SUPPLEMENTARY DATA

Mechanisms behind resistance to PI3K Inhibitor treatment induced by the PIM kinase

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SUPPLEMENTARY METHODS

Chemical and Antibodies

PIM447 was provided by Novartis Oncology. Buparlisib (BKM120), AZD1208 and AZD5363 were purchased from AdooQ Bioscience. H_2DCF-DA was from Molecular Probes (Thermo Scientific). All other chemicals were purchased from Sigma-Aldrich. The antibodies used in the study included anti-β-ACTIN (A3854), anti-FLAG M2 (F3165) from Sigma-Aldrich; anti-phospho-AKT (4060), anti-AKT (9272), anti-phospho-IRS1 (2385), anti-IRS1 (2390), anti-phospho-p70S6K (9205), anti-p70S6K (9202), anti-phospho-S6 (5364), anti-phospho-4EBP1 (9451), anti-phospho-eIF4B (5399), anti-eIF4B (9742), anti-γH2AX (9718), anti-PARP1 (9542), anti-PIM1 (3247) from Cell Signaling Technology; anti-SOD2 (ab13533) from Abcam; anti-HA.11 (MMS-101P-200) from Covance; anti-Ub (sc-8017), anti-PTEN (sc-7974), anti-S6 (sc-74459), anti-KEAP1 (sc-365626), anti-p62 (sc-28359), anti-GCSC (sc-390811), anti-PRDX3 (sc-130336), anti--GCLM (sc-55586), anti-NQO1 (sc-32793) anti-HMOX1 (sc-136960), anti-NRF2 (sc-13032) from Santa Cruz Biotechnology.

Use of Plasmids, siRNA and shRNA

Lentiviral pTRIPz vector encoding Tet-inducible PIM1 and control vectors were previously described (1). FUCRW vector encoding PIM1 and RFP was used as reported previously (2). To knock down expression of NRF2, short hairpin RNAs against human NRF2 (TRC gene set 7555) and TRC lentiviral non-targeting shRNA control were purchased from Dharmacon. Non-silencing small interfering RNA (siRNA) control and HMOX1 RNAi pools have been previously published (3,4). Use of siRNAs targeting PIM1 (5′-GAU AUG GUG UGU GGA GAU A-3′), PIM2 (5′-ACC UUC UUC CCG ACC CUC A-3′) and PIM3 (5′-GCA CGU GGU GAA GGA GCG G-3′) (Dharmacon) were previously described (5). pcDNA3-HA-tagged NRF2, pcDNA3-KEAP1, NQO1-ARE firefly luciferase and *Renilla* luciferase (pGL4.74-hRluc-TK,

Promega) reporter plasmids were provided by Dr. Donna Zhang at the College of Pharmacy, University of Arizona (Tucson, AZ) and previously documented (6-8). The HMOX1 plasmid was a kind gift from Dr. Michal-Ruth Schweiger (University of Cologne, Cologne, Germany) (9).

For lentiviral production and infection of human PCa cells, lentiviruses were produced in 293T cells co-expressing the packaging vectors (pPAX2 and VSVG for TRC and pTRIPz; VSVG, pMDL, pREV for FUCRW), concentrated by ultracentrifugation (20,000 g for 2 hr at 4 °C), and resuspended with culture media for an infection of 48 hr. Transfection of siRNA and cDNA was performed using Lipofectamine 3000 (Invitrogen) and Xfect transfection reagent (Clontech).

Animals and Tissue Culture

Primary cultures of mouse prostate epithelial cells were obtained from $Rb1^{F/F}$:$Trp53^{F/F}$:PB-Cre4 mice, where F represents floxed alleles and PB-Cre4 drives cre expression in the prostate epithelium. Lack of both pRb and p53 in prostate epithelial cells was verified by PCR according to the previous report (10). For isolation of prostate epithelial cell (11), dissected prostate tissue from 8 month-old mice were minced, suspended in collagenase solution, and incubated on a shaker at 37 °C for 2 hr. Then, cells were cultivated in DMEM media supplemented with 2.5% charcoal stripped FCS, 5 μg/mL of insulin/transferring/selenium (Collaborative Research), 10 μg/mL of bovine pituitary extract (Sigma), 10 μg/mL of epidermal growth factor (Collaborative Research), 1 μg/mL of cholera toxin (Sigma), 100 U/mL of penicillin G and streptomycin (Gibco) following the previous report (12). For tumor xenograft studies, 5 $x10^6$ PC3-LN4 cells were injected subcutaneously into the rear flank of SCID mice (male; 8 wk-old) in PBS/Matrigel (v/v). Once tumors reached a volume of approximately 200 mm^3, the mice were randomized to treatment with vehicle, PIM447 (30 mg/kg oral gavage daily), Buparlisib/BKM120 (10 mg/kg by oral gavage daily), or the indicated combinations. Tumor

volumes were monitored over time by caliper measurements. At the end of the study, all tumors were fixed, embedded in paraffin, and sectioned for staining with hematoxylin and eosin (H&E) or Ki67. Additionally, PC3-LN4 tumor xenografted mice were also treated with vehicle, AZD5363 (40 mg/kg oral gavage daily), AZD1208 (30 mg/kg oral gavage daily), or the indicated combinations as previously described (13). To examine the effects of BSO and BKM120 *in vivo*, SCID mice were implanted with 5 $x10^6$ LNCaP/PIM1 (PIM1 overexpressing LNCaP) cells and then were randomized to treatment with vehicle, BSO (400 mg/kg/day), BKM120 (10 mg/kg/day), or the indicated combinations. Tumor volume calculations were obtained using the following formula: tumor volume [mm^3] = (length [mm]) x (width [mm] ^{2})/2 for caliper measurements.

