Published in final edited form as:

Cell Signal. 2018 June ; 46: 1–14. doi:10.1016/j.cellsig.2018.02.008.

p66Shc Regulates Migration of Castration-Resistant Prostate Cancer Cells

Matthew A. Ingersoll¹, Yu-Wei Chou^{1,2}, Jamie S. Lin^{1,3}, Ta-Chun Yuan^{1,4}, Dannah Miller¹, Yan Xie⁵, Yaping Tu⁵, Rebecca E. Oberley-Deegan¹, Surinder K. Batra^{1,6}, and Ming-Fong Lin^{1,6,7,8,*}

¹Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska, United States of America

²Tissue Bank and BioBank, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, 833 Taiwan

³Section of Nephrology, Department of Internal Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, United States of America

⁴Department of Life Science, National Dong Hwa University, Hualien, 974 Taiwan

⁵Department of Pharmacology, Creighton University School of Medicine, Omaha, Nebraska, United States of America

⁶Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska, United States of America

⁷Section of Urology, Department of Surgery, University of Nebraska Medical Center, Omaha, Nebraska, United States of America

⁸College of Pharmacy, Kaohsiung Medical University, Kaohsiung, 807 Taiwan

Abstract

Metastatic castration-resistant (CR) prostate cancer (PCa) is a lethal disease for which no effective treatment is currently available. p66Shc is an oxidase previously shown to promote androgen-independent cell growth through generation of reactive oxygen species (ROS) and elevated in clinical PCa and multiple CR PCa cell lines. We hypothesize p66Shc also increases the migratory activity of PCa cells through ROS and investigate the associated mechanism. Using the transwell assay, our study reveals that the level of p66Shc protein correlates with cell migratory ability across several PCa cell lines. Furthermore, we show peroxide treatment induces migration of PCa cells that express low levels of p66Shc in a dose-dependent manner, while antioxidants inhibit migration. Conversely, PCa cells that express high levels of endogenous p66Shc or by cDNA

*Corresponding Author: Ming-Fong Lin, Ph. D., Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, USA, TEL: (402) 559-6658, FAX: (402) 559-6650, mlin@unmc.edu (MFL).

Conflicts of Interest: None.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

transfection possess increased cell migration which is mitigated upon p66Shc shRNA transfection or expression of oxidase-deficient dominant-negative p66Shc W134F mutant. Protein microarray and immunoblot analyses reveal multiple proteins, including ErbB-2, AKT, mTOR, ERK, FOXM1, PYK2 and Rac1, are activated in p66Shc-elevated cells. Their involvement in PCa migration was examined using respective small-molecule inhibitors. The role of Rac1 was further validated using cDNA transfection and, significantly, p66Shc is found to promote lamellipodia formation through Rac1 activation. In summary, the results of our current studies clearly indicate p66Shc also regulates PCa cell migration through ROS-mediated activation of migration-associated proteins, notably Rac1.

Graphics Abstract

Keywords

Prostate Cancer; p66Shc; Reactive Oxygen Species; Cell Migration; Castration-Resistant; Rac1

1. Introduction

Prostate cancer (PCa)¹ remains the most commonly diagnosed solid tumor and is the third leading cause of cancer-related death in United States men [1,2]. Localized PCa is generally not lethal and effectively treated by means of surgery or radiation therapy. It is not until the tumor metastasizes to vital organs that it becomes life-threatening. While metastatic PCa is initially suppressed by androgen-deprivation therapy (ADT), many PCa patients relapse and develop the lethal castration-resistant (CR) form of the disease for which there are no effective treatments. Thus, new therapeutic targets must be identified. Furthermore, molecules involved in the process of PCa cell migration and proliferation have the potential to be promising biomarkers as well as remedial targets.

p66Shc, a 66 kDa proto-oncogene Src and collagen homologue protein, exhibits oxidase activity and is one of three members of the Shc family, including p52Shc and p46Shc [3,4]. p66Shc differs from the other Shc members in numerous ways. For example, p66Shc protein

