Supplementary Information for

Inhibition of *de-novo* lipogenesis targets androgen receptor signaling in castration resistant prostate cancer.

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This PFD File includes:

1. **Supplementary Materials and Methods**
2. **Supplementary Figures**

   Figs. S1 to S14

Other supplementary materials for this manuscript include the following:

Datasets S1A to S1N

Supplementary Material and Methods

Reagents

Unless stated otherwise, Enza (#NC0482182, Thermo Fisher scientific), MG132 (#C2211, Sigma-Aldrich), TVB-3166 (#SML1694, Sigma-Aldrich), thapsigargin (#T7458, Life Technologies), bapta (#A1076, Sigma-Aldrich), GSK 2606414 (#501016107, Thermo Fisher Scientific), salubrinal (SML0951-5MG, Sigma-Aldrich) were resuspended in DMSO. Doxycycline (#631311, Takara Bio), puromycin (#P7255, Sigma) were resuspended in water. XF Palmitate-BSA FAO substrate (#NC0528933, Thermo Fisher scientific) was used as described by the manufacturer. As transfection agents, Fugene HD (#PRE2311, Thermo Fisher Scientific) or DharmaFECT (T-2001-01, Dharmacon) were used. Polybrene was from EMD Millipore (#TR-1003-G). was from For radiolabeling experiments, ^{14}C-labeled glucose (ARC-0122G), 2-^{14}C-malonyl-CoA (2239094, Perkin Elmer), and palmitic acid [1, 14C] sodium salt (ARC 4007) were used. Matrigel (354234, Corning) was used for *in vivo* cell injection.

Cell culture

LNCaP, 22Rv1, HeK293T, and RWPE-1 cells were obtained from the American Type Culture Collection (ATCC) and cultured in standard conditions. C4-2 and C4-2B cells were kindly provided by K. Knudsen (Thomas Jefferson University) and Dr. J. Lia (Brigham and Women's Hospital), respectively. LNCaP-Abl and LNCaP-95 cells were kindly provided by Dr. M. Brown (Dana-Farber Cancer Institute, DFCI). Cells were maintained in RPMI 1640 (LNCaP, 22Rv1), DMEM (HeK293), supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin. LNCaP-95 and LNCaP-Abl cells were grown in RPMI medium supplemented with 10% CSS. RWPE-1 cells were grown in MEM medium supplemented with 10% FBS, 1 mM MEM non-essential amino acids, 1 mM sodium-pyruvate, 10 ug/ml gentamicin, and 1 mM glutamine. Cells were not used at passage higher than 50, except for LNCaP-Abl. Murine androgen-sensitive MYC-CaP (1) and Pten KO cells (2) were kindly obtained by Dr. Ellis (DFCI). They were grown in DMEM, 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin, 1 mM glutamine, and 1 mM sodium-pyruvate. Cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide. For treatments using ER stress inhibitors, cells (3.5×10^5/ 6-well plate) were seeded for 48 hr. After this, medium was changed and cells were pre-incubated with ER stress inhibitors bapta (10 and 20 uM) and GSK 2606414 (100 nM) for 1hr, prior addition of the ER stressor thapsigargin (1uM) for 12-24 hrs. Samples were collected at the end of treatment and used for immunoblotting.

Cell growth studies

Cells (10^5or 2×10^5 depending on the cell type) were plated in 60mm dishes until they reach 20-30% cell confluence. After 36-48 hrs, cell medium ±IPI-9119 or vehicle (DMSO) was added and cells were grown

for 6 days, without medium replacement. Cells and conditioned medium were collected and cell growth was assessed by counting the number of viable cells using the Vi Cell XR analyzer (Beckman Coulter) based on trypan blue exclusion method. For Enza and IPI-9119 combination experiments, cells were pre-treated with IPI-9119 (0.1 uM) or DMSO for 6 days, then were replated at 200,000/well for 40 hr in the presence of IPI-9119 or DMSO. At end of 40 hr, the medium was changed and single or combinatorial treatments were added. Enza (20 uM) was re-added after 3 days.

Clonogenic assay

22Rv1 (500 cells) and C4-2 (250 cells) were plated in 6-well plates. After 40 hrs, cells were treated with DMSO, 0.1 or 0.5 μM IPI-9119 in 10% FBS containing medium for 3 weeks. Medium was replaced every 7 days. At the end of the 3 weeks, medium was gently removed, colonies were fixed in fixing solution (10 % acid acetic, 10 % ethanol) for 1 hr at RT and stained with 0.1% crystal violet. Wells were carefully washed to remove excess of staining and dried over-night. Pictures were taken using an Epson scanner. Clonogenic ability was quantified by measuring the absorbance (OD= 595) following dye extraction using 10% acid acetic and agitation for 2 hrs at RT.

Cell cycle studies

The percentage of cells in sub-G1, G1, S, and G2-M phase of the cell cycle was analyzed with a BD LSR II flow cytometer (Becton Dickinson), following PI and BrDU staining, using a standard protocol. Briefly, after incubation with BrDU (10 uM, 2 hrs), cells were fixed with ethanol. Cells were incubated with anti BrDu-FITC (BD bioscience) antibody for 20 min, followed by PI (10 ug/ml) labeling (30 min), and FACS analysis. Flow cytometry data were analyzed using the software package FlowJo (version 9.6.4). Cyclins levels were analyzed by immunoblotting. Apoptosis was analyzed by detection of cleaved Parp (c-Parp) in cell lysates and by measuring membrane redistribution of phosphatidylserine using Apo Alert Annexin V-FITC/PI apoptosis kit (Clontech), as described by the manufacturer.

Immunoblotting

Cells were lysed in 1% NP-40 buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40) or RIPA buffer with the addition of phosphatases and protease inhibitor cocktail (Sigma and Roche). For tumor xenograft samples, tissues were homogenized in 1% NP-40 buffer using Tissue-Tearor homogenizer (BioSpec Products). Protein quantification was performed using Bradford protein assay (Bio-Rad). Equal amounts of protein were resolved on precast tris-glycine SDS-polyacrylamide gels (Invitrogen). Bands were quantified by densitometry using the Quantity One software (version 4.6.1, Bio-Rad). Results were normalized to β-actin or vinculin and expressed in arbitrary units. Nuclear and cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Kit (#78833, Thermo Fisher). Protein fractions were quantified and analyzed by immunoblotting, using lysine-specific

demethylase 1 (LSD-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as nuclear and cytoplasmic markers, respectively. Primary antibodies used in this study were the following: FASN (BD transduction laboratories, #610962; CST#3180); β-actin (anti- mouse Sigma, A5316; anti-rabbit CST#4967S); c-Parp (CST# 9541S); cyclin A2 (CST#4656S); cyclin D1 (CST#2978S); Vinculin (anti-mouse Sigma, V9131); AR (Abcam #ab108341, which recognizes both AR-FL and AR-V7); AR-V7 specific (RevMAb Biosciences #31-1109-00); c-MYC (Abcam #ab32072); PSA (Dako, A0562); GAPDH (CST#2118S); LSD-1 (CST#4064S); eiF2α (CST#5324S); p-eIF2α (CST#9721S), eIF4B (CST#13088), puromycin (Kerafast#EQ0001), ERK2 (CST#9108), PERK (CST#5683), p-PERK (CST#3179), BiP (CST#3177), Akt (CST#9272), p-Akt (CST#9275), β-catenin (CST#8480), Active β-catenin (CST#8814), S6RP (CST#2217), p-S6RP (CST#2215), STAT3 (CST#12640), p-STAT3 (CST#9131), 4E-BP1 (CST#9452), p-4E-BP1 (CST#9451), MAPK (CST#9102), p-MAPK (CST#9101), AMPK (CST#2532), p-AMPK (CST#2535), p62 (CST#5114), LC3 A I/II (CST#4599), PTEN (CST#9559).

FASN activity assay

FASN activity in cell lysates was measured by incorporation of 2-^{14}C-malonyl-CoA into lipids. Briefly, cells were washed, pelleted, and resuspended in hypotonic buffer (1mM DTT, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5) for 15 min. Lysate (10 or 20 ug of protein) was mixed with 125 ul of NADPH solution (100 mM potassium phosphate, pH 7, 100mM KCl, 0.5 mM NADPH) and incubated at 37° C for 2 min. A substrate mixture (25nml acetyl-CoA, 25 nmol malonyl-CoA, 0.05 uCi of 2-^{14}C-malonyl- CoA) was then added and incubated at 37° C for 10 min. Reaction was stopped with 1N HCl/methanol (6:4, v/v). Lipids were extracted with petroleum ether. Radioactivity was measured by scintillation counting using the β-counter (Perkin Elmer Tri-carb 3180TR/SL). Counts per minute (cpm) in 1 ml of extract were measured and normalized to protein content.

FASN activity using the purified human FASN protein was performed as following: human FASN protein was purified from SKBR3 cells using procedures modified from those described in (3). SKBR3 cells were obtained from ATCC and grown in DMEM high glucose medium supplemented with 10% FBS, 1 ug/mL bovine pancreas insulin, 100 U/mL penicillin and 100 ug/mL streptomycin. Cells were trypsinized and washed three times with PBS, frozen in liquid N_2, and stored at -80°C. Frozen cells were thawed on ice and resuspended in lysis buffer (25 mM Tris-HCl, pH 7.0, 15 mM NaCl, 1 mM EDTA, and 1 mM DTT) with protease inhibitors. Cells were lysed by sonication, and the cell debris were removed by centrifugation at 20,000 rpm for 30 min. Neutralized saturated ammonium sulfate solution was added (final concentration of 35%) to the supernatant. Solution was left on ice for 1 hr, and the precipitated proteins were harvested by centrifugation at 20,000 rpm for 30 min. Proteins were re-

dissolved in lysis buffer without NaCl and loaded on a mono Q column. Bound proteins were eluted with a linear gradient of NaCl in lysis buffer. Each fraction was analyzed by SDS-PAGE and FASN NADPH consumption assay. The fractions containing FASN were pooled and concentrated. Glycerol was added to 20%, and the protein was frozen in liquid N_2 and stored at -80°C, until use. FASN activity using the purified protein was measured using a FASN Scintillation Proximity Flashplate Assay, as described in (4). Briefly, in a 96-well polypropylene microplate, a dilution series (typical concentrations 60 nM-1.0 mM) of test compounds were prepared in DMSO followed by a 20-fold dilution into FASN assay buffer (50 mM potassium phosphate, pH 7.0, 1.0 mM EDTA, 0.01% NP-40), of which 5.0 uL each was transferred to a FlashPlate® PLUS 96-well plate and mixed with 35 uL FASN assay buffer plus 0.5 mg/mL bovine gamma globulin and 1 mM TCEP. FASN protein (10 uL, 10 nM) was added per well, and the microplate was incubated at 37 °C for 30 min. 10 uL of 20 mM NADPH was added, and the reaction was initiated by addition of 40 uL substrate mixture to final concentrations of 1 nM FASN, 100 uM acetyl-coenzyme A, 6 uCi [3H]-acetyl-coenzyme A, 300 uM malonyl-coenzyme A, 2 mM NADPH, 0.5 mg/mL bovine gamma globulin, and 1 mM TCEP in a volume of 100 uL per well. Assay plates were incubated for 2 hrs at 37 °C. The plates were read in a Wallac 1450 Microbeta Plus liquid scintillation counter (PerkinElmer, Waltham, MA), and cpm were collected over 2 min. Each IPI-9119 concentration well cpm was compared to the maximum FASN enzyme activity (Max) cpm and the background (Min) cpm, as measured by omission of FASN enzyme in the background well. % inhibition values were calculated, and curves were fitted by a four-parameter logistic function to yield IC50 values:

% Inhibition = [1- (Inhibitor- Min)] x 100% / (Max- Min).