Preparation and Culture of Patient-Derived Organoids:

Prostate cancer patient-derived organoids were cultured based on previously established procedures (14,15). Briefly, human prostate biopsy tissues were collected and dissociated with collagenase type 1 (Worthington) and plated in growth factor-reduced matrigel (Invitrogen) in Advanced DMEM/F12 media (Invitrogen) containing EGF, noggin and R-spondin (ENR) (Peprotech) for organoid growth. Organoids were formed in 7-10 days after plating. The patient-derived organoids were subcultured for the desired number of passages by dissociation using TryLE (Invitrogen) and replated in growth factor reduced matrigel along with the ENR media. For organoid viability assays, 5,000 organoid cells in 10 µl of matrigel per well was plated in a 96-well cell culture plate with 100 µl of complete human organoid medium containing vehicle control (DMSO), Buparlisib (1 µmol/L) or PIM447 (3 µmol/L) for 6 days. Viable cells were measured by manually counting the number of organoids remaining per group after 6 days of treatment. All cell viability experiments were conducted in triplicate.

Soft-agar Colony Formation Assay

The soft-agar assay was conducted in 6-well plates in triplicate. For each well, 5,000 cells were placed in growth medium containing 0.7% agarose. Cells were then layered over 1% agarose in medium. After 21 days, colonies were counted under a microscope.

CRISPR-Cas9 Genome Editing

To generate PC3-LN4 cells stably expressing the eIF4A S406A mutation, Alt-R® CRISPR-Cas9 system and reagents were purchased from IDT. Cloning was provided by the Genome Editing Core at the University of Arizona Cancer Center. PC3-LN4 cells were transfected the crRNA/tracrRNA (final concentration 3 μmol/L) and Cas9 protein complex with Lipofectamine®RNAiMAX transfection reagent. The transfected cells were cloned by seeding one cell per one well in 96-well tissue culture plates. The clones were screened by PCR using primers as indicated below. Screening of EIF4B S406A was performed by genomic PCR with S406A screening primer and the PCR fragment was digested by BseYI restriction enzyme (NEB). The BseYI site was introduced during genome editing. The mutation in 406A was confirmed by sequencing of PCR fragments.

crRNA

The guide EIF-g1 sequence is TCTCGTACAGACACCCAAGC **TGG**.

tracrRNA

S406A (AGC -> GCA)

GGTGGCCTTCACTGAGCCAGGCATAATGTGTATTTTGTGAATCTCTCGTACAGACACCCA*G*

*CA*TGGCGAAGTGAAGAAACTCAGGAACGGGAACGGTCGAGGACAGGAAGTGAGTCATCA

CAA

S406A screening primers

EIF4B-F: TAGGCCTGGTAATGAGTGGCT

EIF4B-R: CTCCAAGCCGTTGACTGTTC

For knockout of human eIF4B gene (NM_001417), the following gRNAs were cloned into the pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro vector (ABM Inc).

130 ACGGATGACCTGGAAGGAGA (eIF4B_1)

161 GTCATCGTTACTGTGCCAAG (eIF4B_2)

462 TTACCTCTTCATTGAGACTC (eIF4B_3)

Cell Viability and Focus-forming Assays

To evaluate growth inhibition, cells were plated in 5 mL of RPMI media containing 10 % FBS in a six-well plate at the density of 100 cells and then treated with indicated inhibitors at 37 °C for 7 days before scoring for foci formation. Foci were visualized with crystal violet staining. Cell viability was determined by the MTT assay. Crystal violet staining was previously described (2).

Subcellular Fractionation

For cellular fractionation, nuclear and cytoplasmic extracts were isolated using Pierce Subcellular Protein Fractionation Kit (Thermo Scientific).

Immunoblotting

Cells were lysed in immunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1% NP-40, 5 mmol/L EDTA) supplemented with complete protease/phosphatase inhibitor cocktail (Cell Signaling Cat# 5872S). Following 15 min incubation in lysis buffer at 4°C, lysates were clarified by centrifugation at 15,000 g for 15 min at 4°C, then protein concentrations of the resulting supernatant were determined by DC Protein Assay (Bio-Rad). To detect protein expression in

the total cell lysates, cell lysate (50 µl) in RIPA buffer was mixed with 10 µl of 6 x Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.9], 3 % SDS, 10% glycerol, 5 % β-mercaptoethanol, 0.1 % bromophenol blue) and boiled for 5 min. Aliquots were subjected to SDS-PAGE. Subsequently, proteins were transferred to nitrocellulose membranes and the membrane was blocked by incubation with 5% milk in TBS-T buffer (50 mM Tris-HCl, pH 7.6, 150 mmol/L NaCl, 0.05 % Tween 20) for 1 hr at room temperature. The membrane was then incubated overnight at 4 °C with the indicated primary antibody in 3 % BSA in TBS-T buffer, washed three times in the same buffer, and incubated for 1 hr with HRP-conjugated secondary antibody. The membrane was then washed three times with TBS-T buffer and developed with chemiluminescence reagent kit (Pierce) for visualization on x-ray film.

