Supplemental Experimental Procedures

Materials

Reagents were obtained from the following sources: Antibodies to KEAP1, actin, and HRP-labeled anti-mouse were from Santa Cruz Biotechnology. Antibodies to phospho-T389 S6K1, phospho-T37/46 4EBP1, phospho-Smad2-S465/467, HRP-labeled anti-rabbit secondary antibody and rapamycin were from Cell Signaling Technology. Antibody to Ki-67 was from ThermoScientific. Antibody to BrdU was from Millipore. Antibody to c-Myc [Y69] was from Abcam. Collagen, Matrigel, invasion chambers, NuSerum and antibody to human p62 were from BD biosciences; Antibody to murine p62 was from ThermoScientific. TGFβ and murine IL-6 was obtained from PeproTech. FLAG antibody, Butylated hydroxyanisole (BHA), Gluthatione Ethyl Ester (GEE), torin1, testosterone, methanol, chloroform, norvaline, 5-bromo-2'-deoxyuridine, and the glutathione assay kit were from Sigma Aldrich. TGFβ inhibitor, SB431542 was from StemRD. DMEM and fetal bovine serum were from Hyclone; X-tremeGENE and Dnase were from Roche. OneComp ebeads, antibodies CD31-FITC (clone 390), CD45-FITC (clone 30-F11), Ter119-FITC (clone TER-119), Sca-1-APC (clone D7), CD49f-PE (clone eBioGoH3), and TGF beta ELISA Ready-SET-GO were from eBioscience. αSMA was from DAKO. Trypsin, Insulin and collagenase were from Gibco. Crystal violet and 10% formalin were from Fisher Scientific. RNeasy RNA extraction kit was from Qiagen. High-Capacity cDNA reverse transcription kit was from Applied Biosystems. iTaq Universal SYBR green supermix was from Biorad. NADP/NADPH quantification colorimetric kit was from Biovision. CM-H_2DCFDA was from Molecular Probes. Alexa Fluor 488 tyramide signal amplification kit was from Life Technologies.

Cell Culture

HEK293T and PC-3 were from American Type Culture Collection (ATCC). PNT2 was obtained from Sigma. The Myc-CaP cell line was a generous gift from Dr. Charles Sawyers.

WT and p62 KO stromal prostate fibroblasts were isolated from mouse prostate, as described previously (Tuxhorn et al., 2002). Murine TRAMP-C2Re3 cells were previously described (Olson et al., 2006). Stromal prostate fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (vol/vol) fetal bovine serum (FBS), 5% (vol/vol) NuSerum IV, 1% glutamine, 1% penicillin-streptomycin, 0.01 μM testosterone, and 25 μg ml^{-1} of insulin, in an atmosphere of 95% air and 5% CO_2 at 37°C. Cells were stimulated with inhibitors (rapamycin, 100 nM; torin1, 100 nM; SB431542, 10 μM) or antioxidants (BHA [100 □M] and GEE [0, 2.5, 5.0, 10 mM]) for 12 h.

Mammalian Lentiviral shRNAs and Retroviral Transduction

pWZL-Hygro (Addgene plasmid 18750, donated by Scott, L), pWZL-Blast-Myc (Addgene, plasmid 10674) (Boehm et al., 2005), FLAG pLJM1 RagB 99L (Addgene, plasmid 19315) (Sancak et al., 2008), TRC lentiviral shRNA targeting murine c-Myc (TRCN0000234923) was obtained from Sigma Aldrich. TRC lentiviral shRNA targeting murine IL-6 (TRCN0000067548) was obtained from OpenBioSystems, Thermo Scientific. Actively growing HEK293T cells were co-transfected with shRNA-encoding plasmids, psPAX2 (Addgene, plasmid 12260) and pMD2.G (Addgene, plasmid 12259) packaging plasmids using X-tremeGENE transfection reagent. For retroviruses, pWZL-encoding plasmids were transfected into actively growing Phoenix cells with X-tremeGENE transfection reagent to produce viral particles, which were used to infect cells. Virus-containing supernatants were collected 48 and 72 hr post-transfection and filtered to remove cells. Target cells were infected in the presence of 8 μg ml^{-1} polybrene. Cells were selected with puromycin, hygromycin, or blasticidin after infection.