¹**Abbreviations:** Ab, antibody; ADT, androgen deprivation therapy; AI, androgen-independent; AR, androgen receptor; AS, androgen-sensitive; CA, constitutively active; cPACP, cellular prostatic acid phosphatase; CR PCa, castration-resistant prostate cancer; DHT, 5 α -dihydrotestosterone; DMSO, dimethyl sulfoxide; DN, dominant-negative; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; NAC, N-acetylcysteine; PCa, prostate cancer; PSA, prostate-specific antigen; ROS, reactive oxygen species.

level is, in part, regulated through post-translational stabilization via steroids, including androgens, which play a critical role in the process of PCa development [4–6]. While other Shc members are ubiquitously expressed, p66Shc protein level is higher in epithelial cells compared to stromal tissues and has both cytosolic and mitochondrial localization. Structurally, p66Shc protein has an additional N-terminal CH2 domain which contains serine phosphorylation sites that can regulate p66Shc activity [3,4,7]. For instance, serine-36 phosphorylation by ERK/JNK in response to stress has been observed to induce translocation of p66Shc from the cytosol into the mitochondria [8, 9]. In the mitochondrial intermembrane space, p66Shc binds and oxidizes cytochrome C, uncoupling the electron transport chain and inducing production of reactive oxygen species (ROS) [10]. Additionally, p66Shc has been reported to induce Rac1 activation in mouse fibroblasts and breast cancer, though their interaction in PCa is unknown [11]. Rac1 is a key regulator of cell motility and can also increase ROS production via interaction with NOX family of NADPH oxidases [12]. Furthermore, Rac1 protein level is higher in androgen-sensitive prostate cancer compared to benign epithelium, and further increases as tumors progress to castration-resistance [13]

ROS molecules are natural by-products of cellular respiration and contribute to essential signaling pathways; local ROS production stimulated by external growth factors and hormones mediates the transduction of signals from the cell membrane to the nucleus through the oxidation and reduction of proteins [14,15]. However, when ROS molecules are produced in excess, they also readily oxidize a number of cellular targets causing DNA, lipid, and protein damage, which facilitate various mutations and cancer development [16]. Furthermore, ROS is known to regulate processes like angiogenesis, cell adhesion, proliferation, and migration, all of which are critical to cancer metastasis [17–20]. Results of several studies have indicated oxidation of protein tyrosine phosphatases mediated by increased cellular levels of ROS can shown that cell migration in mouse fibroblasts [21,22].

p66Shc protein levels have been found elevated in prostate, thyroid, ovarian, and colon adenocarcinomas compared to corresponding non-cancerous cells [6,23–25]. In androgen-sensitive PCa cells, the p66Shc protein is stabilized by androgens, and thus mediates androgen-induced growth via generation of ROS [6,26]. Moreover, p66Shc protein is elevated in multiple androgen-independent (AI) PCa cell lines which correspond to advanced metastatic CR PCa. For example, in the LNCaP PCa cell line, androgen-sensitive (AS) LNCaP cells (LNCaP-AS/C-33) possess relatively low levels of p66Shc protein [6,26,27]. In contrast, as LNCaP cells progress to androgen-independence, meaning they maintain a similar growth rate regardless of the presence of external androgens (LNCaP-AI/C-81), these cells have much higher levels of p66Shc protein and exhibit many biochemical properties seen in clinical CR PCa [7,26–29]. These same phenomena are also observed in human MDA-PCa2b PCa cells, which become AI upon passage and possess increased levels of p66Shc [26,30–31]. Nevertheless, while results of intensive studies have clearly shown that elevated p66Shc protein via ROS production promotes cell proliferation and supports AI PCa cell progression [7,26,32,33,34], it is not known if p66Shc also regulates biological functions other than cell growth. The current study is the first to report that p66Shc also mediates PCa cell migration, a vital process for tumor metastasis, and we further elucidate its signaling mechanism.