Immunoprecipitation

Cells were transfected with empty vector or a vector expressing HA-NRF2, KEAP1, PIM1, or FLAG-tagged ubiquitin. Cell lysates were collected at 48 h post-transfection in RIPA buffer. Cell lysates were incubated with 1 µg of antibody and 20 µl of protein A-agarose beads on a rotator at 4°C overnight. For HA-NRF2 immunoprecipitation, monoclonal anti-HA−agarose (Sigma) was used. The immunoprecipitated complexes were washed with RIPA buffer three times and eluted in sample buffer by boiling for 5 min. Samples were then resolved by SDS-PAGE and subjected to immunoblot analysis.

Quantitative Real-time PCR (qRT-PCR) Analysis

Total RNA was extracted from cells by using Qiagen QIAshredder and RNeasy Mini kit. Equal amounts of total RNA (1 µg RNA) was subjected to first-strand cDNA synthesis using iScript cDNA synthesis kit according to the manufacturer's protocol. qRT-PCR reactions with Bio-Rad SsoAdvanced Universal SYBR Green Supermix were performed using a Bio-Rad CFX96 Touch System. Predesigned primer sets (KiCqStart® SYBR® Green Primers; Sigma) for

each of the following genes were purchased for qRT-PCR analysis of gene expression: *HMOX1*, *NQO1*, *GCLM*, *NRF2*, and *PIM1.* For data analysis, raw counts were normalized to the housekeeping gene averaged for the same time point and condition (ΔC_t). Counts are reported as fold change relative to the untreated control ($2^{-\Delta\Delta}C^t$).

The primers used for qRT-PCR are listed below.

NRF2 forward 5'-CGTTTGTAGATGACAATGAGG-3'

NRF2 reverse 5'-AGAAGTTTCAGGTGACTGAG-3'

HMOX1 forward 5'-AAC TTT CAG AAG GGC CAG GT-3'

HMOX1 reverse 5'-CTG GGC TCT CCT TGT TGC-3'

NQO1 forward 5'-CGC GAC TCC CAC AAG GTT-3'

NQO1 reverse 5'-GTC CGA CTC CAC CAC CTC C-3'

GCLC forward 5'-CCG CTG AGC TGG GAG GAA A-3'

GCLC reverse 5'-CCA GAC AGG ACC AAC CGG ACT-3'

GCLM forward 5'-GGG GAA CCT GCT GAA CTG G-3'

GCLM reverse 5'-GGC TGT AAA TGC TCC AAG GAA A-3'

ME1 forward 5'-CTACGGCAGAGAAGAGTAAG-3'

ME1 reverse 5'-GCCATACTTGGAAGAAACTG-3'

IDH1 forward 5'-TAAAGGTTTACCCAATGTGC-3'

IDH1 reverse 5'-CTGTAGACCTAGTTACCAAAAG-3'

PGD forward 5'-CATACCACCTGATGAAAGAC-3'

PGD reverse 5'-GCTCTGTCTTATTCCAATCC-3'

G6PD forward 5'-AAGGTCAAGGTGTTGAAATG-3'

G6PD reverse 5'-TCTCATTCTCCACATAGAGG-3'

GPD2 forward 5'-ATGTTGAAGTGAGAAGAGGG-3'

GPD2 reverse 5'-CAACATGATTTCGGGAGATAG-3'

KEAP1 forward 5'-GCACAACTGTGTATCTATGCTG-3'

KEAP1 reverse 5'-CTCCAAGGACGTAGATTCTC-3'

MAF forward 5'-GTACAAGGAGAAATACGAGAAG-3'

MAF reverse 5'-TATGAAAAACTCGGGAGAGG-3'

PIM1 forward 5'-CGACATCAAGGACGAAAACATC-3'

PIM1 reverse 5'-ACTCTGGAGGGCTATACACTC-3'

PIM2 forward, 5'-GAACATCCTGATAGACCTACGC -3'

PIM2 reverse, 5'-CATGGTACTGGTGTCGAGAG -3'

PIM3 forward, 5'-GACATCCCCTTCGAGCAG-3'

PIM3 reverse, 5'-ATGGGCCGCAATCTGATC-3'

18S forward, 5'-GTAACCCGTTGAACCCCATT-3'

18S reverse, 5'-CCATCCAATCGGTAGTAGCG-3'

ACTIN forward 5'-GACGACATGGAGAAAATCTG-3'

ACTIN reverse 5'-ATGATCTGGGTCATCTTCTC-3'

Microarray

The Genomics Facility Core at University of Arizona Cancer Center performed quality control using the Agilent Bioanalyzer 2100 to confirm all RNA samples had RNA Integrity Numbers (RINs) greater than seven, and quantitate concentration. From the RNA, the Genomics Core produced labeled DNA target using the WT PLUS reagent kit and hybridized it to the Affymetrix® Mouse Gene 1.0 ST Array according to the manufacturer's instructions. Arrays were washed and scanned with the GeneChip Hybridization, Wash, and stain kit and an Affymetrix® Scanner 3000 following manufacturer's instructions. The Affymetrix® Transcriptome Analysis Console v3.0 software was used to analyze resulting data file to identify differentially expressed genes between control (empty vector, EV) and PIM1. The datasets generated during the current study are available in the GEO repository (http://www.ncbi.nlm.nih.gov/geo/) under the following accession number, GSE118786.

