGFP-positive cells were transfected with chemical siRNAs targeting either a control sequence (siLacZ) or the 3'-UTR of AR (eliminates endogenous receptor). A, a western blot characterization of the resultant cell lines is shown at the right using antibodies for v5 (recognizes only exogenous AR), AR (recognizes both exogenous and endogenous AR) or GAPDH (loading control). B, LNCaP cells used in the AR replacement experiments were also subjected to qPCR analysis using primers targeting the AR 3'UTR (monitors endogenous AR levels). The expression of AR was normalized to 36B4 levels and results are expressed as relative mRNA levels of AR compared to mock-transfected cells + SE (n = 2). *, $P < 0.05$ indicates significant changes from mock-transfected cells. C, cells were then subjected to a migration assay as described in Fig. 4. *, $P < 0.05$ indicates significant changes from vehicle-treated cells.

Supplementary Figure S6. Identification of the ARE that regulates *CaMKK β* expression. A, two *CaMKK β* enhancer (fragments D and E from Fig. 4C) luciferase reporter constructs were transfected into LNCaP cells and then pretreated for 30 minutes with vehicle or 10⁻⁶ M Casodex followed by treatment overnight with vehicle or various concentrations of R1881 (0, 0.1, 1 and 10 nM). After treatment, cells were harvested and assayed for luciferase activity. Luciferase values were normalized to β -galactosidase control. Data are the mean relative light units (RLUs) + SEM for one representative experiment performed in triplicate (n = 3). *, $P < 0.05$ indicates significant changes from vehicle (no R1881)-treated cells. # $P < 0.05$ indicates significant changes from vehicle (no Casodex)-treated cells.

B, VCaP cells were transfected, treated and assayed for luciferase activity as in A using the *PSA* enhancer and *CaMKK β* enhancer fragments D and E. C, *CaMKK β* enhancer deletion constructs were transfected into LNCaP cells and then treated and assayed for luciferase activity as in A (n = 2). Emp Vec, empty vector.

Supplementary Figure S7. Androgen-mediated migration occurs through a CaMKK β -AMPK-dependent pathway. A, VCaP cells were treated for 24 h +/- 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (right). Phospho-CaMKI (p-CaMKI) levels were normalized to total CaMKI. Phospho-AMPK (p-AMPK) levels were normalized to total AMPK. Results are expressed as fold induction/phosphorylation over vehicle-treated cells + SE (n = 2). *, $P < 0.05$ indicates significant changes from vehicle-treated cells. B, selection of optimal AMPK α 1 and α 2 siRNAs. LNCaP cells were transfected as described in Fig. 5 with mock or Stealth siRNAs targeting LacZ (negative control) or AMPK α 1 or α 2. The expression of AMPK was assessed using qPCR and normalized to 36B4 levels. Results are expressed as fold induction over mock-transfected cells + SE (n = 2). *, $P < 0.05$ indicates significant changes from mock-transfected cells. C and D, densitometry results for western blots in Figs. 5C and D, respectively. For AMPK α knockdown (C), siAMPK α 1-#1 and siAMPK α 2-#1 from B were selected since they produced the greatest knockdowns. *, $P < 0.05$ indicates significant changes from control (siLacZ)-transfected cells.

Supplementary Figure S8. AMPK is required for prostate cancer cell migration. LNCaP cells stably expressing either GAL4 or CaMKK β were transfected with indicated siRNAs and subjected to a migration assay (top) or western blot analysis (bottom) as in Fig. 3C (n = 2). *, $P < 0.05$ indicates

significant changes from LNCaP-GAL4 cells. #, $P < 0.05$ indicates significant changes from control (siLacZ)-transfected cells.