^{14}C-glucose incorporation into lipids

LNCaP cells were plated (4×10^5) in 60 mm dish and treated with IPI-9119 for 72 hrs. Twenty-four hrs before harvesting, cells were incubated with 1uCi of ^{14}C-labeled glucose. Medium was removed, cells washed and collected in a glass vial. A mixture of methanol: chloroform: water (2: 1: 0.5) was added and the lipids were extracted as described by Bligh & Dyer (5). ^{14}C-glucose incorporation into cellular lipids was quantitated by scintillation counting and normalized to mean protein content. Five biological replicates were used (3 for lipid extraction and 2 for protein normalization).

FAO analysis

LNCaP cells were seeded in 12-well plates for 48 hrs. 7 biological replicates were used for the experiments (4 for FA oxidation measurement, 3 for protein normalization). Cells were treated for 6 days with IPI-9119 or DMSO and 2.5 hrs before harvesting ^{14}C-palmitate-complexed with BSA was added to the cell medium. At the end of the incubation period, 100 ul of 70% perchloric acid were added to each well, firmly covered with a piece of Wathman filter paper, previously saturated with phenylethylamine.

Plates were firmly covered with saran wrap and rocked for 2.5-3 hrs at RT. FA oxidation was measured as the production of ^{14}C-CO_2 trapped in the Wathman filter paper. Radioactivity was measured by scintillation counting and normalized to mean protein content.

Oil Red O staining

LNCaP cells were treated with IPI-9119 or DMSO for 6 days, washed carefully with PBS and fixed for 30 min with fresh 10% neutral buffered formalin. Six replicates/condition were used (three for Oil Red O staining, three for protein normalization). After washing with PBS and dry the dish, cells were incubated for 30 min with Oil red O in 0.5% isopropanol solution (O1391, Sigma). Dishes were washed to remove the excess of staining. Dishes were dried and pictures were taken using an Olympus CK40 microscope. For quantitative analysis, cell-bound Oil red O was extracted in 1 ml of 100% isopropanol in agitation for 1 hr and absorbance was measured (OD= 510 nm). Absorbance value from similarly treated plates but without cells was used as blank and subtracted from the experimental values.

Cholesterol measurement

Cholesterol levels in murine tissue were measured using the Amplex Red Cholesterol Assay Kit (Thermo Fisher Scientific), as per manufacturer's instruction.

RNA extraction and RT-qPCR

Total RNA was extracted with the RNeasy Plus MiniKit (Qiagen). Two micrograms of RNA were retrotranscribed with a High-Capacity cDNA Reverse Transcription Kit (Applied biosystems), as described by the manufacturer. For single gene mRNA analysis, 10 ng of cDNA were used. The relative gene expression was determined by the delta-delta Ct method (6) using β-actin as reference gene for LNCaP, 22Rv1 cells, and 22Rv1 xenograft experiments, while glucuronidase beta (*GUSB*) was used for LNCaP-95 cells. The list of Taqman probes used is provided in supplementary Materials and Methods. For AR-V7 gene signature rescue experiment, a custom-made 384-well TaqMan Array microfluidic card (Applied Biosystems) was designed. Two micrograms of cDNA per sample were loaded and processed according to the manufacturer's instructions using a 7900HT Fast Real-Time PCR instrument (Applied Biosystems). *GUSB* was used as housekeeping gene. The list of genes included in the array is provided (**Dataset S1N**). For single gene expression analysis, the following Taqman probes (Applied Biosystems) were used:

FASN: Hs00188012_m1

AR-FL: Hs00171172_m1

AR-V7: AI89MD2

KLK3: Hs02576345_m1

c-MYC: Hs00153408_m1

TMPRSS2: Hs01120965_m1

NKX3-1: Hs00171834_m1

FKBP5: Hs01561006_m1

HMGCR: Hs00168352_m1

ACTB: Hs99999903_m1

hu GUS: 4326320E-0502004

Lentiviral infections

0.65×10^6 of 293T cells were seeded in 60 mm dishes. The following day, 293T cells were transfected with lentiviral constructs (PLKO.1, or lenticrispr V2, or lentivirus PLVX-TetOne-Puro-Vector) together with packaging plasmids Delta 8.9 and VSV-g, using the Fugene HD transfection reagent (Promega). Lentivirus-containing surnatant was collected after 48 hrs and used to infect target cell-lines (LNCaP, C4-2, 22Rv1, and LNCap-95) overnight in the presence of 5 μg/ml polybrene. Target cells were selected with 2ug/ml of puromycin (Sigma Aldrich) for 6 days and then lysed for immunoblotting analysis. Infections were performed 3 times (biological triplicates), showing comparable results.

To stably knockdown FASN, we infected cells with pLKO.1 lentiviral constructs containing two different shRNA sequences generated by the RNAi Consortium (Broad Institute, Cambridge) (sh-FASN1: CATGGAGCGTATCTGTGAGAA; sh-FASN2: CGAGAGCACCTTTGATGACAT), using the Fugene HD (Promega, #E2311). To control for off-target effects, we used two shRNA against green fluorescence protein (GFP) (sh-Ctrl1: TGACCCTGAAGTTCATCTGCA, sh-Ctrl2: ACAACAGCCACAACGTCTATA). To induce a stable knockout of FASN, cells were infected with lenticrispr V2 constructs containing 2 gRNAs targeting FASN (FASN CRISPR gRNA 1: CCTTCAGCTTGCCGGACCGC; FASN CRISPR gRNA 2: GGATGGTGGCGTACACCGC, GenScript). To control for off target effects, a gRNA sequence targeting Lac Z (CCCGAATCTCTATCGTGCGGG) was cloned into lentiCRISPRV2 construct (Addgene) using a protocol available online (Zhang_lab_LentiCRISPR_library_protocol.pdf). Single clones were generated by serial dilution in 96 wells and culturing the cells in the presence of puromycin for 3 weeks, followed by screening by immunoblotting. To transiently knockdown AR-FL, we transfected LNCaP cells with 50 nM of a pool of 4 siRNAs targeting AR-FL or control (SMART Pool, ON TARGET plus AR siRNA, L-003400-00-0005; ON-TARGETplus Non-targeting Pool, D-001810-10-05, Dharmacon), using DharmaFECT (T-2001-01, Dharmacon), as described by manufacturer for 96 hrs. To stably overexpress AR-V7 in AR-negative LNCaP cells, doxycycline-inducible lentivirus PLVX-TetOne-Puro-Vector (Systembio) containing AR-V7 cDNA (kindly provided by Dr. Plymate, University of Washington) was used. The expression of AR-V7 was induced by incubating cells with 50 ng/ml of doxycycline.

Luciferase assay

LNCaP cells were seeded at $2x10^4$ cells/well in 24-well plates and treated with IPI-9119 or DMSO the following day. After incubation for 72 hrs, each well was transfected with 375ng of PSA-Luc and 125ng of SV40-REN constructs, using Fugene HD as manufacturer's recommendations. Twenty-four hrs post-transfection, cells were treated for additional 24 hrs with 10 μM Enza or vehicle (0.1% v/v ethanol). Cells were harvested in 1x passive lysis buffer provided in a Dual Luciferase Assay Kit (Promega). Activities of the firefly and Renilla luciferase reporters were assayed by Dual Luciferase Assay, as per manufacturer's recommendations. Data were normalized by dividing firefly luciferase by Renilla luciferase activity.

RNA-sequencing and data analysis

RNA Quality Control (QC): RNA quality was assessed by running all samples on the Agilent Bioanalyzer, using the RNA 6000 Nano assay. Any sample with a RNA integrative number (RIN) value greater than 6.0 passed QC and was used for library preparation. The concentration of total RNA was determined using the RNA High-Sensitivity Qubit assay. The concentration of dsDNA was also measured using the DNA High-Sensitivity Qubit assay to ensure that less than 10% DNA contamination was present in the samples before library preparation.

Library preparation: mRNA was isolated from 100 ng of total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module, which was then reverse transcribed and library prepped using the NEBNext Ultra Directional RNA Library Prep kit (Illumina). The resulting libraries were run on the bioanalyzer on a High-Sensitivity DNA chip to check the library size distribution. The concentration of the library was determined using the DNA High-Sensitivity Qubit assay, and a final functional library concentration was determined through qPCR using Illumina adaptor-specific primers with the KAPA SYBR FAST Universal qPCR kit (Sigma-Aldrich). All indexed libraries were normalized to 2 nM and pooled at equal volumes for sequencing. The library pools were denatured and diluted to a loading concentration of 2 pM for Paired End 75 cycle sequencing on a high-throughput flowcell on the NextSeq500 sequencer.

Data alignment: Fastq files were aligned to the human 19 genome using STAR aligner with default parameters (version 2.3.1z4), producing BAM files as output, which were passed through several processing steps. The *.sort.primary.bam files, which retain only the primary alignments, were used for further analysis.

RNA-Sequencing (RNA-Seq) QC: RNA-Seq quality metrics were produced using the Broad Institute's RNA-SeQC tool (7) (http://www.broadinstitute.org/cancer/cga/rna-seqc).

Read quantification and normalization: Reads and differential expression analysis were treated at the gene level, as opposed to inference about specific transcript abundance. The feature Counts software,

which produces integer counts for all genes, was used (8). For read normalization, DESeq normalization software, as part of the DESeq package (7), was used.

Differential expression testing: For differential expression testing, DESeq software was used, as previously described (9). The output files were named according to the contrast performed and described in **Table S1**. Normalized_count_matrix.sort.primary.counts were used for heatmap generation.

Gene set enrichment analysis (GSEA)

GSEA (10) using RNA-seq data was performed with the Pre-ranked option and defaults parameters, as recommended. Ranked list was created by using sign fold change x [-log10(p value)] from the DSEq-2 output. Hallmarks (h.all.v5.2s symbols.gmt) and Canonical pathways (CP; C2.cp.v5.2) molecular signature databases (MSigDB) or previously published signatures were run with 1,000 permutations. Pathways were considered significantly enriched or depleted at $p<0.05$ and FDR<0.15.

Metabolic profiling

Sample preparation and metabolomics techniques: Dry pellet of $5x10^6$ cells in dry-ice was sent to Metabolon for analysis. A recovery standard was added prior to the first step in the extraction process for QC purposes. Proteins were precipitated with methanol under vigorous shaking for 2 min, followed by centrifugation. Extracts were divided into five fractions: one for Ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) with positive ion mode electrospray ionization (IMEI); one for (UPLC-MS/MS) with negative IMEI; one for liquid chromatography (LC) polar platform; and one Gas Chromatography (GC)-MS. An additional sample was reserved as a backup. Samples were placed briefly on a TurboVap® (Zymark), to remove the organic solvent. For LC, samples were stored overnight under nitrogen before analysis. For GC, each sample was dried under vacuum overnight before analysis.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS): All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried and then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more

hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions with a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z.

QA/QC: Several types of controls were analysed together with the experimental samples: 1) a pooled matrix sample was generated by taking a small volume of each experimental sample (or alternatively, a pool of well-characterized human plasma) and served as a technical replicate throughout the data set; 2) extracted water samples served as process blanks; 3) a cocktail of QC standards that were carefully chosen to not interfere with the measurement of endogenous compounds were spiked into every analysed sample to monitor instrument performance; and aided in chromatographic alignment. Instrument variability was also evaluated during the entire procedure. Experimental samples were randomized across the platform, and run with QC samples spaced evenly among the injections.