^{13}C-Glucose Labeling and Metabolite Measurement

LNCaP cells expressing EV or PIM1 were seeded at the density 3 x10^6 cells in 10 cm tissue culture dishes. After overnight culture, cells were placed in glucose-free RPMI media containing U-^{13}C glucose (11.1 mmol/L; Cambridge, CLM-1396-PK) and oleate (0.1 mmol/L oleate dissolved in 0.1 % fatty acid-free BSA) for 0, 1, and 3 hr. At the end, cell plates were washed with 150 mmol/L ammonium acetate and then immediately liquid nitrogen was poured onto the plate and they were placed on dry ice (16). The incorporation of ^{13}C-labeled glucose (Sigma) into the glycolysis, pentose phosphate pathway (PPP) and tricloroacetate (TCA) cycle metabolites in cells was measured by the University of Michigan Regional Comprehensive Metabolomics Resource Core (http://mrc2.umich.edu) using liquid chromatography–mass spectrometry performed on an Agilent 6520 high-resolution Q-TOF (quadrupole–time of flight) instrument coupled with an Agilent 1200 HPLC system (Agilent Technologies, New Castle, DE), equipped with an electrospray source.

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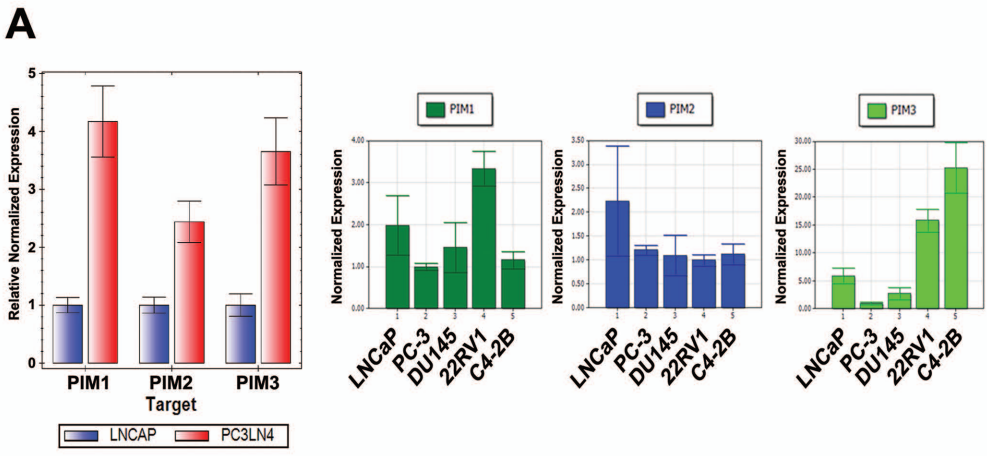
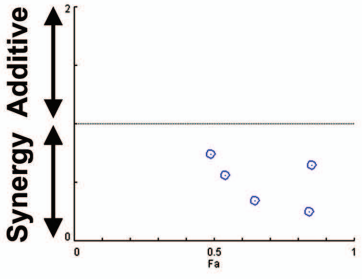
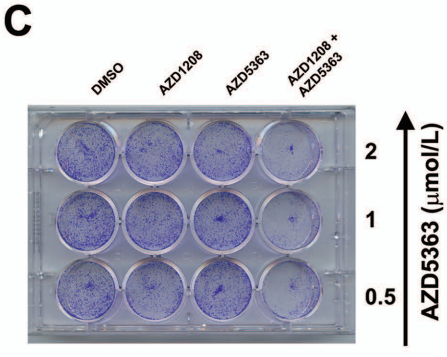
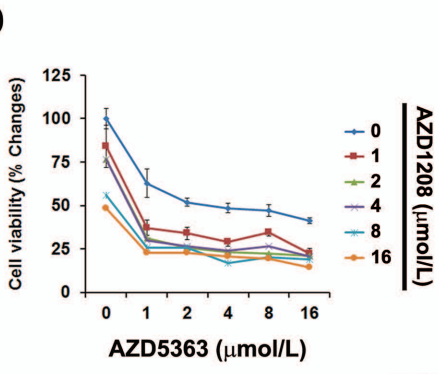


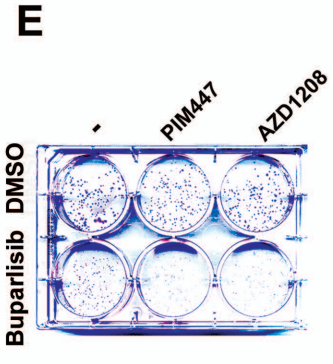
B

Buparlisib (μmol/L)	PIM447 (μmol/L)	Effect	CI
0.01	3	0.49	0.74090
0.1	3	0.54	0.56057
0.3	3	0.647	0.34744
1.0	3	0.84	0.24649
3.0	3	0.85	0.64616