2. Materials and Methods

2.1 Materials

RPMI 1640 medium, Keratinocyte SFM medium, DMEM medium, gentamicin, anti-p66Shc (#180S0105A) Ab, rhodamine phalloidin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA). FBS and charcoal-treated FBS were obtained from Atlanta Biologicals (Lawrenceville, GA). Molecular biology-grade agarose was procured from Fisher Biotech (Fair Lawn, NJ). Protein molecular weight standard markers, acrylamide, and Bradford protein assay kit were purchased from Bio-Rad (Hercules, CA). Anti-CDC25B (#D2810, 1:1000), anti-cyclin B₁ (#K1907, 1:1000), anti-phospho-ErbB-2 (Y1221/2) (#B2212, 1:1000), anti-ErbB-2 (#E3110, 1:1000), and horseradish peroxidase-conjugated anti-mouse (#C2011, 1:5000), anti-PAcP (#D0209, 1:1000), anti-PYK2 (#F061, 1:1000), anti-Rac1 (#G1905, 1:1000), anti-rabbit (#D2910, 1:5000), anti-goat (#J0608, 1:5000) IgG Abs, and AKT inhibitor (MK2206) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-AKT (Ser473) (#GA160, 1:1000), anti-AKT (#C1411, 1:2000), anti-FOXM1 (#5436S, 1:500), anti-HA-Tag (#C29F4, 1:3000), anti-phospho-mTOR Ser2448 (#5536S, 1:1000), and anti-mTOR (#2972S, 1:1000) Abs were from Cell Signaling Technology (Beverly, MA). Anti-GTP-Rac1 (#G052YWF2, 1:1000) Ab was obtained from New East Biosciences (Malvern, PA). Anti-phospho-PYK2 Y402 (#CDRO0114121, 1:1000) Ab was obtained from R&D Systems (Minneapolis, MN). Anti-Shc (#06-203, 1:5000) Abs was obtained from Upstate Biotech. Inc. (Lake Placid, NY). Anti-β-actin (#99H4842, 1:10000) Ab, FOXM1 inhibitor (FDI-6), Rac1 inhibitor (EHop-016), PYK2 inhibitor (PF-431396), mTOR inhibitor (Rapamycin), N-acetyl-cysteine (NAC), hydrogen peroxide (H₂O₂) was procured from Sigma (St. Louis, MO). DAPI Hard-Mount Medium was obtained from Vector Laboratories (Burlingame, CA). ErbB-2 inhibitor (AG879), PI3K inhibitor (LY-294002), and ERK inhibitor (PD-9805) were obtained from Calbiochem (San Diego, CA).

2.2 Cell Culture

Human prostate carcinoma cell lines LNCaP, MDA-PCa2b, PC-3, and DU145 cells were originally purchased from the American Type Culture Collection (Rockville, MD, USA). LNCaP, PC-3, and DU145 were routinely maintained in RPMI 1640 medium containing 5% FBS, 2 mM glutamine, and 50 µg/ml gentamicin [26–29,35]. MDA PCa2b cells were maintained in BRFF-HPC1 medium containing 20% FBS, 2 mM glutamine and 50 µg/ml gentamicin [31,38]. As reported previously, we established LN-AI (C-81) and MDA-AI cells which obtain many biochemical properties of clinical CR PCa including the expression of functional AR as well as PSA secretion and rapid cell proliferation in androgen-depleted conditions [27–29,35]. LNCaP-AI cells also possess the enzymatic capacity to synthesize androgens from cholesterol [28]. Most importantly, both AI cell lines have elevated basal growth rates as well as increased levels of p66Shc protein compared to their respective AS cell lines [7,26].

2.3 Transwell Migration Assay

Cell migration was assessed via Boyden Chamber transwell assay as described previously [31,37]. Cells were plated at a density of 5×10^4 cells into the upper chamber of 24-well

plate transwell inserts and allowed to migrate for 24 hours. In experiments with small molecule inhibitors, inhibitor compounds were added to the bottom chamber for a final concentration of their IC₅₀ in LNCaP cells prior to the addition of cells. After a 24-hour incubation, cells were stained with 0.2% crystal violet solution in 50% methanol and cells remaining in the upper chamber were removed via cotton swab. Cells which had migrated to the lower chamber were counted at 40× magnification under a microscope. For experiments using small molecule inhibitors, to distinguish cell migration from cell growth, the results were normalized to growth inhibition in which 24-hour change in cell migration was divided by 24-hour change in cell growth.