Data extraction and compound identification: Raw data were extracted, peak-identified, and QC-processed, using Metabolon's hardware and software, Compounds were identified by comparison to library entries of purified standards, or to recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contain the retention time/index (RI), *m/z*, and chromatographic data (including MS/MS spectral data) for all molecules present in the library. More than 3300 commercially available purified standard compounds have been acquired and registered into the Metabolon laboratory management information systems, for distribution to both the LC-MS and GC-MS platforms for determination of their analytical characteristics. The QC and the curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those entities that represent system artifacts, mis-assignments, and background noise.

Metabolite quantification and data pre-processing: Peaks were quantified using area-under-the-curve. Data were normalized, to correct variations that resulted from differences in the inter-day tuning of the instruments. Essentially, each compound was corrected in run-day blocks, by registering the medians to equal one, and normalizing each data point proportionately. Further information is provided in (11, 12).

Lipidomics

As described in (13), lipid samples were re-suspended in 30 µL of 1:1 LC/MS grade isopropanol: methanol prior to LC-MS/MS analysis, 5 µL were injected. A Cadenza 150 mm ×2 mm 3 µm C18 column (Imtakt) heated to 40 °C at 260 µL/min was used with a 1100 quaternary pump HPLC with room temperature autosampler (Agilent). Lipids were eluted over a 20 min gradient from 32% B buffer (90% IPA/10% ACN/10 mM ammonium formate/0.1% formic acid) to 97% B. A buffer consisted of 59.9% ACN/40% water/10 mM ammonium formate/0.1% formic acid. Lipids were analyzed using a high-resolution hybrid QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific) in DDA mode (Top 8) using positive/negative ion polarity switching. DDA data were acquired from m/z 225-1450 in MS1 mode and the resolution was set to 70,000 for MS1 and 35,000 for MS2. MS1 and MS2 target values were set to 5e5 and 1e6, respectively. Lipidomics data were analyzed using LipidSearch 4.1.9 software (Thermo Fisher Scientific).

Metabolite set enrichment analysis

Metabolite set enrichment analysis (MSEA) was carried out using the GSEA algorithm. The metabolite sets were built using the lipid classification provided by the LipidSearch 4.1.9 software. The ranking in the MSEA was performed by using the Spearman correlation coefficient (Spearman's Rho values), calculated between each lipid metabolite and the drug concentration (**Dataset S5-S9**). Lipid class enrichment was considered significant at $p<0.05$ and FDR<0.15.

Animal studies

22Rv1 ($7.5x10^6$) or LNCaP-95 cells (10^6) were injected with matrigel (1:1) into the flanks of 8-10-week Male Ncr Nu Castrated mice (Taconic) or castrated NOD male SCID (Charles River). When the tumor reached a volume of 100 mm^3, mice were randomly assigned to IPI-9119 or vehicle-treated groups, and ALZET osmotic pumps (containing IPI-9119 or vehicle) were implanted subcutaneously. Pumps were replaced after 2 weeks until the end of the study. Tumors were measured using calipers three times a week and tumor volumes were calculated (V= length x $width^2$ x 0.5). Mouse weight and general health were checked every other day. At the end of the study, tumors were collected and homogenated for analysis. Cholesterol was measured in xenograft tumor homogenates using the Amplex Red Cholesterol Assay Kit (Thermo Fisher Scientific), as described by the manufacturer.

Human samples

mCRPCs from 61 patients (up to 4 metastatic sites per patient) were collected at University of Washington within 8 hrs of death. Six neuroendocrine cases were excluded from the analysis. The 55 patients analyzed were treated with androgen deprivation therapy (all), chemotherapy (50 cases), and immunotherapy (9 cases) and a subset received also Enza and/or Abi (22 cases: 2

treated with Enza, 6 with Abi, 13 with both Enza and Abi, 1 with Abi and ARN-509). All specimens were formalin-fixed (bone specimens decalcified in formic acid) and paraffin-embedded. Eight TMAs were constructed with 1 mm-diameter triplicate cores from CRPC (including 81 visceral metastases and 109 bone metastases). Multiplex (3-color markers: FASN, AR-FL, AR-V7) immunofluorescence staining was performed on the Leica Bond Rx automated immunostainer. Positivity at the cell level was defined in a semi-automatic fashion for each marker (see below). Metastatic sites were defined as positive if at least 5% of tumor cells were scored positive. Each patient was classified as positive for each of the investigated markers (i.e., FASN, AR-FL, and AR-V7) if at least one of his metastatic sites was scored positive for that protein.

TSA-plus Fluorescence Immunohistochemistry

A multiplexed tyramine signal amplification (TSA) method was performed on 4-μm sections of the TMAs for detection of FASN, AR-V7, and AR-FL proteins on the Leica Bond Rx automated immunostainer. The staining approach consisted of a multi-step protocol of sequential TSA-amplified immunofluorescence labels for each target, and DAPI was applied as a nuclear counterstain. The slides were incubated with antibodies against FASN (A301-323A, Bethyl Laboratories, dilution 1: 3,000), AR-V7 (Clone RM7, 31-1109-00, RevMAb Biosciences, dilution 1: 300), and AR-FL (Clone SP242, M5420, Spring Bioscience, dilution 1: 100) for 30 min. FASN antibody dilution used was specifically adjusted to capture the dynamic range of protein expression. TSA conjugated to a unique fluorophore was used for each target in the multiplexed panel (CY5 for FASN, CY3 for AR-V7, FITC for AR-FL) and incubated for 15 min. DAPI was applied as a nuclear counterstaining. For spectral library construction, single-stained slides for each antibody, a slide counterstained only with DAPI, as well as a slide without primary antibody (herein called blank slide), were included.

Spectral Imaging

Each TMA/tissue was scanned on a Perkin Elmer Vectra 3 imaging workstation. Each single-stained control slide was imaged with the established exposure time to generate the spectral library for un-mixing the fluorescence channels. The blank slide was used to define background and autofluorescence. A tissue and cell segmentation algorithm was developed in a PerkinElmer InForm software by training representative areas to classify tumor, non-tumor, and other categories that included folded, non-specific staining and background areas. Pathological visual inspection was provided to check for the accuracy of the classification training. Nuclei were identified by segmenting objects in the DAPI channel based on tuning-defined parameters. A 2-pixel radius around the nuclei was defined as the cell membrane. The algorithm was then applied to all the core images within the TMA batch. All segmented images from the batch were reviewed by a pathologist and manually edited for accuracy before the final segmentation

data were generated. TMA cores with severe tissue folding, major staining artefacts or no viable tumour were excluded from the analysis.

Intensity scoring per metastatic site

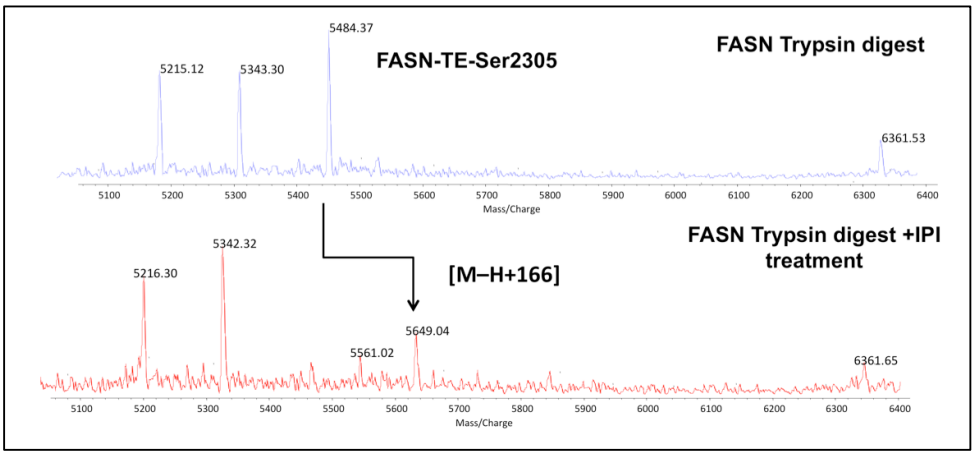
Signal units were expressed as fluorescent count normalized to exposure time, as given by InForm software. Based on a combined approach that considers background noise, *bona-fide* negative signal (e.g., neuroendocrine cases), and visually-inspected weak specific signal, thresholds for positivity were established at the cell level for each fluorophore. Signal thresholds (expressed as total weighted mean) were found to be: 0.14 for FASN (cytoplasm); 3.07 for AR-V7 (nucleus); 3.84 for AR-FL (nucleus). After merging the information from the replicate cores, each metastatic site was defined as positive if the rate of positive-scored cells exceeded 5% of the segmented tumour area.

Statistical analyses

Mean comparison in IPI-9119 treated group/groups *vs* vehicle group was performed using Student t-test or one-way ANOVA followed by Tukey's post-hoc test to correct for multiple comparisons. When two independent variables were tested, two-way ANOVA was used, followed by Sidak's post-hoc test to correct for multiple comparisons. Non-parametric Mann Whitney test was used to compare tumor volume at the end of the treatment (IPI-9119 or vehicle) period. GraphPad Prism 7.0 was used for these statistical analyses and graphical representation. For RNA-seq analyses, differential expression analysis was performed using DESeq software. Details are provided in supplementary Material and Methods (RNA-seq section). GSEA and MSEA were performed using the GSEA algorithm (10). Details for these analyses are provided in supplementary Material and Methods. Probabilistic quotient normalization method was used to normalize both metabolomics and lipidomics data (14). For metabolomics analysis, metabolites with more than 70% of missing values in each cell line set were removed from analyses to allow the inclusion of those specifically reduced under the detectability threshold in IPI-9119 treated samples. Then, the number of missing metabolites in the 5 replicates of each condition (i.e., drug and cell line) was counted. When the number was higher than 3, missing values were imputed with zero; otherwise, missing values were imputed using the k-nearest neighbor (kNN) algorithm (15), with k=3. The kNN imputation is an efficient method to fill in missing data by a value obtained from related cases in the whole set of records. By limiting the kNN imputation to the metabolites with at least 2 values out of 5/condition, imputation using the information from different conditions (e.g., treated and non-treated) was avoided. Principal component analysis (PCA) was used to visualize the metabolomic data. Data were log-transformed, mean-centered and scaled to unit variance before PCA (Qlucore Omics Explorer version 3.1). Normalized, imputed data were used for univariate analyses and box-plot representations of key metabolites. Student t-test was used to identify significantly altered metabolites for each drug

concentration. In both RNA-seq, metabolomics, lipidomics analyses, multiple comparisons were corrected using false discovery rate (FDR). Differences were considered significant with $p<0.05$ and, if multiple comparisons were done, FDR <0.05. For GSEA and MSEA, an FDR threshold of 0.15 was used. RNA-seq and metabolomics/lipidomics analyses were done using R software. Heatmaps were ordered according to hierarchical clustering (Ward linkage) on the basis of the KODAMA dissimilarity matrix, implemented in R package KODAMA (16).