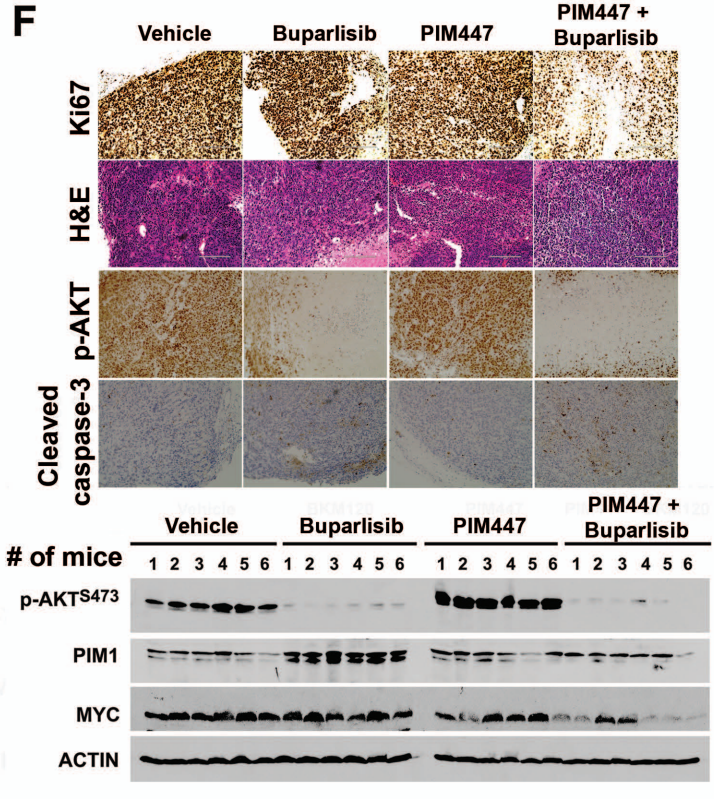
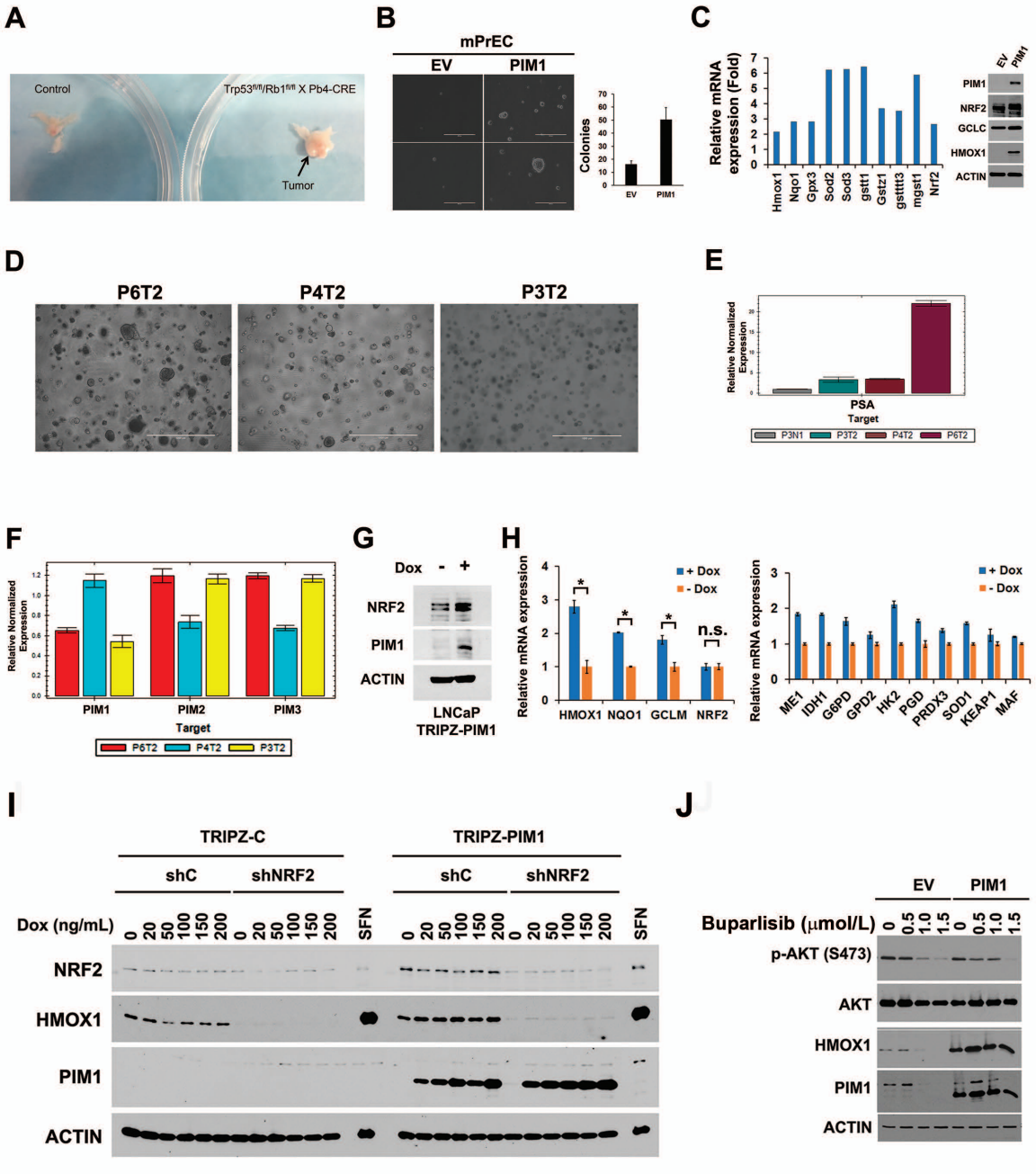


Fig. S1



(G) LNCaP cells expressing doxycycline-inducible PIM1 (TRIPZ-PIM1) were stimulated with 20 ng/mL doxycycline (+ Dox) or unstimulated (- Dox) for 72 hr. Immunoblots were probed with the listed antibodies.

