Supplementary materials and methods

Cell culture, plasmids and reagents

LNCaP, 22Rv1 and HEK293T cell lines were obtained from American Type Culture Collection (Baltimore, MD, USA) (CRL-1740, CRL-2505, CRL-3216). C4-2 cells were obtained from Dr. Nancy Weigel (Baylor College of Medicine). LNCaP-*CAMKK2* and 22Rv1-sh*CAMKK2* cells have previously been described (1). 22Rv1-fLuc cells were created by pBABE-fLuc-YFP plasmid (a gift from Dr. Christopher Counter, Duke School of Medicine) with retroviral transduction strategy (2). Cells were maintained as previously described (3) and validated by STR profiling (University of Texas MD Anderson Cancer Center Cell Culture Core). All cells were confirmed to be mycoplasma-free by MycoAlert Mycoplasma Detection Kit (Lonza, Morristown, NJ USA; Cat #: LT07-118). Cells were steroid-starved in phenol red-free medium containing 10% charcoal stripped-FBS (5% CS-FBS for C4-2 cells) for 72 hours before treatment unless otherwise noted. pCW-Cas9 and pLX-sgRNA were gifts from Drs. Eric Lander & David Sabatini (Addgene, Watertown, MA, USA; plasmids #: 50661, 50662). pcDNA4-VPS34-Flag was a gift from Dr. Qing Zhong (Addgene plasmid #: 24398). pcDNA3.1-hULK1 and 4SA mutant were gifts from Dr. Mondira Kundu (St. Jude Children's Research Hospital). Enhanced GFP-LC3 and mCherry-GFP-LC3B constructs have been previously described(4). The synthetic androgen methyltrienolone (R1881) was purchased from PerkinElmer (Naperville, IL, USA; Cat #: NLP005005MG). Chloroquine (Cat #: C6628), doxycycline hyclate (Cat #: D9891), puromycin (Cat #: P8833), BrdU (5-bromo-2-deoxyuridine, Cat #: B5002) and polybrene (Cat #: TR-1003) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

G418 sulfate was purchased from Gold Biotechnology (St. Louis, MO, USA; Cat #: G-418-25). Blasticidin was purchased from Millipore Sigma (St. Louis, MO, USA; Cat #: 203350).

Xenografts, histology and immunostaining

Xenografts were performed on 6-8 weeks male NSG mice obtained from either The Jackson Laboratory (Bar Harbor, ME, USA; Cat #: 005557; Fig. 1&3E) or MDACC Experimental Radiation Oncology Breeding Core (Fig. 3A). Castrations were conducted one week before injections. $1x10^6$ cells in 200 μl DPBS: Matrigel® 1:1 vol/vol (Corning, Corning, NY, USA; Cat #356231) were injected subcutaneously into flanks. Tumor size was measured by calipers until tumor lengths in the control group reached 1.5 cm or signs of morbidity at which point tumors were removed for further analyses.

For 22Rv1-sh*CAMKK2* xenografts, mice were randomized into normal/control or doxycycline-containing (625 mg/kg, Envigo, IN, USA) diet groups. Then, shRNA expression with surrogate red fluorescent protein (RFP) was tracked by fluorescence (IVIS Spectrum *In Vivo* Imaging Station, PerkinElmer). For chloroquine xenograft experiments, mice were randomly grouped into vehicle control or chloroquine IP treatment when the tumor volume reached 100 mm^3. One hour before tissue/tumor collection/sacrifice, mice were injected with 100 mg/kg BrdU. Half of the tumor sample was snap frozen while the other half was immediately fixed in 4% PFA overnight at 4°C. For staining, samples were dehydrated and embedded in paraffin. Paraffin slides were then rehydrated and further processed with antigen retrieval in citrate buffer (DAKO, Santa Clara, CA, USA; Cat #: S169984-2). Peroxidase blocking was performed in 1%