2.4 Cell Growth Assay

Cells were seeded in regular culture medium and allowed to attach for 3 days, after which cells were treated with small-molecule inhibitors at their IC₅₀ in LNCaP cells or solvent DMSO alone and allowed to grow for 24–72 hour as noted. Cells were harvested via trypsinization and live cell numbers were counted by Trypan Blue dye exclusion assay using a Cellometer Auto T4 Image-based cell counter (Nexcelom, MA, USA) [7,37].

2.5 Immunoblot Analysis

All cells were rinsed with ice-cold HEPES-buffered saline, pH 7.0, harvested via scraping, and lysed in ice-cold lysis buffer containing protease and phosphatase inhibitors. Total cellular lysates were prepared as previously described [38–39]. The protein concentration of the supernatant was determined using a Bio-Rad Bradford protein-assay. For immunoblotting, an aliquot of total cell lysate was electrophoresed on SDS-polyacrylamide gels (7.5%–12%). After being transferred to nitrocellulose membrane, membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 for 30 minutes at room temperature. Membranes were incubated with the corresponding primary Ab overnight at 4°C. Membranes were then rinsed and incubated with the appropriate secondary Ab for 60 minutes at room temperature. Proteins of interest were detected by an ECL reagent kit and β-actin was used as a loading control. The intensity of the protein bands were analyzed with NIH ImageJ software [38].

2.6 Transfection

For transient transfection experiments, LNCaP cells were plated at a density of 1×10^4 cells per cm² and transfected using Lipofectamine and Plus reagents. Five hours after transfection, the cells were fed with RPMI media containing 10% FBS for 24 hrs. The cells were then used for transwell assays and whole cell lysates harvested for immunoblot analysis. Stable subclones of LNCaP cells overexpressing p66Shc were established as described previously [26]. DN (T17N) and CA (G12V) Rac1 cDNA were prepared as previously described [40]. For knockdown of p66Shc expression, transient transfection of pSUP-p66 plasmid-based small interfering RNA system targeted against the CH2 domain was used for cDNA transfection as described previously [7,26].

2.7 Protein Microarray

Cell lysates were prepared from an equally mixed population of stable p66Shc cDNA-transfected subclones and V1 vector-alone control cells as previously described and sent to Kinexus Protein Profiling Services (Vancouver, BC) where the company performed analysis via the KAM-900P microarray. The Kinex™ KAM-900P microarray uses validated polyclonal and monoclonal antibodies and consists of 2 identical fields, allowing two samples to be analyzed side by side at a time. Within each field, there are 16 subgrids of 11 × 10 spots. Diameters of spots average between 120 and 150 μm. A complete list of unique target proteins and phospho-sites tracked in the Kinex™ KAM-900P antibody microarray is available for downloading from the Kinexus website (www.kinexus.ca).

An aliquot of 100 μg of lysate protein from each sample is covalently labeled with biotin. Free biotin molecules are then removed at the completion of labeling reactions by gel filtration. After blocking non-specific binding sites on the array, an incubation chamber is mounted onto the microarray to permit the loading of 2 samples (normally one control and one matching treated sample) side by side on the same chip and prevent mixing of the samples. Following sample incubation, unbound proteins are washed away and the array is then probed with anti-biotin antibody that is labelled with a proprietary fluorescent dye combination. Each array produces a pair of 16-bit images, which are captured with a Perkin-Elmer ScanArray Reader laser array scanner (Waltham, MA).

Signal quantification is performed with ImaGene 9.0 from BioDiscovery (El Segundo, CA) with predetermined settings for spot segmentation and background correction. Z scores are calculated by subtracting the overall average intensity of all spots within a sample from the raw intensity for each spot, and dividing it by the standard deviations (SD) of all of the measured intensities within each sample. For ease of reading, results are reported as percent change from control (%CFC).