(H) Quantitative real-time PCR (qRT-PCR) analysis of mRNA expression of NRF2 target genes in Dox-inducible PIM1 expressing LNCaP cells. The data shown is the mean of measurements ± SD (n=3).

(I) Immunoblot analysis of Dox-inducible PIM1 (TRIPZ-PIM1) or control vector (TRIPZ-C) PC-3 cells expressing control shRNA (shC) or shNRF2. Cells stimulated with 5 μmol/L sulforaphane (SFN) were positive sample.

(J) LNCaP/EV and LNCaP/PIM1 cells were treated with varying doses of BKM120 for 24 hr. Lysates of these cells were subjected to immunoblot analysis.

Fig. S2

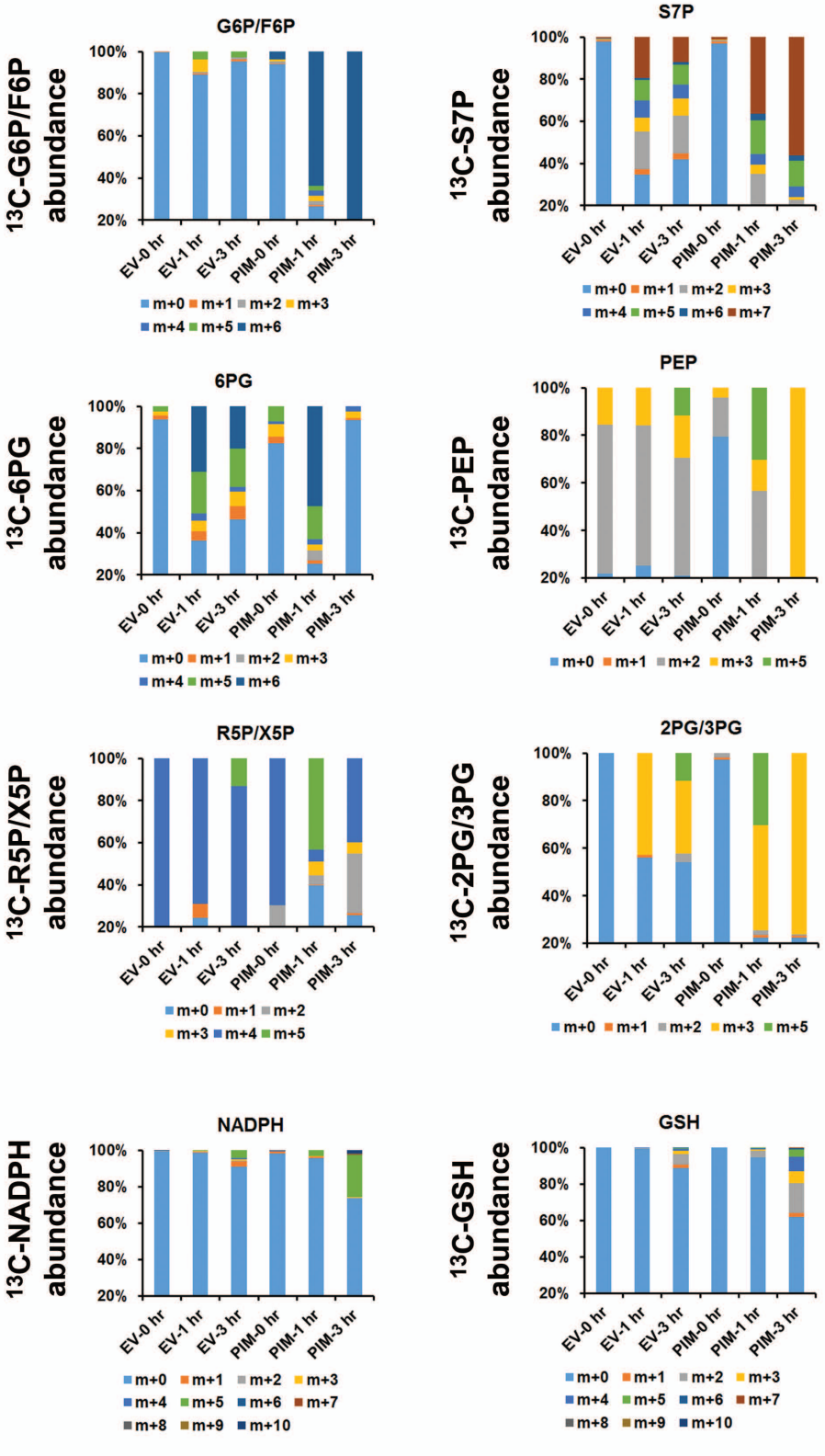


Fig. S3. PIM1 promotes glucose carbon flux in various metabolic pathways. Flux analysis of LNCaP/PIM versus LNCaP/EV cells treated with labeled 13C-glucose at the indicated times (duplicates are shown).