Gene-expression analyses

RNA was isolated using RNeasy mini kit from Qiagen (Valencia, USA). Total RNA (1 µg) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using iTaq Universal SYBR green supermix (Biorad) on a Biorad CFX96 detection system. PCR primers were designed using the online primer tool Primer3 and purchased from Integrated DNA Technologies. 18S was used as the housekeeping gene for normalization, with a melting curve performed after each reaction.

Histological analysis

Excised prostates were fixed in 10% formalin, dehydrated, and embedded in paraffin. Samples were sectioned at 5 µm and subjected to staining with hematoxylin and eosin. For immunohistochemistry, samples were dewaxed and stained with anti-αSMA (clone 1A4, 1:400 dilution), with a ready to use anti-Ki-67 (1:10 dilution), or with anti-BrdU (clone PRB-1, 1:200 dilution). Secondary biotinylated antibody and ABC complex (Vectastain Elite, Vector Labs) were used for detection following manufacturer's instructions. Human prostate tissue microarrays (TMA) were obtained from US Biomax. This study had Institutional Review Board exempt status due to de-identification of the human samples. TMA were stained with anti-p62. TMA slides were scanned and imaged using the Scanscope XT system (Aperio) and Aperio ImageScope software, respectively. The scoring of staining intensity was done by a pathologist (E.C) in a blinded fashion. For immunofluorescence, sections were deparafinized as described above and incubated with anti-p62 antibody (1:100) overnight at 4°C. Alexa Fluor 488 tyramide signal amplification kit for p62 antibody was used. Stained sections were examined under an inverted laser scan microscope (LSM 710 NLO, Zeiss, Germany).

FACs-sorted murine stromal isolation

Prostates from 12-week-old mice were harvested and dissected. Prostate cell isolation has been described previously (Lukacs et al., 2010). Cells were stained with fluorescently labeled lineage markers (CD31-FITC, CD45-FITC, and Ter119-FITC), CD49f-PE, and Sca-1-APC. Unstained prostate cells were used as the control to set the background fluorescence. Single-color stained OneComp ebeads (eBioscience) were used for FACS Aria cell sorter equipment compensation. FMO compensations were also performed. Stromal cells were sorted at 4°C into FACS Collection media using the FACS Aria cell sorter and FACS Diva software (BD Biosciences). Stromal cell fractions were sorted based on the Lin^-, $Sca\text{-}1^+$, $CD49f^-$ profile.

Determination of ROS Levels

Cells were washed once with warm PBS and were incubated with 10 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Molecular Probes) in warm PBS. After 30 min at 37°C, cells were washed, trypsinized, and re-suspended in PBS. Detection of ROS levels was carried out through flow cytometric analyses using a FACS Canto flow cytometer (Becton-Dickinson) with FlowJo software (BD Biosciences).

Measurement of NADPH/NADP ratio and GSH intracellular levels

Fibroblast cells were washed with cold PBS and extracted with NADP/NADPH extraction buffer. Quantification was carried out using the NADP/NADPH quantification colorimetric kit (Cat. No. K347-100, BioVision) according to the manufacturer's protocol. GSH was measured using the Glutathione Assay Kit (Cat No. CS0260, Sigma Aldrich). The plate reader was set to 412 nm with kinetic reads at 1 min intervals for 5 min. At least three independent measurements were carried out.

ELISAS

Culture supernatants were harvested, clarified by centrifugation, and frozen for the subsequent determination of IL-6 concentration by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BD Bioscience). TGFβlevels in culture supernatants were quantified using the Human/Mouse TGF beta 1 ELISA Ready-SET-Go! (eBioscience).