A



B

Features of IPI-9119	
Human purified FASN IC_{50}	0.3 nM
Ricerca Serine Hydrolase Panel Screen (10 serine hydrolases)	> 100 fold selectivity
CEREP Receptor Panel Screen (54 receptors)	1 receptor (CCK1) with > 50% inhibition at 10 uM

C

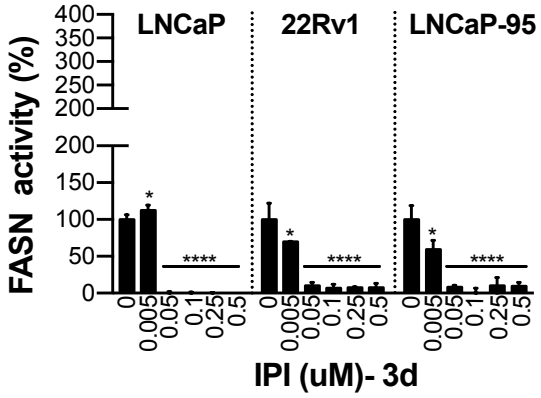


Fig. S1. IPI-9119 is a potent and selective inhibitor of FASN. (**A**) Mass spectrometry profile shows that IPI-9119 (IPI) irreversibly inhibits FASN activity by inducing the acylation of serine 2305 in the thioesterase domain (TE). The molecular weight shift is indicated by the black arrow. (**B**) Table summarizing the main properties of IPI. CCK1= Cholecystokinin A receptor. (**C**) FASN activity in AD (LNCaP) and AI (22Rv1, LNCaP-95) cells, following 3-day (3d) treatment with IPI at the indicated concentrations. Data are expressed as the mean activity ± SD (n=3) and plotted as % DMSO. $^{****}p<0.0001$, $^{*}p<0.05$, one-way ANOVA followed by Tukey's post hoc test.

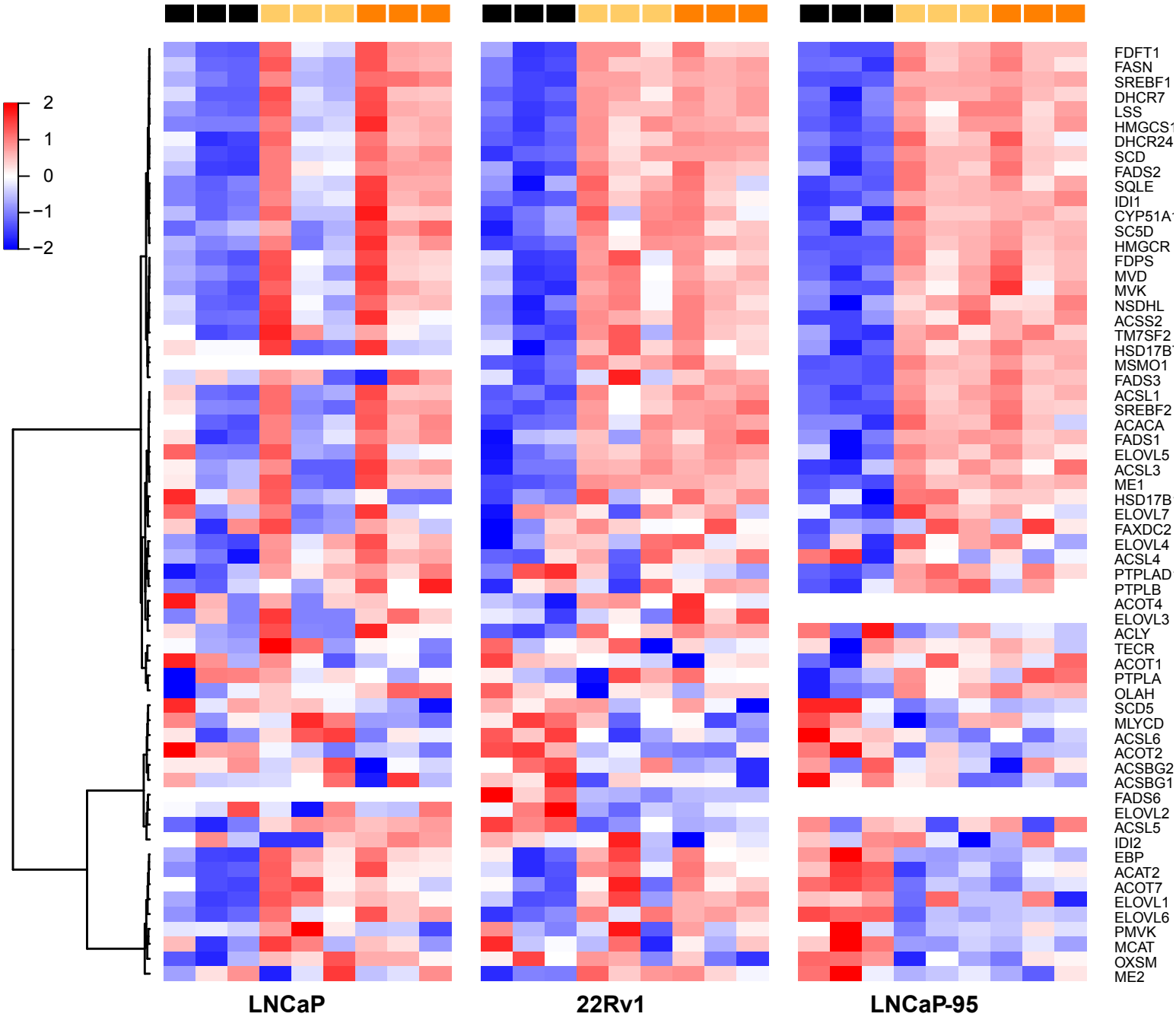


Fig. S2. Expression of key genes of lipid metabolism is increased following treatment with IPI-9119. Heatmaps of RNA-seq data showing the expression of key genes involved in lipid metabolism. Normalized counts are shown (n=3). n= number of independent samples.

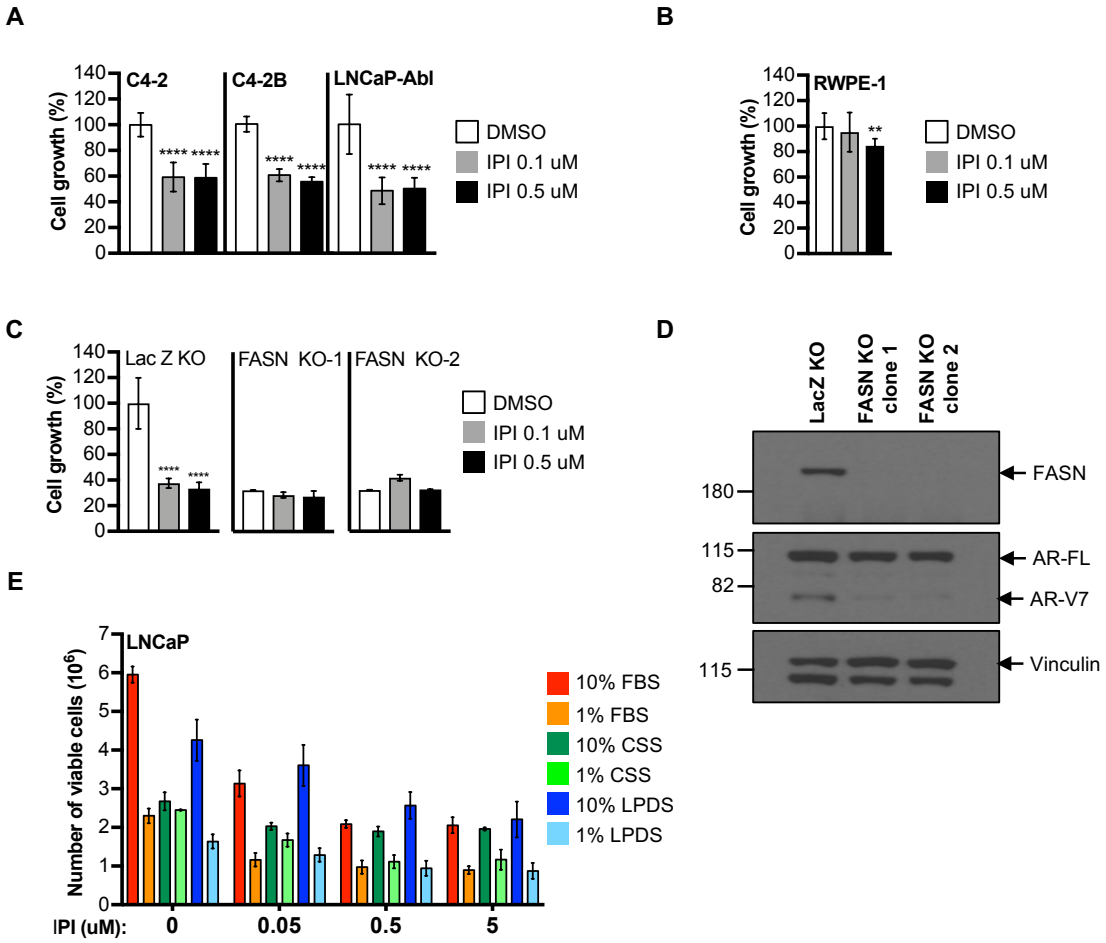


Fig. S3. IPI-9119 specifically inhibits FASN-mediated cell growth in PCa cells. (**A**) Cell growth of AI cells treated with IPI-9119 (IPI) for 6 days. Data are expressed as the mean number of viable cells ±SD (n/concentration= 12 for C4-2; 9 for C4-2B; 12 for LNCaP-Abl) and plotted as % DMSO. ****p<0.0001 IPI vs DMSO, one-way ANOVA followed by Tukey's post hoc test. (**B**) IPI minimally inhibits non-transformed epithelial prostate RWPE-1 cells. Data are expressed as the mean number of viable cells ± SD (n/ concentration: 3 (0.1 uM) and 9 (DMSO and 0.5 uM), and plotted as % DMSO. **p<0.01 IPI 0.5 uM vs DMSO, one-way ANOVA followed by Tukey's post hoc test. (**C**) Cell growth of FASN KO-1 (clone 1) and KO-2 (clone 2) LNCaP-95 and control (Lac Z KO) cells, following treatment with IPI for 6 days. Data are expressed as the mean number of viable cells ± SD (n/concentration= 3), and plotted as % DMSO of LacZ KO cells. ****p<0.0001 IPI vs DMSO, two-way ANOVA followed by Sidak's post hoc test. (**D**) Immunoblotting showing the complete absence of FASN in single FASN KO clones (1 and 2). (**E**) Effect of different media on IPI efficacy. Cell growth was assessed after 6 days of treatment with IPI in the presence of different sera composition and % in the media. FBS: fetal bovine serum; CSS: charcoal stripped serum; LPDS: lipoprotein depleted serum. Data are expressed as the mean ± SD of 9 independent samples (n=9).