Supplementary Figure S9. AMPK is required and sufficient for androgen-mediated prostate cancer cell migration. A, LNCaP cells were pretreated for 1 h with vehicle or increasing concentrations of compound C (10 or 40 μ M) prior to overnight treatment +/- 10 nM R1881 or 1 mM AICAR. Cells were then subjected to a migration assay as described in Fig. 3. The results are expressed as mean \pm SE (n = 2). *, $P < 0.05$ indicates significant changes from double vehicle-treated cells. #, $P < 0.05$ indicates significant decreases from vehicle (no compound C)-treated cells. B, LNCaP cells were pretreated for 1 h with vehicle, 1, 10 or 40 μ M compound C prior to overnight treatment +/- 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (top). ACC is a direct target of AMPK and thus, was used as a readout of AMPK catalytic activity. Phospho-ACC (p-ACC) levels were normalized to total ACC. Results are expressed as fold induction/phosphorylation over double vehicle-treated cells + SE (n = 2). *, $P < 0.05$ indicates significant changes from double vehicle-treated cells. C, LNCaP cells were pretreated for 1 h with vehicle, 10 or 40 μ M compound C prior to overnight treatment +/- 10 nM R1881. PSA or SGK1 mRNA levels were quantitated using qPCR. Results are expressed as fold induction over vehicle (no R1881)-treated cells \pm SE (n = 2). *, significant changes from double vehicle-treated cells. #, $P < 0.05$ indicates significant decreases from vehicle (no compound C)-treated cells. D, LNCaP cells were treated overnight +/- 1 mM AICAR and then subjected to western blot analysis and densitometry (top) as in B. Phospho-AMPK (p-AMPK) levels were normalized to total AMPK. *, $P < 0.05$ indicates significant changes from vehicle-treated cells.

Supplementary Table 1. Primers and siRNA sequences used in these studies

<i>Primer/siRNA</i>	<i>Sequence</i>
qPCR primers	
36B4	Forward: 5'-GGACATGTTGCTGGCCAATAA-3' Reverse: 5'-GGGCCCAGACCAGTGTT-3'
CaMKK β	Forward: 5'-TCCAGACCAGCCCAGCATAG-3' Reverse: 5'-CAGGGGTGCAGCTTGATTTC-3'
CXCR4	Forward: 5'-TGGCCTTATCCTGCCTGGTAT-3' Reverse: 5'-AGGAGTCGATGCTGATCCCAA-3'
AR 3'UTR	Forward: 5'-CCATGGCACCTTCAGACTTT-3' Reverse: 5'-ACTGGGCCATATGAGGATCA-3'
AMPK α 1	Forward: 5'-CTCAGTTCCTGGAGAAAGATGG-3' Reverse: 5'-CCCAGTCAATTCATGTTTGCC-3'
AMPK α 2	Forward: 5'-ATGGAATATGTGTCTGGAGGTG-3' Reverse: 5'-TGGTTTCAGGTCTCGATGAAC-3'
PSA	Forward: 5'-CCTCCTGAAGAATCGATTCC-3' Reverse: 5'-GAGGTCCACACACTGAAGTT-3'
SGK1	Forward: 5'-TATTATGTCGGAGCGGAATGTTTC-3' Reverse: 5'-CAGCGTTCCCTCTGGAGAT-3'
CaMKKβ enhancer CHIP primers	
distal upstream control	Forward: 5'-GCACAGTTTGCACACCTGAA-3' Reverse: 5'-GCTTTGGATTTAGGCCCTGT-3'
CaMKK β enhancer	Forward: 5'-AACAGGAAAGGACACCCAAA-3' Reverse: 5'-AAACCATTCTTAGCAGGCCAT-3'
CaMKKβ enhancer and promoter reporter gene primers	
promoter	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTTGAGACAGGGTCTCTGTGTTGC-3'
fragment A enhancer	Forward: 5'-CGCTAGCGAATTGCAACTGTGAGACCAGGCA-3' Reverse: 5'-CAAAGCTTGTGGCCTTGGGCAAATGACTTGAT-3'
fragment B enhancer	Forward: 5'-CGCTAGCATCAAGTCATTTGCCAAGGCCAC-3' Reverse: 5'-CAAAGCTTAACACTGTAGCTCACACAGGCAGA-3'
fragment C enhancer	Forward: 5'-CGCTAGCATCAAGTCATTTGCCAAGGCCAC-3' Reverse: 5'-CAAAGCTTTATTTGATGCTCAGCCACCTCCCT-3'
fragment D/598 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTAAATGTGAAAGGCCAGGTGTGGTG-3'
fragment E enhancer	Forward: 5'-CGCTAGCTGCCTGTGTGAGCTACAGTGTCT-3' Reverse: 5'-CAAAGCTTAAATGTGAAAGGCCAGGTGTGGTG-3'
fragment F enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTAACACTGTAGCTCACACAGGCAGA-3'
fragment G enhancer	Forward: 5'-CGCTAGCCACCACACCTGGCCTTTCACATTT-3' Reverse: 5'-CAAAGCTTGCACCTTAAGGCAGGGTCAGCAAA-3'
fragment H enhancer	Forward: 5'-CGCTAGCGTTTCAAGCGATTCTCCTGCCTCA-3' Reverse: 5'-CAAAGCTTTCACGCCTGTAATCCCAGCACTTT-3'
566 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTTACACGGGTGATTACAATCTTAGC-3'
487 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTTGGACAACATGGCAAGACCCATCT-3'
312 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTCTGGATCTCTTTTCCTGGTACTTG-3'
233 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTACACTGTAGCTCACACAGGCAGAA-3'
152 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTTACAAATCCAAACCCTAGCTCAAG-3'