Fig. S3

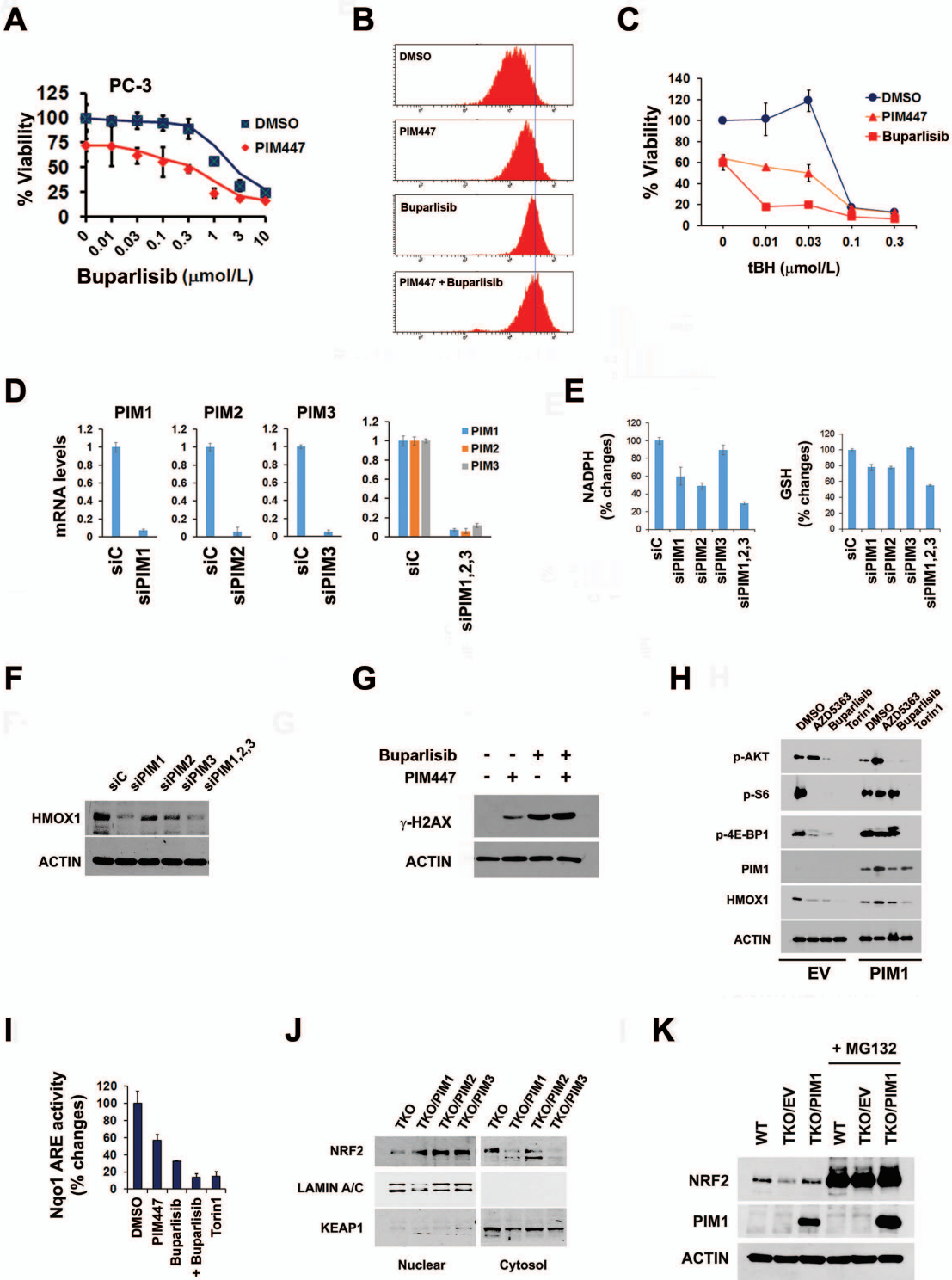

A
PC-3
% Viability
DMSO
PIM447
Buparlisib (μmol/L)
B
DMSO
PIM447
Buparlisib
PIM447 + Buparlisib
C
% Viability
DMSO
PIM447
Buparlisib
tBH (μmol/L)
D
PIM1
PIM2
PIM3
mRNA levels
siC
siPIM1
siPIM2
siPIM3
siPIM1,2,3
E
NADPH (% changes)
GSH (% changes)
F
HMOX1
ACTIN
G
Buparlisib
PIM447
γ-H2AX
ACTIN
H
DMSO
AZD5363
Buparlisib
Torin1
p-AKT
p-S6
p-4E-BP1
PIM1
HMOX1
ACTIN
EV
PIM1
I
Nqo1 ARE activity (% changes)
DMSO
PIM447
Buparlisib
PIM447 + Buparlisib
Torin1
J
TKO
TKO/PIM1
TKO/PIM2
TKO/PIM3
NRF2
LAMIN A/C
KEAP1
Nuclear
Cytosol
K
+ MG132
WT
TKO/EV
TKO/PIM1
NRF2
PIM1
ACTIN

Fig. S4

Supplementary Figure Legend

Figure S1. PIM and PI3K/AKT inhibitors synergizes to suppress PCa cell growth and survival.

(A) qRT-PCR analysis of PIM1, PIM2 and PIM3 mRNA expression in LNCaP versus PC3-LN4 cells. LNCaP cells were also compared to PC-3, DU145, 22RV1 and C4-2B.

(B) Combination index (CI) values after treatment with varying doses of PIM447 and Buparlisib by Combosyn software analysis.

(C) Colony focus formation was assessed by crystal violet staining. PC3-LN4 cells (100 cells) were seeded and then treated with for 7 days with the AK inhibitor, AZD5363, at the doses indicated either with or without 3 μmol/L AZD1208.

(D) Dose-response analysis of PC3-LN4 cells exposed to AZD5363, AZD1208, or the combination for 72 hr. Data shown are the means +/- S.D. (n=4).