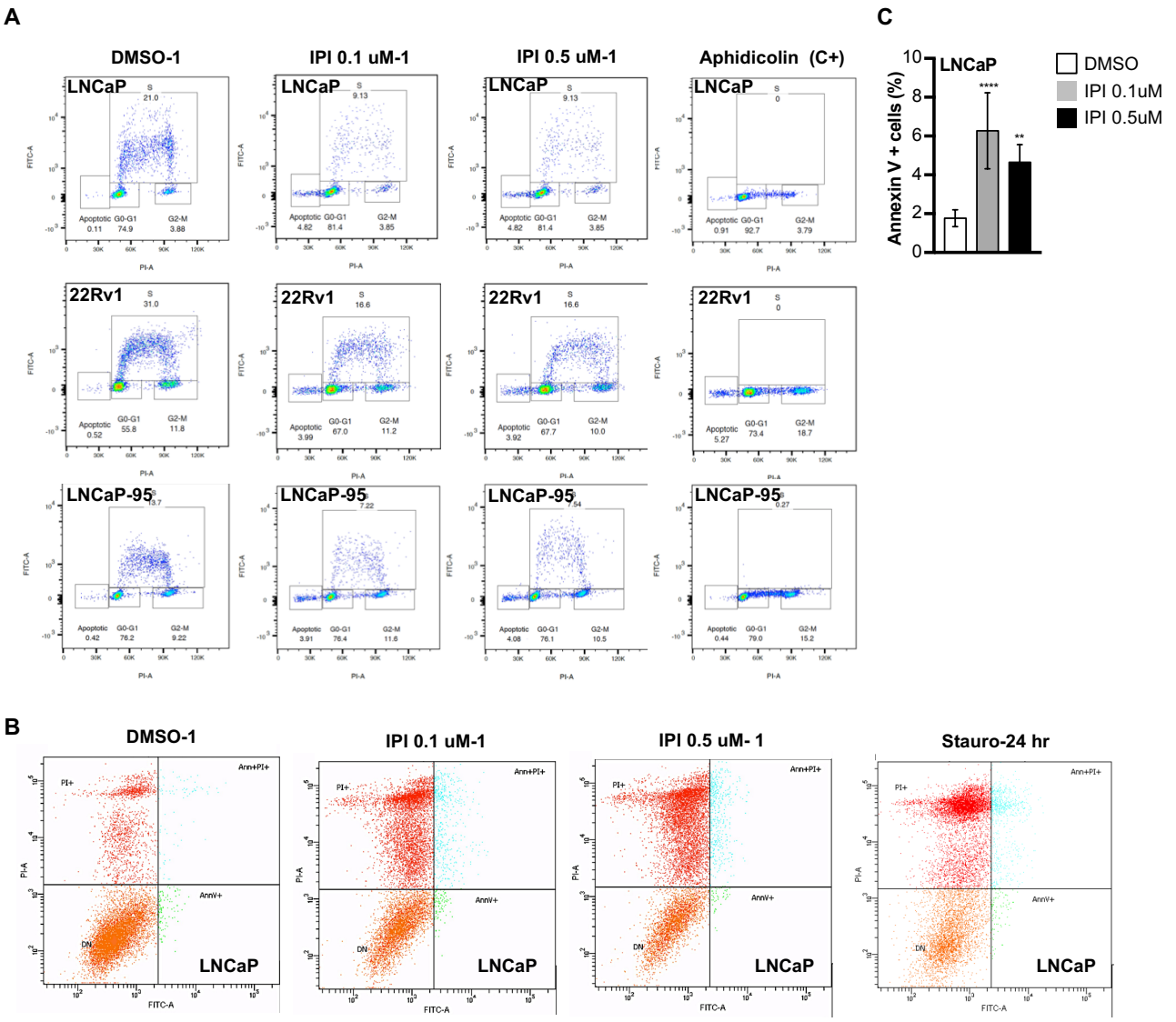


Fig. S4. IPI-9119 induces cell cycle arrest and apoptosis. (**A**) Cell cycle distribution under IPI-9119 (IPI) treatment for 6 days. Representative bivariate plots showing DNA content (propidium iodide staining, PI, x axis) and BrdU incorporation (FITC staining, y axis) are shown. Treatment with the Sphase inhibitor, aphidicolin (0.3 ug/ml, 3 hrs) was used as positive control. (**B**) Representative flow cytometry plots of Annexin V (FITC, x axis)/PI (y axis) assay in LNCaP cells, following 6 day-treatment with IPI. LNCaP treatment with staurosporine for 24 hr (Stauro) was used as positive control. Color code: orange, healthy cells; red, necrotic cells, green: early apoptotic cells, light blue: late apoptotic cells. (**C**) Quantification of apoptotic cells. Bar graph shows the % of Annexin V positive cells (both early and late apoptotic cells). Data are expressed as the mean % +SD of 2 independent experiments, each performed in biological triplicate (n=6). ****p<0.0001, **p<0.01, One-way Anova, followed by Tukey post hoc test.

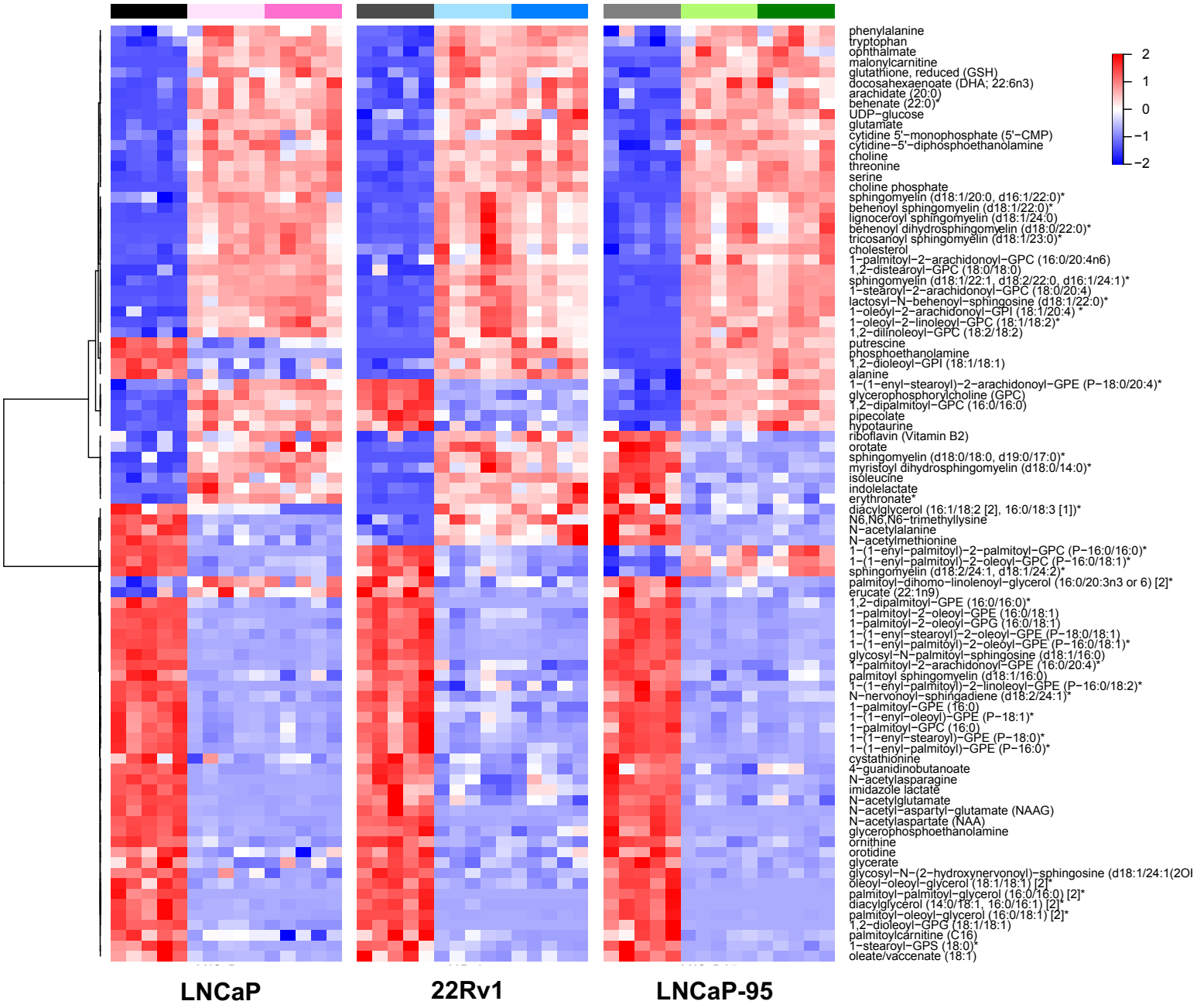


Figure S5. IPI-9119 alters the expression of key metabolites involved in lipid metabolism, TCA cycles, amino acid, carbohydrate, and nucleotide metabolism.

Heatmap representing the metabolites significantly altered under IPI-9119 (IPI) treatment at both drug concentrations (0.1 and 0.5 μM) in all the 3 cell lines ($p<0.05$, FDR <0.05). $\log_2$ (normalized values +1) are plotted.

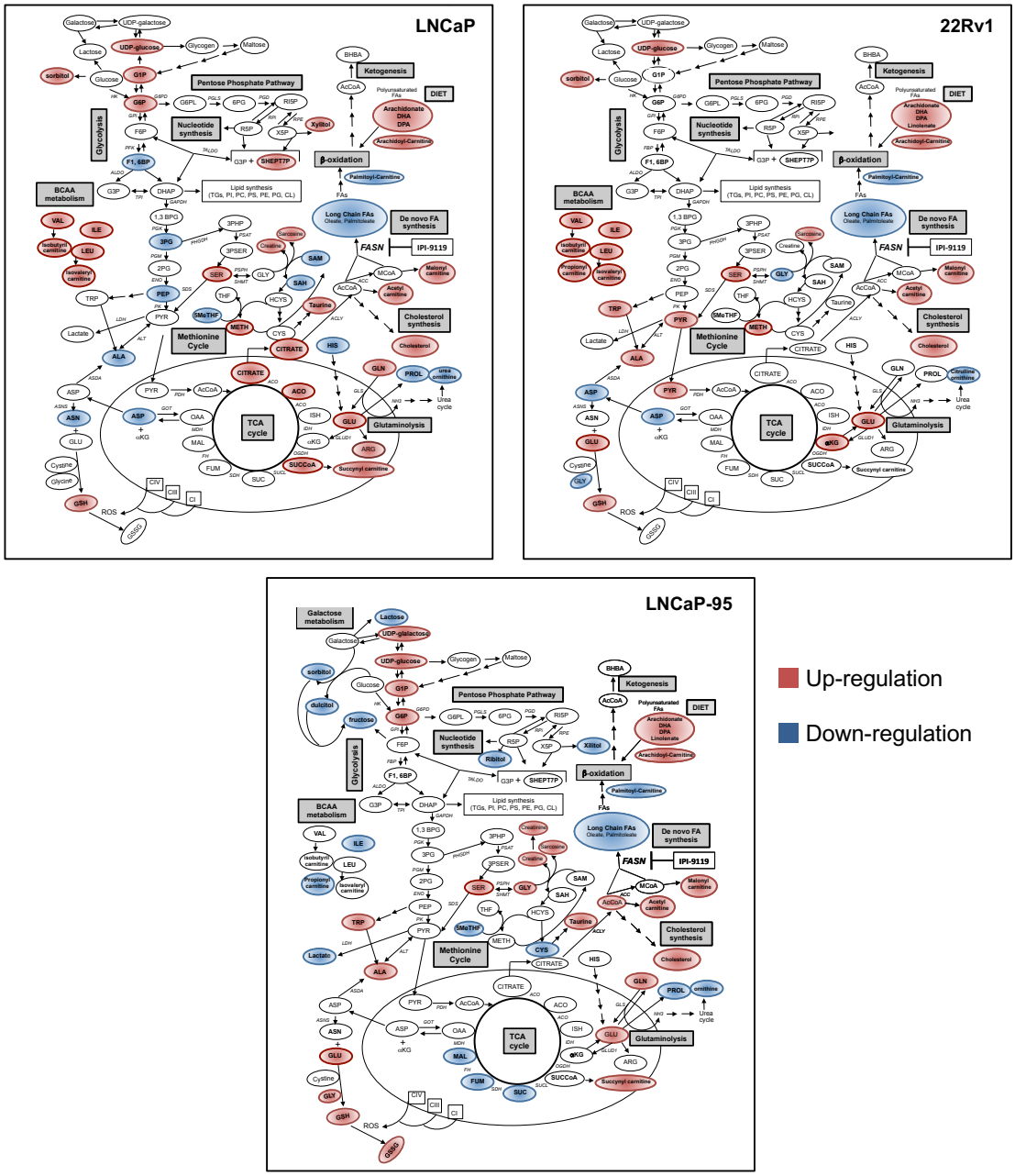


Fig. S6. Graphical representation of the key metabolites involved in central carbon metabolism. Metabolites that were significantly increased or decreased under IPI-9119 treatment (6 days) at both the concentration (IPI 0.1 vs DMSO and IPI 0.5 uM vs DMSO, p value <0.05, FDR < 0.05; Student t-test) are depicted in red or blue, respectively (n=6/ condition).