H_2O_2 plus 10% methanol solution. Proliferative cells were detected by BrdU staining. For this, slides were blocked with goat serum (DAKO; Cat #: X090710-8) and incubated overnight with anti-BrdU antibody (Calbiochem: Part of Millipore Sigma; Cat #: NA61), anti-LC3A/B (Cell Signaling Technology, Cat #: 12741) or anti-p62 (Enzo Biochem, Farmingdale, NY, USA; Cat #: BML-PW9860-0100). After washing with PBST (PBS with 0.02% Tween 20), secondary antibodies (Mouse-on-Mouse HRP Polymer, Biocare Medical, CA, USA, Cat#: MM620; SuperBoost™ Goat anti-Rabbit Poly HRP, Thermo Fisher Scientific, Waltham, MA, USA; Cat#: B40962) were incubated for 30 minutes. Sections were developed by DAB (Vectorlabs, Burlingame, CA, USA; Cat #: SK-4100). Cell death was detected by TUNEL staining using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Madison, WI, USA; Cat #: 11684795910) following the manufacturer's instructions. Hematoxylin and eosin staining as well as cleaved caspase-3 staining (Biocare Medical, Pacheco, CA, USA; Cat #: CP229a,b,c) were performed by the University of Texas MD Anderson Cancer Center Department of Veterinary Medicine and Surgery Research Animal Support Facility. Microscopy was done with an Olympus BX51 microscope and cellSens imaging software/Nikon C-TEP3 microscope. For the evaluation of p62 and LC3, the staining intensity of DAB (target protein) and hematoxylin (nuclei) were measured using Image J. The percentage of the positive signals were calculated by DAB intensity divided by hematoxylin intensity. Analysis for all IHC was done on 3-10 acquired fields per section and data were averaged.

Cell pellet preparation for IHC

$1x10^8$ treated LNCaP cells were collected and fixed with 10% neutral buffered formalin (with 1 drop of eosin) for overnight (5). After PBS wash, an equal amount of 4% agarose was added to immobilize the cells. Cell plugs were then removed from tubes and placed in biopsy cassettes. Paraffin embedding and sectioning processes were done by the University of Texas MD Anderson Cancer Center Department of Veterinary Medicine and Surgery Research Animal Support Facility. IHC staining was performed using the above-described IHC protocol.

Generation of CRISPR/Cas9 *CAMKK2* knockout cells.

pCW-Cas9 was co-transfected with lentiviral packaging plasmids into actively growing HEK293T cells using Lipofectamine 2000 transfection reagent. After 48 hours, medium containing virus was collected, filtered and added to the target cells with 8 μg/ml polybrene. After 48 hours, fresh medium with 1 μg/ml puromycin was used to select doxycycline-inducible Cas9 expressed target cells. The gRNAs targeting *CAMKK2* were designed by http://crispor.tefor.net/(6) and synthesized by Sigma (listed in Supplementary Table 1). The sgRNA oligos were cloned into pLX-sgRNA. pLX-*CAMKK2* sgRNAs were transfected into Cas9-inducible expressing cells by the same lentivirial transduction strategy before selection with 10 μg/ml blasticidin. Cells expressing inducible Cas9 and sgRNA were first treated with doxycycline for 7 days. This method limited the Cas9 activation window and therefore greater potential for off-target CRISPR effects. After, single clones were isolated and screened to establish *CAMKK2* knockout cells. Parental Cas9-inducible cells were used as control. Each clone was validated by sequencing and western blot.

Western blot analysis

Western blot analysis was performed as previously described(1, 3, 4, 7). Briefly, cells were harvested in RIPA lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Sigma, Cat #: 11697498001) and PhosSTOP phosphatase inhibitor (Roche, Cat #: 4906845001). Primary antibodies were purchased from the following sources: Cell Signaling Technology (Danvers, MA, USA): ULK1 (Cat #: 4773), p-ULK1(S555) (Cat #: 5869), p-ULK1(S757) (Cat #: 6888) , LC3B (Cat #: 2775), p-AMPK(T172) (Cat #: 2535), p-VPS34(S249) (Cat #: 13857), p-p70S6K(Thr389) (Cat #: 9205), p70S6(Cat #: 2708), p-S6 (S235/236) (Cat #: 4856), S6 (Cat #: 2317) ; Sigma: CAMKK2 (Cat #: HPA017389), GAPDH (Cat #: G8795), FLAG (Cat #: F1804); Novus Biologicals (Littleton, CO, USA): ULK1 (Cat #: JA58-36); Abcam (Cambridge, United Kingdom): ULK1 (Cat #:ab203207); Thermo Fisher Scientific: ULK1 (Cat #: MA532699).