2.8 F-Actin Staining

Cells were plated on sterile round coverslips at 3×10^4 cells per coverslip and allowed to attach for 24 hours. Cells were then washed with pre-warmed phosphate-buffered saline and fixed with a 3.7% formaldehyde solution for 10 minutes at room temperature. Cells were then washed, permeabilized with 0.1% Triton X-100 solution for 5 minutes, and again washed before blocking with 1% BSA solution for 30 minutes. At this time, cells were stained with fluorescent phallotoxins, which directly binds to F-actin, in 1% BSA for 20 minutes followed by washing. To stain HA-tagged DN Rac1 transfected cells, cells were then incubated with primary anti-HA-tag Ab in 1% BSA for 1 hour, followed by washing and secondary Ab incubation for 30 minutes and a final wash. Coverslips were then mounted using VectorShield hard-mount medium containing DAPI stain and allowed to set overnight before images were captured via confocal microscopy [30]. The relative area of cell lamellipodia to total cell area was semi-quantified using NIH ImageJ software. Results were repeated in three separate experiments in which 20 cells were quantified (For a total of 60) for each cell-line/treatment [38].

2.9 ROS Analysis

Changes in cellular ROS levels upon transfection of LNCaP-AI or p66Shc cDNA subclones with p66Shc shRNA or redox-inactive dominant-negative mutant W134F cDNA were determined via DCF-DA dye analysis [32,37]. 24 hours after transfection, cells were harvested and incubated with phenol red-free medium containing 20 μ M DCF-DA dye in darkness for 30 minutes. Determination of cell cycle distribution and DCF-DA fluorescence was carried out using the Becton-Dickinson fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA, USA) at the UNMC Flow Cytometry Core Facility.

2.10 Statistical Analysis

Each set of experiments are conducted in duplicate or triplicate as specified in the figure legend, and experiments are repeated independently at least three times, denoted as $n=3\times 3$. All results are presented as mean $\pm$ standard error measurement. Correlation coefficient r was calculated using Microsoft Excel. Statistical significance was determined using a paired two-tailed student-t test assuming unequal variance where appropriate unless otherwise stated. $p<0.05$ was considered statistically significant.

3. Results

3.1 p66Shc Protein Level Correlates with Androgen Independence and PCa Cell Migration

We examined p66Shc protein level across multiple PCa cell lines via immunoblot analysis as well as their migratory potential, an important step of metastasis, using the Boyden Chamber transwell assay. As shown in Figure 1A, LNCaP-AS cells have relatively low levels of p66Shc protein and possess correspondingly low migratory activity when compared to androgen receptor (AR)-negative AI PC-3 and DU145 cells that exhibit higher levels of p66Shc as well as migration. Since most of advanced, metastatic clinical PCa still express functional AR, we investigated the role of p66Shc in regulating cell migration using AR-positive PCa progression models in LNCaP and MDA PCa2b cells. As shown in Figures 1B–C, across both progressive PCa cell models, p66Shc protein level and migratory activity are elevated in AI cells compared to respective AS cells. Together, the data clearly supports the hypothesis that p66Shc protein level correlates with the AI/CR phenotype as well as cell migratory activity.

3.2 p66Shc Promotes PCa Cell Migration

To determine p66Shc's role in PCa cell migration, the migratory activities of parental LNCaP-AS, vector-alone transfected (V1) cells, and p66Shc cDNA-transfected S32 and S36 stable subclones were investigated. As shown in Figure 2A, an increase in p66Shc protein resulted in increased migratory activity. A reversal experiment was then conducted in which stable p66Shc cDNA-transfected subclones were transiently transfected with p66Shc shRNA for analysis. For this set of experiments, to diminish the possible effects of variation between stable subclones, three individual p66Shc-stable subclones (S31, S32, and S36) were combined in equal number prior to shRNA transfection. As shown in Figure 2B, while mixed subclones exhibited elevated migratory activity, upon shRNA transfection both p66Shc protein levels and cell migration were decreased in a dose-dependent manner.

Correspondingly, the overall ROS levels (RFU) were decreased as measured by DCF-DA analysis. Moreover, LNCaP-AI (C-81) cells, which possess relatively high levels of p66Shc protein as well as migratory activity (Fig. 1B), were transiently transfected with increasing amounts of p66Shc shRNA and then analyzed via western blot and transwell assays. As exhibited in Figure 2C, upon shRNA transfection both p66Shc protein levels and cell migratory activity decreased in a dose-dependent manner, in addition to the decreased levels of cellular ROS. Taken together, the data shows p66Shc protein as well as ROS level is associated with cell migratory activity and thus indicates p66Shc can directly regulate PCa cell migration.