Invasion Assay

PCa cells (5 x 10^4) were plated onto 8 µm-pore Matrigel Invasion chambers (BD Biosciences) in 24-well plates. Conditioned media from WT, p62 KO, IL-6 KO, p62/IL-6 DKO, p62/IL-6 DKO + IL-6, WT shNT, p62 KO shNT, p62 KO shIL-6 fibroblasts were added to the lower chamber as a chemoattractant. Cells were allowed to invade for 22 hr at 37°C. Non-invading cells in the upper surface were removed and those on the lower surface fixed in methanol and stained with crystal violet. Cells in at least five randomly selected fields from each of three experiments were counted.

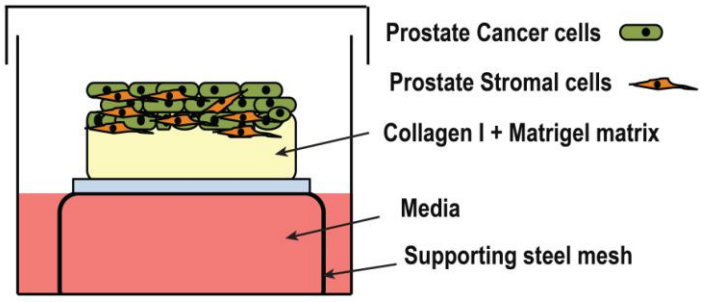
Co-culture invasion assays

Invasion assays were performed as described above with a few modifications. WT shNT or WT shMyc fibroblast (7.5 x 10^4) were seeded in TC-treated 24-well plates and allowed to attach for 6 hr before PCa cells were seeded on top of the invasion chamber. The chamber was then placed in the pre-seeded 24-well plate. Invasion was quantified after 22 hr.

Organotypic cultures

3D air liquid organotypic culture cultures have been used extensively in skin (Gaggioli et al., 2007), esophageal (Okawa et al., 2007), pancreas (Coleman et al., 2014), colon (Henriksson et al., 2011), breast (Chioni and Grose, 2012) and more recently in prostate cancer (Kim et al.,

2013), to determine the mechanisms of tumor invasion and the role of the stromal compartment (Chioni and Grose, 2008). To study the interaction between the prostate cancer cells and surrounding stroma to impact cancer cell proliferation and invasion, a 3D air liquid organotypic culture was set up as previously described (Kim et al., 2013) (Coleman et al., 2014). Briefly, gels were composed of one ml of a mixture of 1.75 volumes of Matrigel, 5.25 volumes of collagen type I, 1 volume of 1x DMEM, 1 volume of 10x DMEM, and 1 volume of filtered FBS. The mixture was plated onto 24-well plates coated with diluted collagen type I. Gels were allowed to equilibrate with 1 ml of 1x DMEM overnight at 37°C. 5 x 10^5 cells PCa cells and prostate stromal cells (50:50) were then seeded on top of the matrix as shown in the scheme below. For organotypic cultures with macrophages, whole bone marrow cells were obtained from WT and p62 KO mice and differentiated as previously described (Lee et al., 2010). Differentiated macrophages were embedded into the organotypic gels and PCa cells were seeded on top of the gel. Gel rafts were placed onto collagen-coated nylon sheets and lifted using a sterile supporting steel mesh to set up a raised air-liquid culture. Normal medium was changed in alternate days and organotypic cultures were allowed to grow for 14 days. Afterwards, organotypic gels were harvested, fixed in 10% neutroal buffered formalin, bisected and embedded in paraffin. H&E stained sections were analyzed with a Zeiss light microscope supplemented with Axiovision40 software. Quantification of the invasion assays was performed as described previously (Nystrom et al., 2005) using ImageProPlus software.



Schematic representation of air-liquid organotypic cultures

In vivo orthotopic tumor assay

Murine TRAMP-C2Re3 prostate carcinoma cells (5×10^4) were injected orthotopically into the prostates of WT, p62 KO, and p62/IL-6 DKO mice and harvested after 60 days, as previously described (Olson et al., 2006).

Xenograft experiments

Cell mixtures (1×10^6 cells) of equal ratios of PCa cells with fibroblasts in a 100 µl of BD Matrigel (BD Biosciences) were injected into the flanks of WT mice. Tumors were allowed to grow for 6 weeks. Tumor volume was measured every week.