Immunofluorescence microscopy

For LC3 assays, GFP-LC3/mCherry-GFP-LC3 fusion constructs were expressed in cells as previously described (3, 4). Following treatments, cells were fixed with 4% PFA for 15 min at RT and DAPI was used as a counterstain.

For ULK1 antibody validation tests, LNCaP cells were treated with siRNA targeting scramble or ULK1 for 72 hours. Cells were then fixed with 4% PFA, permeabilized with 0.1% Triton-100, and blocked with BSA. Following blocking, cells were incubated with various ULK1 antibodies overnight and then goat anti-rabbit IgG (H+L) cross-adsorbed

secondary antibody, Alexa Fluor 594 (Thermo Fisher Scientific; Cat #: A11012) for one hour.

Images were captured using the Olympus BX51 fluorescence microscope and cellSense imaging software. For LC3 quantification, samples were analyzed by Image J where LC3 puncta per cell were counted for 50 cells per cell line and averaged.

Transmission electron microscopy (TEM)

Cells were plated at 100,000 cells/well in 6-well plates and treated as indicated in figures/figure legends. Samples were fixed with a Karnovsky's fixative solution (3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3) at 4 °C. Samples were further processed by the University of Texas MD Anderson Cancer Center High Resolution Electron Microscopy Core Facility.

Proliferation assays

Proliferation assays were carried out as previously described by measuring the cellular double-stranded DNA content using a fluorescent DNA stain (4).

Clonogenic assays

Cells were plated at 5000 (LNCaP) or 1000 (C4-2, 22Rv1) cells/well in 6-well plates. Colonies were formed for 3-4 weeks. Media and treatments were refreshed every week. Cells were fixed with acetic acid/methanol 1:7 (vol/vol) and then stained with 0.5% crystal violet. The number of visible colonies were counted. The data were representative of three independent experiments with similar results.

REFERENCES

1. White MA, Tsouko E, Lin C, Rajapakshe K, Spencer JM, Wilkenfeld SR, et al. GLUT12 promotes prostate cancer cell growth and is regulated by androgens and CaMKK2 signaling. Endocr Relat Cancer. 2018;25(4):453-69.
2. Blessing AM, Ganesan S, Rajapakshe K, Ying Sung Y, Reddy Bollu L, Shi Y, et al. Identification of a Novel Coregulator, SH3YL1, That Interacts With the Androgen Receptor N-Terminus. Mol Endocrinol. 2015;29(10):1426-39.
3. Blessing AM, Rajapakshe K, Reddy Bollu L, Shi Y, White MA, Pham AH, et al. Transcriptional regulation of core autophagy and lysosomal genes by the androgen receptor promotes prostate cancer progression. Autophagy. 2017;13(3):506-21.
4. Shi Y, Han JJ, Tennakoon JB, Mehta FF, Merchant FA, Burns AR, et al. Androgens promote prostate cancer cell growth through induction of autophagy. Mol Endocrinol. 2013;27(2):280-95.
5. Koh CM. Preparation of cells for microscopy using 'cell blocks'. Methods Enzymol. 2013;533:249-55.
6. Haeussler M, Schonig K, Eckert H, Eschstruth A, Mianne J, Renaud JB, et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 2016;17(1):148.
7. Tennakoon JB, Shi Y, Han JJ, Tsouko E, White MA, Burns AR, et al. Androgens regulate prostate cancer cell growth via an AMPK-PGC-1alpha-mediated metabolic switch. Oncogene. 2014;33(45):5251-61.