3.3 p66Shc Promotes PCa Cell Migration via Increasing ROS Production

p66Shc is an authentic oxidase and promotes ROS generation, at least in part, via oxidation of cytochrome C in the mitochondria. Moreover, p66Shc-mediated ROS production induces PCa cell proliferation [32]. To determine whether p66Shc also promotes cell migration through ROS production, we first investigated the effects of ROS on PCa migration. We examined the effect of ROS on cell migration via transwell assay utilizing LNCaP-AS and MDA-AS cells which possess low migratory potential (Fig. 1B & 1C). As shown in Figure 3A, hydrogen peroxide treatment increased cell migration over 24 hours in a dose-dependent manner with 10 μ M inducing the optimal effect on both cell lines. To further investigate the effect of ROS on cell migration, competitive inhibition transwell experiments were conducted on the same cell lines using 10 μ M hydrogen peroxide, 10mM antioxidant N-acetylcysteine (NAC), or combined treatments. While hydrogen peroxide treatment significantly increased cell migration, Figure 3B demonstrate its enhanced effect is mitigated by combination treatment with NAC. NAC alone also reduces the basal migratory activity of both cell lines (Fig. 3B).

ROS can directly increase cell migration, therefore we determined whether p66Shc promotes cell migration via ROS production. We conducted 24-hour transwell assays using p66Shc stable subclones, i.e., S31, S32 and S36, and the corresponding vector-alone control cells were treated with 10mM NAC (Fig. 3C). NAC treatment completely mitigated the elevated migratory activities of the subclones, indicating ROS generation is a key mechanism of p66Shc-induced migration. To further explore p66Shc's reliance on ROS to promote cell migration, LNCaP-AI cells were transiently transfected with the redox-deficient DN mutant p66Shc W134F cDNA [26,32], and cell migration was then analyzed. This p66Shc W134F mutant has been demonstrated to reduce overall cellular ROS levels in PCa cells [7,32]. As shown in Figure 3D, upon transfection, LNCaP-AI cell migration and cellular ROS decreased in a dose-dependent manner. The migration assay was again conducted using an equally mixed population of p66Shc cDNA-transfected stable subclones and the corresponding vector-alone control. Figure 3E shows that upon transfection with the redox-deficient DN p66Shc W134F cDNA, the elevated migration and cellular ROS induced by WT cDNA in the mixed subclone population was mitigated in a dose-dependent manner. Collectively, the data (Figs. 2 & 3) a clearly shows that p66Shc promotes PCa migration via its ROS-production mechanism.

3.4 Identification of p66Shc Down-Stream Targets and Signaling Profile

p66Shc plays a role in regulating PCa cell migration, thus we analyzed its downstream signaling targets to elucidate its mechanism of action. To investigate changes in overall protein phosphorylation signaling initiated by p66Shc expression, we prepared whole cell lysates from an equally mixed population of p66Shc cDNA-transfected subclones (S31, S32, and S36) as well as V1 control cells and analyzed the molecular profile via a KinexTM Antibody Microarray KAM900-P performed by the company. As shown in Figure 4A in the format of a percent change-from-control (%CFC) heat-map, a number of proteins had elevated activation through phosphorylation in the p66Shc cDNA-transfected subclones compared to vector-transfected control cells. Interestingly, the phosphatase PTEN, which plays a critical role in advanced CR PCa progression and is responsible for inactivation of some of these proteins, was found to be down-regulated in the subclones. Of the potential downstream-targets, we chose ErbB-2, AKT, mTOR, ERK, and PYK2 for further validation via western blot due to their association with PCa cell migration [41–46]. We also examined ROS-sensitive FOXM1 and its downstream target CDC25B, as well as Rac1 which are redox-sensitive and associated with migration [11,12,33,44,46–47]. We first performed immunoblot analysis to validate key molecules in V1 and mixed cell lysates used in the array analysis (Figure 4B) as well as individual subclone cell lysates (Figure 4C) to examine possible individual variation. We also analyzed the level of cellular prostatic acid phosphatase, cPAP, because it is redox-sensitive [7] and shown to function as a prostate-specific tumor-suppressor-gene in part by inactivating ErbB-2 [29,35,38]; thus its decreased expression is associated with PCa tumorigenicity and clinical progression. Notably, as shown in Figure 4B–C, cPAP protein level was down-regulated in p66Shc-subclones. Consequently, protein tyrosine kinase ErbB-2 and its down-stream targets AKT/mTOR, ERK, PYK2, and Rac1 were shown to have increased activity, by phosphorylation or GTP-binding, in the subclones compared to V1 cells [30–31,48]. The total protein level of FOXM1, which is regulated by AKT and ERK [49], was also elevated in subclones along with its downstream target CDC25B (Fig. 4B–C). Additionally, cell proliferation protein Cyclin B1 was also elevated in the subclones. Thus, the array analysis data is validated by immunoblot, and together the data clearly reveals migration-associated downstream targets of p66Shc.