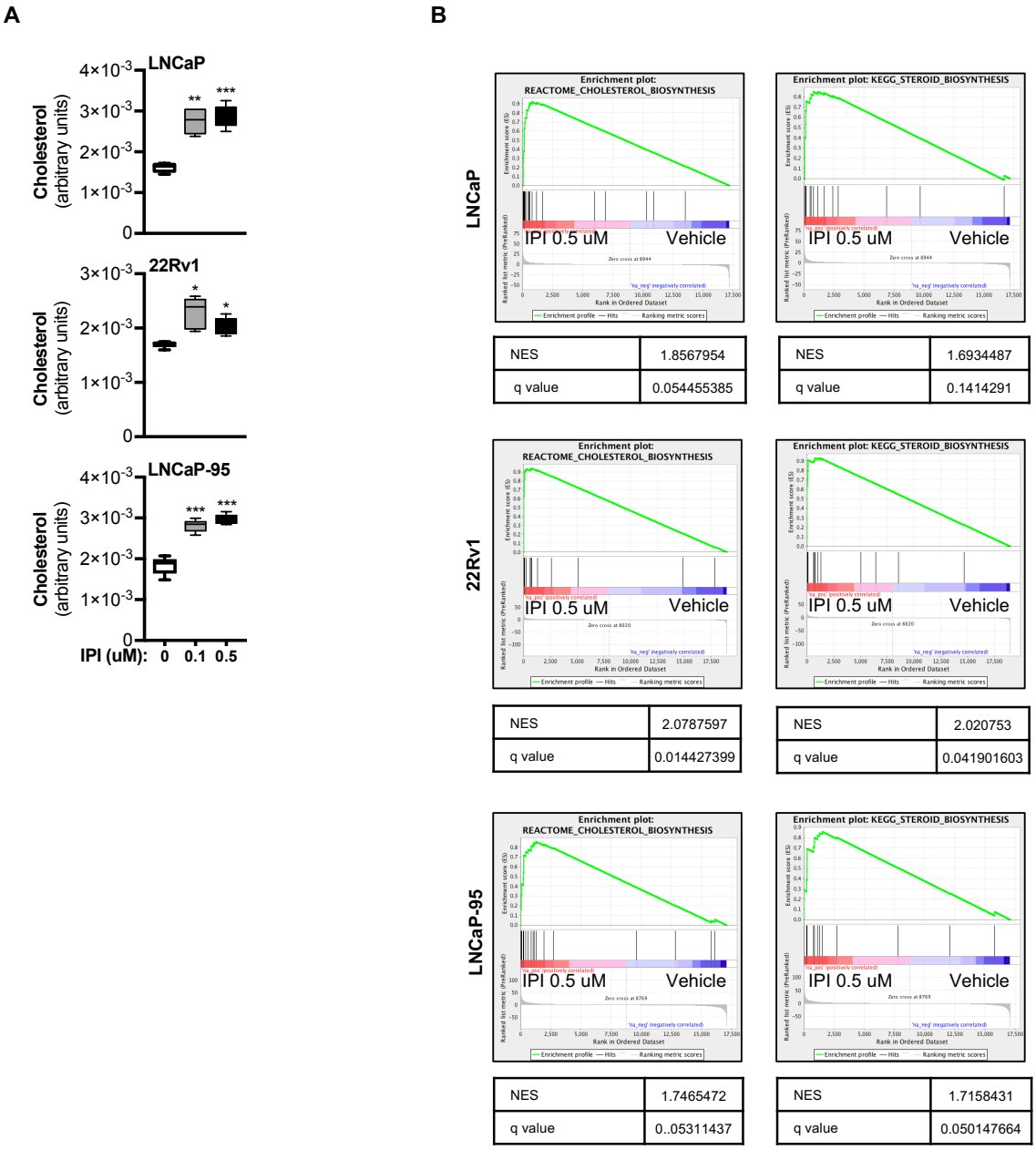


Fig. S7. IPI-9119 induces cholesterol synthesis. (**A**) Box plots showing cholesterol levels under IPI-9119 (IPI) treatment for 6 days. FDR values from metabolomics analysis are indicated (*q<0.05, **q<00.1, ***q<0.001, Student t-test) (n=6/condition). (**B**) Pre-ranked GSEA shows IPI-mediated upregulation of gene sets associated with cholesterol synthesis and steroidogenesis in AD LNCaP cells and AI 22Rv1 and LNCaP-95 cells (Canonical pathways; C2.cp.v5.2). Normalized enriched score (NES) and FDR (q values) are indicated under each plot.

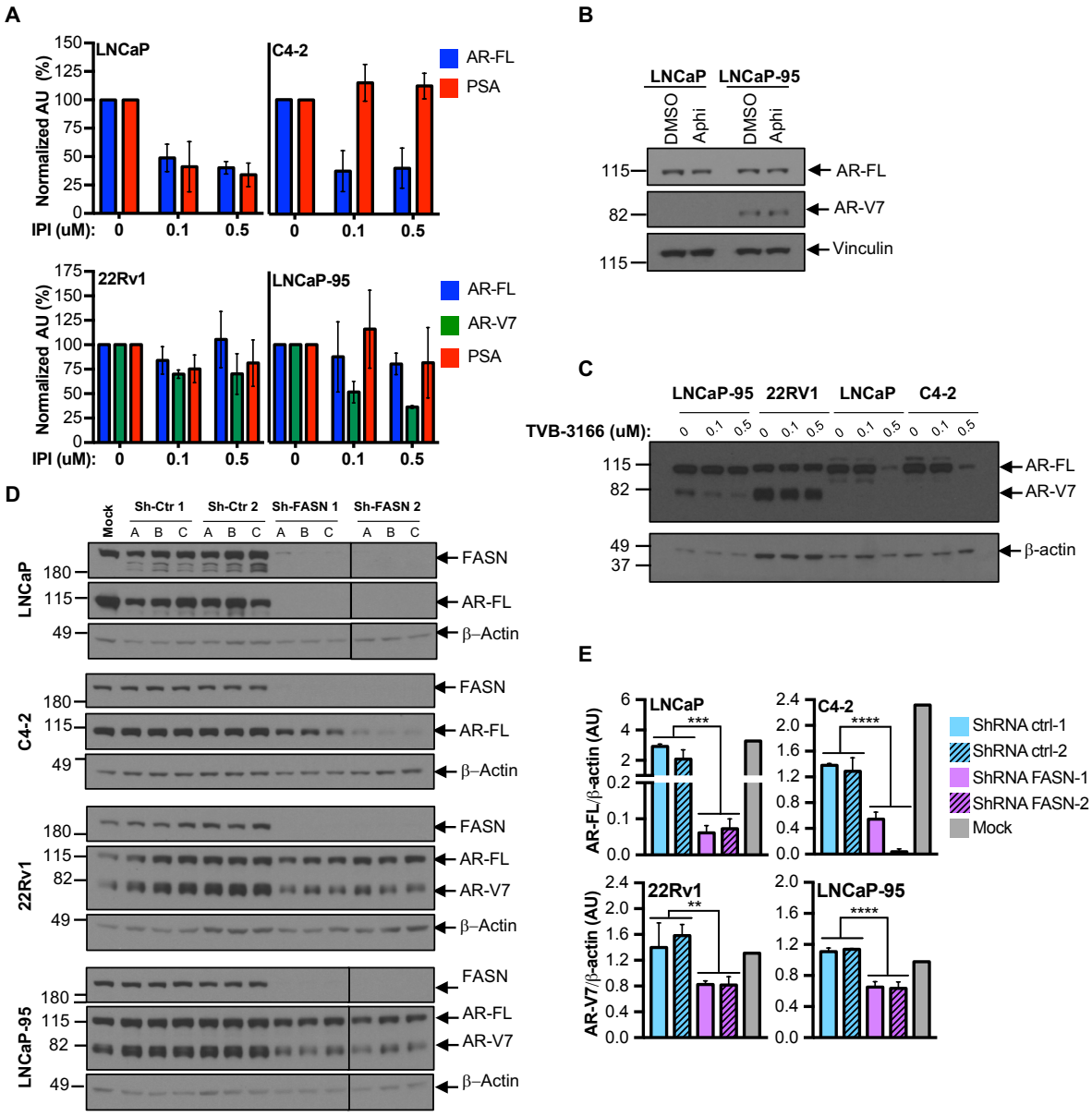


Fig. S8. Pharmacological and genetic suppression of FASN inhibits AR-FL and AR-V7 protein expression. (**A**) Densitometry of AR-FL, AR-V7, and PSA protein expression under IPI-9119 (IPI) treatment for 6 days. Immunoblotting of 3 independent experiments were used to perform densitometric analysis. Data are expressed as normalized AR-FL and AR-V7 (AR-FL/β-actin; AR-V7/β-actin) arbitrary units (AU) ± SD and plotted as % DMSO. (**B**) Inhibition of cell cycle S phase using aphidicolin (Aphi, 0.3 ug/ml, 3 hr) does not reduce AR/AR-V7 levels. (**C**) Immunoblotting showing the inhibition of AR-FL and AR-V7 using the commercially available FASN inhibitor (TVB-3166). (**D**) Immunoblotting showing the inhibition of AR-FL and AR-V7 using two different shRNA targeting FASN, after 6 days of infection. Two different shRNA targeting GFP were used as control. Three biological replicates (n=3, A-C) were used. A non-infected sample (Mock) was also included in the experiment. (**D**) Densitometry of AR-FL and AR-V7 protein expression following FASN KD for 6 days in all the PCa cell analyzed (n=3). Data are expressed as normalized AR-FL and AR-V7 (AR-FL/β-actin; ARV7/β-actin) arbitrary units (AU) ± SD. Statistical analysis were performed by comparing the FASN shRNA values (hairpins FASN 1 and 2) vs. control values (hairpins ctrl 1 and 2), using Student t-test, ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. Non-infected cells (Mock) were used as control.