90 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTTGCTGTGAGCCAGGCCCTCCCTGC-3'
69 bp enhancer	Forward: 5'-CGCTAGCAGGGAGGTGGCTGAGCATCAAATA-3' Reverse: 5'-CAAAGCTTCTGCCCCGCTCCTCTCTCTGATGTC-3'
promoter-ARE deletion	Forward: 5'-CATACAGAATTGTTTAAACAAGTACC-3' Reverse: 5'-TAAATTGCCTGTGTTTTATTAGAACACTG-3'
CaMKKβ RT-PCR primers	
F(1-6)	5'-ACCTGTAATCCCAGCACTTTCGGA-3'
R(1-6)	5'-CGATCTCGGATCACTGCAACCTCT-3'
F(7)	5'-TGAGCCGAGCCGAGCCGAGCTG-3'
R(1-7)	5'-TCACAGGGCTTCTGGCTTTCGCT-3'
F1	5'-AGCTGAGGACTTGAAGGACCTGAT-3'
R1	5'-AGGTTGTCTTCGCTGCCTTGCTT-3'
R2	5'-ACCTGGGCTGGCTATGTGTATGAA-3'
siRNA sequences	
CaMKK β #1	5'-GGACCAUCUGUACAUGGUGUUCGAA-3'
CaMKK β #2	5'-GCUGACUUUGGUGUGAGCAAUGAAU-3'
CaMKK β #3	5'-CACCUGGGCAUGGAGUCCUUCAUUG-3'
AR 3'UTR	5'-CAGAUGUCUUCUGCCUGUUUAACU-3'
AMPK α 1 #1	5'-CCCAUCCUGAAAGAGUACCAUUCUU-3'
AMPK α 1 #2	5'-CCCUCAAUAUUUAAAUCCUUCUGUG-3'
AMPK α 1 #3	5'-ACCAUGAUUGAUGAUGAAGCCUUA-3'
AMPK α 2 #1	5'-GAUGGUGAAUUUCUGAGAACUAGUU-3'
AMPK α 2 #2	5'-CCGUAUGACAUUAUGGCUGAAGUUU-3'
CaMKI #1	5'-GGAGATACAGCTCTAGATAAGAATA-3'
CaMKI #2	5'-CCATAGGTGTCATCGCTACATCTT-3'