3.5 p66Shc Regulates Migration-Associated Proteins via ROS

To further validate down-stream proteins associated with p66Shc/ROS signaling, LNCaP-AI cells and mixed p66Shc subclone cells were transiently transfected with p66Shc shRNA and whole cell lysates were analyzed by immunoblot. As shown in Figures 5A and 5B, knockdown of p66Shc has the reverse, dose-dependent effect on each previously identified signaling target in Figure 4. To determine whether p66Shc regulates these proteins through ROS production, LNCaP-AI and mixed p66Shc-subclone cells were transiently transfected with increasing amounts of DN p66Shc redox-deficient mutant W134F cDNA (Myc-Tag) for immunoblot analysis. As shown in Figures 5C and 5D, inhibition of cPAP protein and activation of all other proteins were found to be dependent on p66Shc's ability to generate ROS. The ratio of phosphorylated or GTP-activated protein to respective total protein was quantified using the NIH ImageJ software. Collectively, the data validates p66Shc signaling

targets via rescue experiments and demonstrates the signaling mechanism's reliance on p66Shc-oxidase activity.

3.6 Determination of Functional Molecules in p66Shc-Regulated PCa Cell Migration

To determine the functional molecules that play a critical role in p66Shc-mediated migration, p66Shc mixed subclone cells and V1 control cells were treated with small molecule inhibitors and evaluated by transwell migration assay (Fig. 6A). Due to the potentially significant impact of small molecule inhibitors on both cell proliferation and migration, the results were normalized to the growth inhibition in which cells' 24-hour change in migration was divided by 24-hour change in growth. While inhibition of most functional proteins decreased the migration of p66Shc-subclones and V1 control cells to a similar level, inhibition of ERK and Rac1 had significantly greater impact on p66Shc subclone cells compared to V1 cells. In addition, as shown in Figure 6B, while no impact on cell migration was observed when cells were treated with FOXM1 inhibitor FDI-6 for 24 hours, pretreatments for 24 and 48 hours (i.e. for a total of 48 and 72 hours respectively) selectively inhibited subclone cell migration compared to V1 control cells (Fig. 6B). To determine if effects of FDI-6 on cell migration is entirely due to cell growth effect, a similar experiment was conducted measuring the FDI-6's effect on cell growth over 24, 48, and 72 hours. The results showed that the inhibitory effect of FDI-6 on migration is greater than cell proliferation, thus showing FOXM1 contributes to PCa cell migration (Fig. 6B).