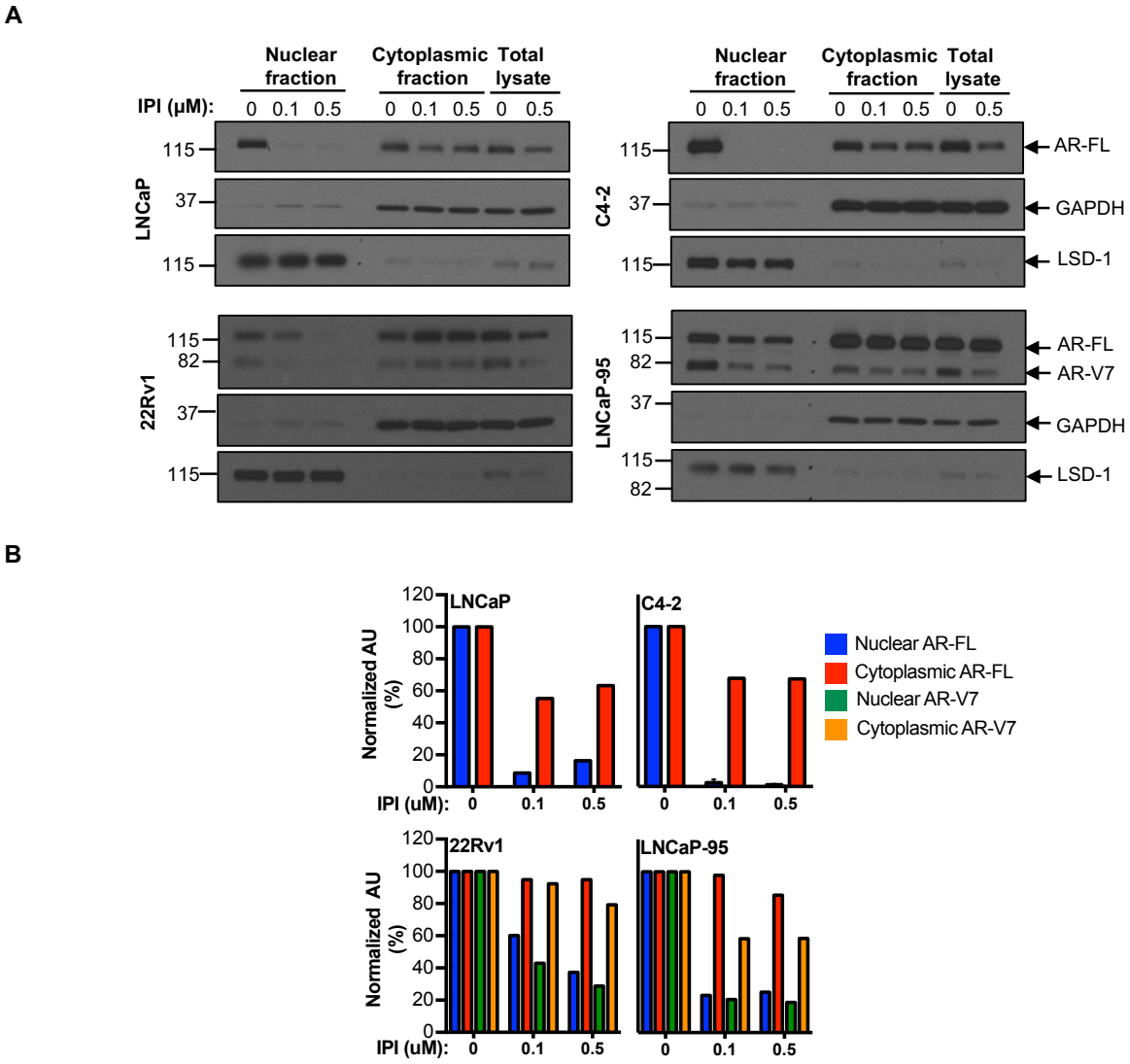


Fig. S9. IPI-9119 inhibits nuclear and cytosolic AR-FL and AR-V7 proteins. (**A**) Representative immunoblotting showing the reduction of nuclear AR-FL in AD LNCaP and AI C4-2 cells and the reduction of both nuclear AR-FL and ARV7 in 22Rv1 and LNCaP-95 cells, following treatment with IPI-9119 (IPI) for 6 days. Two independent experiments were performed with similar results. (**B**) Densitometry of nuclear and cytoplasmic AR-FL and AR-V7 protein expression. Immunoblotting of two independent experiments were used to perform densitometric analysis. Data are expressed as the mean of normalized AR-FL and AR-V7 (AR-FL/GAPDH; AR-V7/LSD-1) expression in arbitrary units (AU) and plotted as % DMSO.

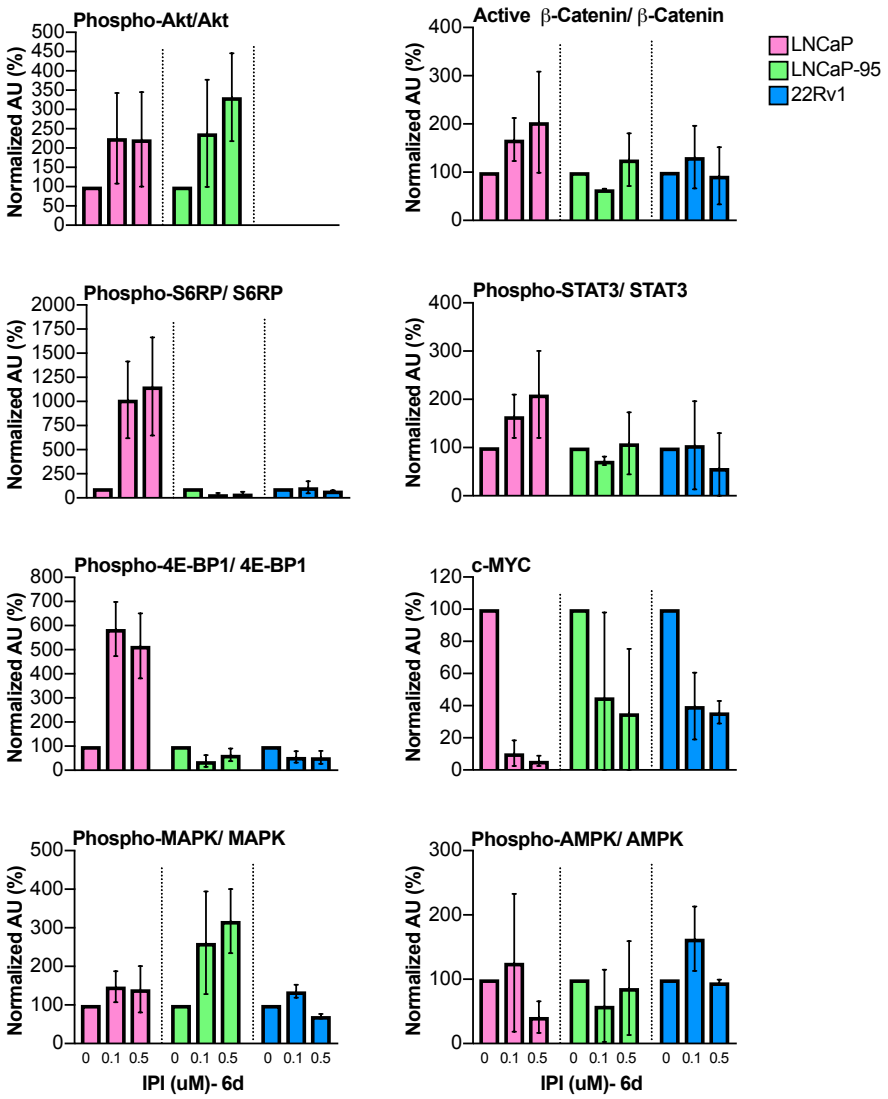


Fig. S10. IPI-9119 does not cause a general downregulation of survival/proliferation pathways. Densitometric analysis of key proteins involved in proliferation/survival, and energy stress pathways. Data are expressed as normalized arbitrary units (AU) and expressed as % of phospho-protein/ total-protein of two independent experiments ± SD.

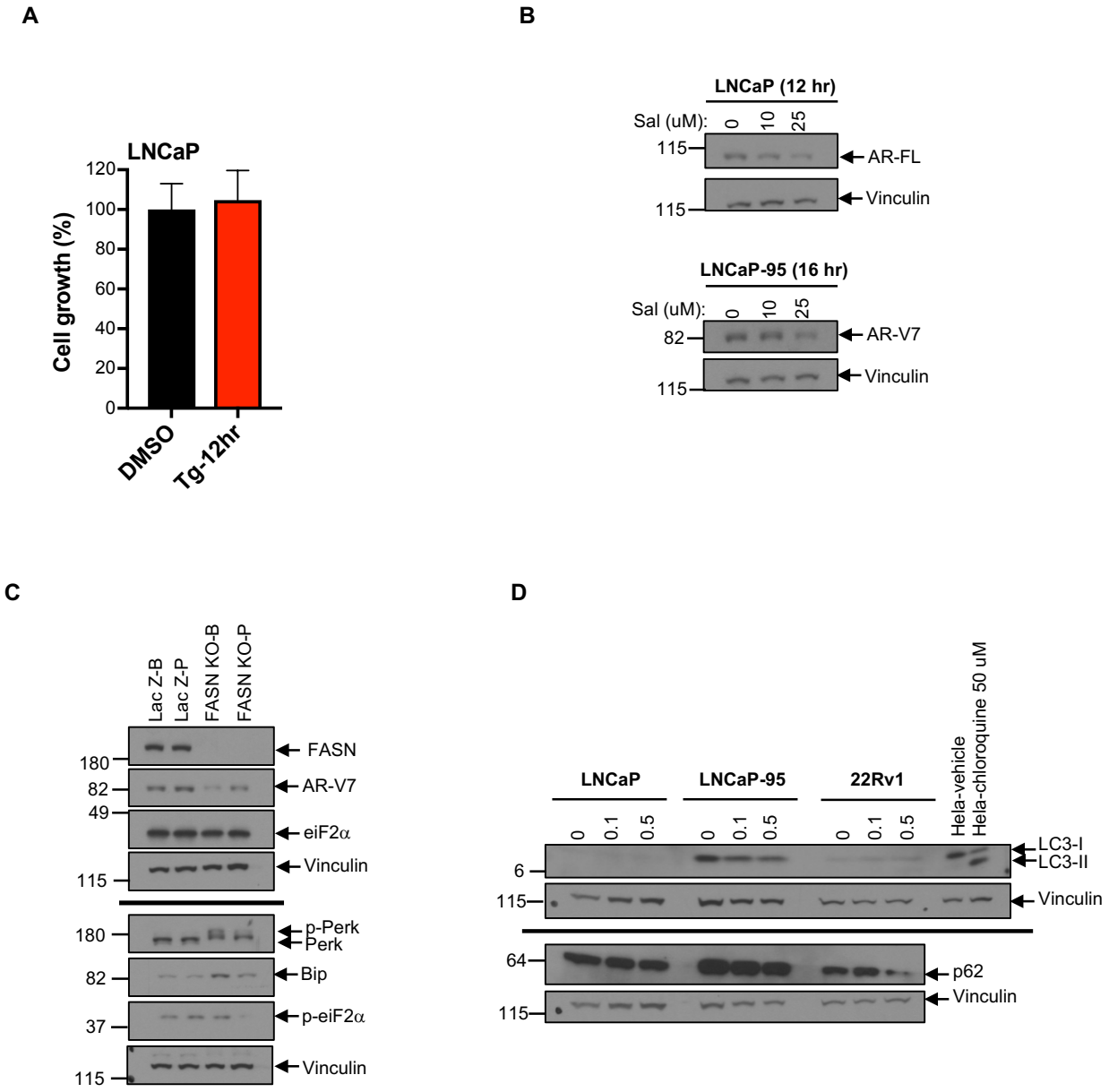


Figure S11. IPI-9119-mediated FASN inhibition is associated with ER stress induction. (**A**) Evaluation of cell growth under thapsigargin (Tg, 1 uM) treatment for 12 hrs. (**B**) Immunoblotting showing the reduction of AR and AR-V7 proteins, under treatment with the specific inhibitor of eif2a dephosphorylation, salubrinal (Sal, 10 and 25 uM). Duration of treatment is indicated. (**C**) Immunoblotting showing amelioration of FASN KO-induced ER stress, following incubation with palmitate (P, 50uM) for 3 days. Equal molar amount of BSA (B) was used as control. (**D**) Representative immunoblotting showing the expression of autophagy markers under treatment with IPI-9119. Commercial lysate from Hela cells treated with chloroquine (50 uM, overnight) was used as positive control. Experiment was repeated twice.