Metabolic Extraction

For labeling experiments, 2×10^5 cells were seeded in 6-well plates. After 16 hr of incubation, cells were washed with PBS and incubated with DMEM, 10% dialyzed serum, and either 4 mM [U-$^{13}C_5$]glutamine and 25 mM unlabeled glucose or [1,2$^{13}C_2$]glucose and unlabeled glutamine. Spent medium from labeled cells was collected and analyzed for glucose, glutamine consumption, and lactate production using the YSI2950 analyzer. Cells were rinsed in ice-cold saline solution, and then ice-cold 100% HPLC-grade methanol was added to the cells. An equal volume of water containing norvaline as standard was added to the plate. Cells were scraped and mixed with ice-cold chloroform. Samples were vortexed for 10 min at room temperature and centrifuged at 3000 g for 10 min. Polar metabolites were separated and evaporated in a refrigerated vacuum centrifuge.

Derivatization, gas chromatography/mass spectrometry (GC/MS) analysis, and flux calculations

Polar metabolites were derivatized to form methoxime-tBDMS derivatives by first dissolving the evaporated samples in 20 µl of 2% (w/v) methoxylamine hydrochloride (MP Biomedicals, OH) in pyridine and incubating at 37°C for 60 min. Samples were then silylated by addition of 30 µl of N-tertbutyldimethylsily-N-methyltrifluoroacetamide (MTBSTFA) with 1% tert-butyldimethylchlorosilane (TBDMCS) (Regis Technologies, IL) and incubated at 37°C for 45 min. Samples were centrifuged at 14,000 rpm for 5 min and clarified supernatant was transferred to GC sample vials for analysis. Derivatized samples were analyzed by GC-MS using a DB-35MS (Agilent J&W Scientific) installed in an Agilent 7890A GC interfaced with an Agilent 5975C MS operating in electron impact mode scanning over the range 100-650 m/z. The GC method and metabolite peaks used for integration have been previously described (Grassian et al., 2011) (Metallo et al., 2012). Uptake and secretion fluxes of glucose, glutamine, and lactate were then calculated using an exponential growth model. PPP flux was determined by multiplying lactate secretion flux by the ratio of M1 to M2 lactate labeled from [1,2-$^{13}C_2$]glucose as described above.

Array and Gene Set Enrichment Analysis

Microarray studies were performed in the Genomics and Microarray Laboratory at the Department of Environmental Health, University of Cincinnati Medical Center. Briefly, total RNA was extracted from six independent orthotopic tumors from WT and p62 KO mice and hybridized on Affymetrix mouse ST 1.0 microarrays. Scanning of the images and the first pass processing of probe-level fluorescence intensities was performed using the Microarray Suite 5.0 software (MAS 5.0; Affymterix, Santa Clara, CA). The data was normalized, and the calculation of the gene-specific summary measures was performed by the robust multi-array average

(RMA) procedure (Irizarry et al., 2003) based on the Entrez gene-centric probe set definitions provided by the University of Michigan "brainarray" group (Dai et al., 2005). Statistical significance of genes differentially expressed between WT and p62 KO orthotopic tumors were assessed using Empirical Bayes linear model (Sartor et al., 2006). Gene set enrichment analysis was performed using GSEA v2.0.14 software (http://www.broadinstitute.org/gsea/index.jsp) with 5000 gene set permutations using the metric Signal-to-Noise ratio (S2N) and the collection C5.bp.v3.1.symbols or C2.all.v4.0.symbols (Subramanian et al., 2005Subramanian et al., 2005). Probe sets were collapsed using MoGene_1_0_st.chip. Meta-analyses to identify overlapping and associated genes with publically available data set were performed using the NextBio^{TM} (NextBio, Cupertino, CA) on-line search engine (www.nextbio.com). Differentially expressed genes were further analyzed with the Ingenuity Pathways Analysis program (http://www.ingenuity.com/index.html).

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