(E) Colony focus formation of PC3-LN4 cells was assessed by crystal violet staining after PIM447 or AZD1208 treatment with or without Buparlisib addition. Representative images are shown.

(F) Representative images of PC3-LN4 xenograft tumors with H&E staining and immunohistochemistry for KI67, p-AKT^{S473} and cleaved capase-3 (scale bar,100 μm). Immunoblot analysis of p-AKT ^{S473}, PIM1 and c-MYC expression in the tumors that were excised from the mice at the end of treatment.

Figure S2. PIM1 induces expression of ROS scavengers by NRF2 transcription activity.

(A) Spontaneous occurrence of tumors in mouse prostate of Tp53 and Rb1 double knockout mouse (Trp53$^{loxP/loxP}$; Rb1$^{loxP/loxP}$; Pb-CRE4) at the age of 8 months.

(B) A representative image of anchorage independent growth of mouse prostate epithelial cancer (PrEC) cells expressing EV or PIM1. PrECs were placed in soft agar for 3 weeks prior to imaging.

(C) Fold induction of NRF2 mRNA by PIM1 expression compared to control PrEC/EV (triplicates are shown). Immunoblot analysis of total lysates of PrEC expressing EV or PIM1.

(D) Phase contrast images of patient-derived prostate cancer organoid cultures.

(E) qRT-PCR analysis of PSA mRNA expression in patient-derived organoids including three from patient tumors (P6T2, P4T2 and P3T2) and one from normal (P3N1) tissue.

(F) qRT-PCR analysis of PIM1, PIM2 and PIM3 mRNA expression in three patient-derived tumor organoids (P6T2, P4T2 and P3T2). Data shown are the mean ± S.D. (n=4).

(G) LNCaP cells expressing doxycycline-inducible PIM1 (TRIPZ-PIM1) were stimulated with 20 ng/mL doxycycline (+ Dox) or unstimulated (- Dox) for 72 hr. Immunoblots were probed with the listed antibodies.

(H) Quantitative real-time PCR (qRT-PCR) analysis of mRNA expression of NRF2 target genes in doxycycline-inducible PIM1 expressing LNCaP cells. The data shown is the mean of measurements ± S.D. (n=3).

(I) Immunoblot analysis of Dox-inducible PIM1 (TRIPZ-PIM1) or control vector (TRIPZ-C) PC-3 cells expressing control shRNA (shC) or shNRF2. Cells stimulated with 5 μmol/L Sulforaphane (SFN) were positive sample.

(J) LNCaP/EV and LNCaP/PIM1 cells were treated with varying doses of Buparlisib for 24 hr. Extracts of these cells were subjected to immunoblot analysis.

Figure S3. PIM1 promotes glucose carbon flux in various metabolic pathways.

Flux analysis of LNCaP/PIM versus LNCaP/EV cells treated with labeled ^{13}C-glucose at the time indicated (duplicates are shown).

Figure S4. PIM and PI3K/AKT inhibitors downregulate NRF2 antioxidant response leading to oxidative DNA damages.

(A) Dose-response analysis of PC-3 cells exposed to Buparlisib or DMSO for 72 hr in the absence or presence of 3 μmol/L PIM447. Data shown are the mean ± S.D. (n=4).

(B) Flow cytometry for detection of ROS. PC3-LN4 cells treated for 24 hr with DMSO, 1 μmol/L Buparlisib, 3 μmol/L PIM447 or the combination were incubated with H_2DCF-DA for 30 min prior to the analysis.

(C) Dose-response analysis of LNCaP/PIM1 cells exposed for 72 hr to tert-butyl hydroperoxide (tBH) alone with 1 μmol/L Buparlisib, 3 μmol/L PIM447, or DMSO. Data shown are the means ± S.D. (n=4).

(D) PC3-LN4 cells were transfected with siRNA targeting PIM1, 2, 3 or all three PIM isoforms and then analyzed for PIM1, 2, 3 mRNA expression by qRT-PCR (triplicates).

(E) After 72 hr transfection of PC3-LN4 cells with siRNAs used in (D), glutathione (GSH) and NADPH levels were determined (triplicates).

(F) After 72 hr transfection of PC3-LN4 cells with siRNAs used in (D), cell lysates were subjected to immunoblot analysis of HMOX1 expression.

(G) Immunoblot analysis of γ-H2AX (S139) expression. PC3-LN4 cells were treated with DMSO, 1 μmol/L Buparlisib, 3 μmol/L PIM447 or the combination for 24 hr.

(H) Immunoblot analysis of LNCaP/EV and LNCaP/PIM1 cells exposed to AZD5363 (2 μmol/L), Buparlisib (1 μmol/L), or Torin (0.5 μmol/L) for 24 hr.

(I) NRF2 promoter activity. PC3-LN4 cells were co-transfected with Nqo1-ARE firefly and *Renilla* luciferase reporters. Transfectants were treated with PIM447, Buparlisib or combination for 16 hr. Dual luciferase activities were measured, and are expressed as the mean ± S.D. (triplicates).

(J) Immunoblot analysis of NRF2, LAMIN A/C and KEAP1 expression in nuclear and cytosolic extracts of TKO and PIM1-, PIM2- or PIM3-expressing MEFs.

(K) Immunoblot analysis of NRF2 expression. WT, TKO and PIM1 MEF cells were treated with 10 μmol/L MG132 for 4 hr.