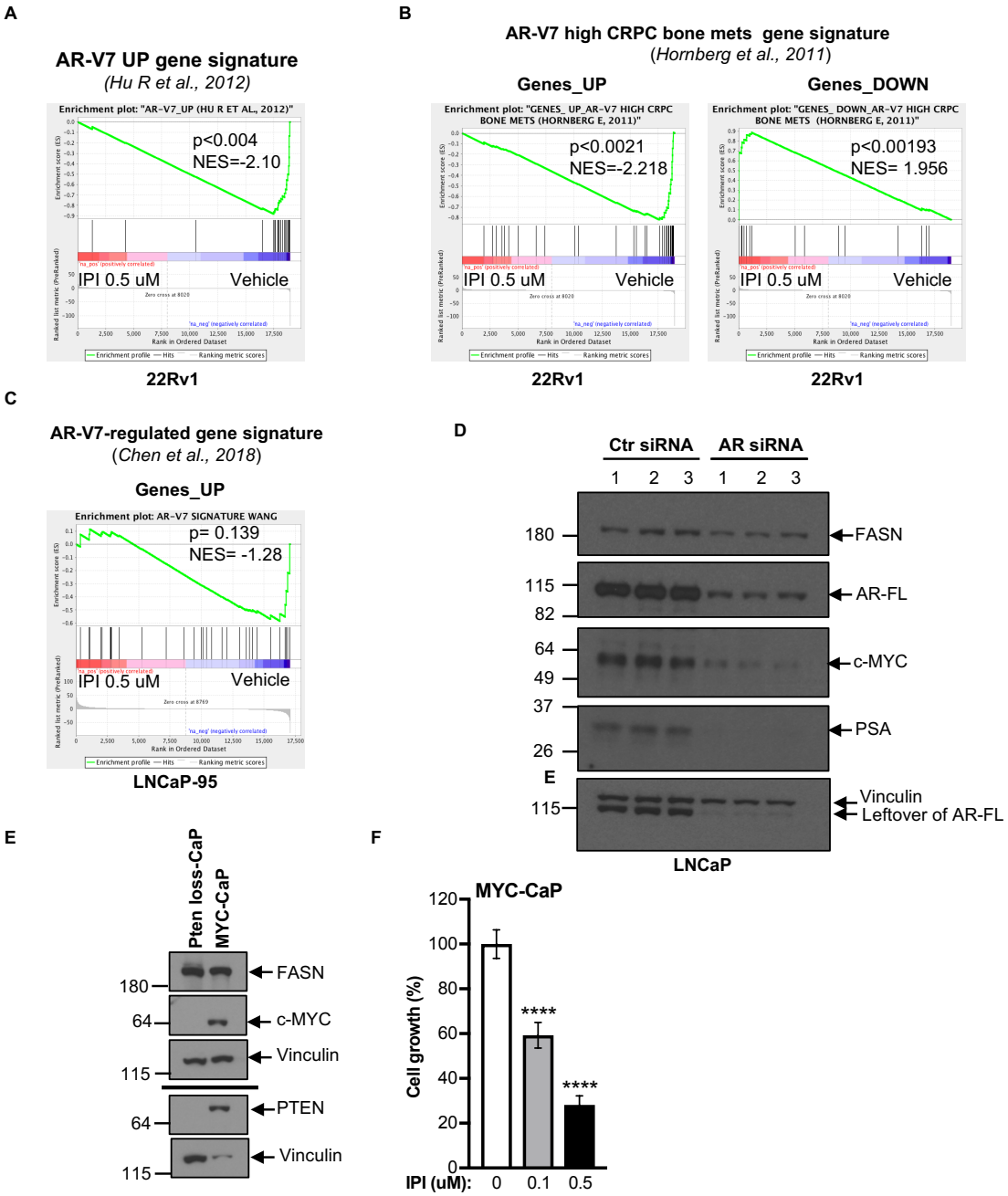


Fig. S12. IPI-9119 down-regulates AR-V7 associated gene signatures and c-MYC expression. (**A**) Pre-ranked GSEA analysis showing IPI-9119 (IPI)-mediated down-regulation of the gene signature associated with ARV7 previously described (ref. main text: 32). P value is indicated. (**B**) Pre-ranked GSEA analysis showing IPI-mediated reversion of the gene signature associated with CRPC bone mets expressing high levels of AR-V7 (ref. main text: 33). (**C**) Preranked GSEA analysis showing IPI-tendency to downregulate AR-V7-regulated gene set (33 genes up in both 22Rv1 and LNCaP-95) (ref. main text 34). (**D**) Immunoblotting showing reduction of c-MYC, following AR-FL KD. AR-FL KD was achieved following 96 hrs-transfection with a pool of 4 siRNA targeting AR-FL. Non-targeting pool control siRNA was used to rule out off-target effects. (**E**) Immunoblotting showing c-MYC overexpression in MYC-CaP cells. (**F**) Cell growth analysis of MYC-Cap cells treated with IPI for 6 days. Data are expressed as % of DMSO treated cells ± SD. ****p<0.0001, One-way ANOVA, followed by Tukey post hoc test (n=9).

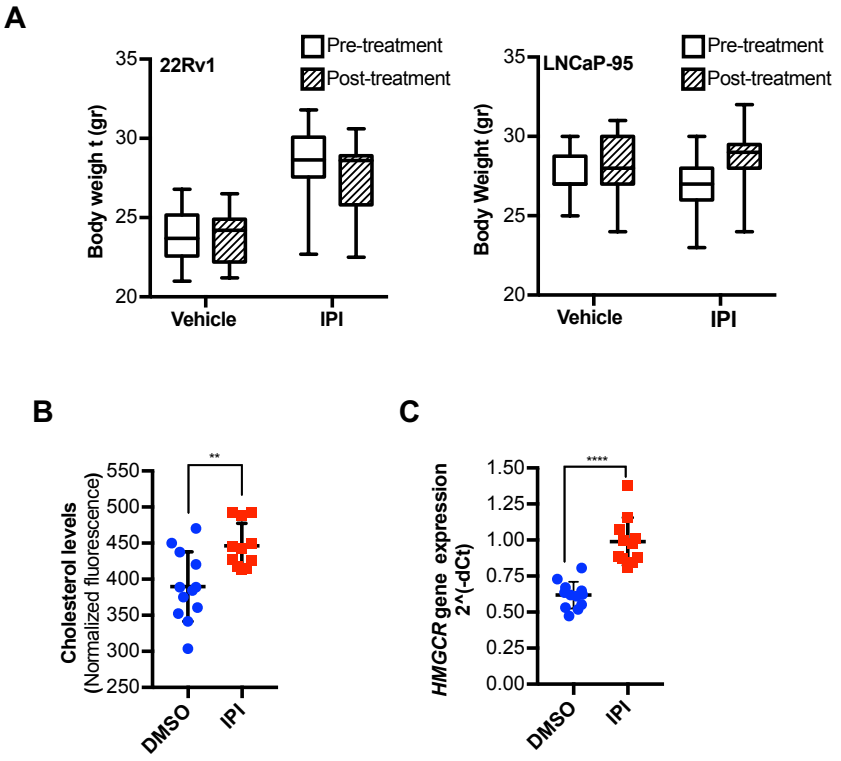


Fig. S13. IPI-9119 effects in preclinical models. (**A**) Mouse body weight under treatment with IPI-9119 (IPI) using subcutaneous pump infusion for four weeks. (**B**) Cholesterol levels, measured with the fluorescent Amplex Red Cholesterol Assay Kit in xenograft tumors collected at the end of the treatment period (28 days), **p= 0.0033 IPI vs DMSO, Student t-test. (**C**) HMGCR mRNA levels (expressed as 2^-dCT) measured in xenograft tumors at the end of the treatment period (28 days). ****p<0.0001, IPI vs DMSO, Student t-test.

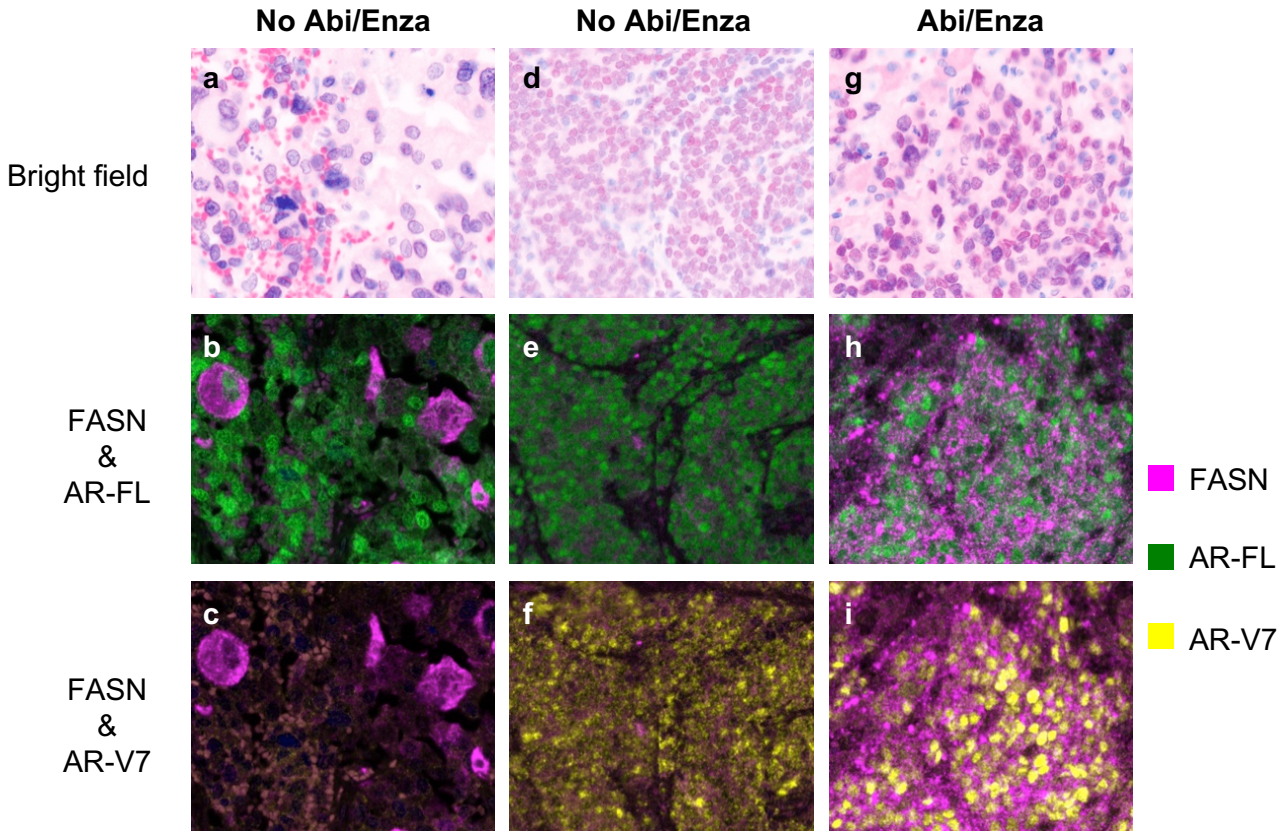


Fig. S14. FASN is co-expressed with AR-FL and AR-V7 in visceral metastases. Representative images of FASN co-expression with AR-FL and AR-V7 (20X) in liver metastases: Bright field (a), FASN/AR-FL staining (b), FASN/ AR-V7 staining in AR-V7-negative liver metastasis from a mCRPC Enza/Abi-naïve patient (c); Bright field (d), FASN/AR-FL staining (e), FASN/ AR-V7 staining in AR-V7-positive liver metastasis from a mCRPC Enza/Abi-naïve patient (f); Bright field (g), FASN/AR-FL staining (h), FASN/AR-V7 staining in AR-V7-positive liver metastasis from a mCRPC Enza/Abi-treated patient (i).

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