We then validated the results via a molecular approach. We focused our efforts on determining the role of Rac1 in the mechanism of migration because its inhibition resulted in both potent and selective suppression of migration of p66Shc subclones, compared to V1 control cells (Fig. 6A). V1 control and mixed p66Shc subclones were transiently transfected with either vector-alone or HA-tagged dominant negative (DN) Rac1 T17N cDNA and a 24-hour transwell migration assay was then performed. As shown in Figure 6C, while both transfected V1 and subclone cells had significantly reduced migration, subclone cells' migration was reduced by 67% compared to only 33% of V1 cells. Conversely, parental LNCaP-AS (C-33) cells were transiently transfected with either vector-alone or HA-tagged constitutively active (CA) Rac1 G12V cDNA and assessed by a 24-hour transwell migration assay. LNCaP-AS cells transfected with CA Rac1 G12V possessed significantly increased migration capability compared to the vector alone-transfected cells. Immunoblot analysis of HA-tag was conducted to ensure cDNA transfection. Taken together, the data confirms that all identified p66Shc-downstream proteins participate in regulating cell migration signaling. Furthermore, cDNA transfection experiments demonstrate Rac1 activation is critical to the mechanism of p66Shc-mediated migration.

3.7 p66Shc Promotes Lamellipodia Formation via Rac1 Activation

Rac1 is a well-established regulator of lamellipodia formation, which is essential to cell motility [41,50]. Rac1 activation mediates p66Shc-induced migration, therefore we investigated the effect of p66Shc on lamellipodia formation. Initially, LNCaP-AS and – AI as well as V1 control and mixed p66Shc-cDNA transfected subclone cells were immunocytochemically stained with rhodamine phalloidin to visualize F-actin, which is enriched in the lamellipodia, and cells were observed using confocal microscopy (Fig. 7A).

The ratio of lamellipodia to total cell area was semi-quantified using NIH ImageJ software. Figure 7A shows that in the LNCaP cell line progression model, as cells progressed from AS to AI, there is an observed approximately 50% increase in lamellipodia size which correlates with p66Shc protein level and cell migration (Fig. 1B). Additionally, p66Shc cDNA-transfected subclones possess about 50% larger lamellipodia compared to V1 control cells (Fig. 7A), which correlates with observed migration (Fig. 2B). The data together demonstrates that p66Shc expression promotes lamellipodia formation.

To validate whether p66Shc-induced lamellipodia formation is mediated through Rac1, V1 and p66Shc cDNA-transfected stable subclones were transiently transfected with DN Rac1 T17N cDNA. As shown in Figure 7B, cells were then stained with rhodamine phalloidin to visualize F-actin and with anti-HA-Tag to identify transfected cells. Upon DN Rac1 T17N cDNA transfection, the lamellipodia area of both V1 and p66Shc-overexpressing subclones was reduced by about 17% and 33%, respectively, thus a greater effect was observed in the p66Shc subclones. The combined data demonstrate p66Shc promotes lamellipodia formation in PCa cells through activation of Rac1, which increases their migratory activity.

4. Discussion

Localized PCa is not life threatening until it has metastasized to vital organs. Moreover, AS PCa is effectively treated by ADT, while CR PCa is a lethal disease with limited therapeutic options. Thus, to identify novel therapeutic targets for the treatment of metastatic CR PCa, in this report, we investigate the functional molecules that regulate PCa cell migration – a key part of metastasis. The p66Shc protein has been demonstrated to promote AI proliferation of PCa cells through generation of ROS and its protein level correlates with acquisition of the CR phenotype of PCa cell lines [6,7,26,32]. Additionally, it is proposed that ROS can promote the motility of PCa cells, suggesting p66Shc also has the potential of regulating PCa cell migratory activity during metastasis [17–20]. Our results for the first time clearly show that p66Shc via ROS production plays a critical role in regulating PCa migration, at least in part by activating Rac1.

Initially, we found p66Shc protein level correlates with the migratory activity of PCa cells, including AR-null PC-3 and DU145 cell lines as well as AR-positive LNCaP and MDA PCa2b cell progression models (Fig. 1A–C) [51,52]. Further analysis demonstrates that p66Shc can regulate PCa cell migratory ability. While p66Shc cDNA transfection of low migratory-potential LNCaP-AS cells results in significantly enhanced migration, knockdown of p66Shc in high migratory-potential LN-AI cells with p66Shc shRNA significantly decreases cell migration and ROS levels in a dose-dependent manner (Fig. 2A,2C). Moreover, the elevated migratory activity and ROS levels of p66Shc subclones was reduced in a dose-dependent manner upon p66Shc shRNA transfection (Fig. 2B). Together, the data demonstrates p66Shc, via ROS, has a key regulatory role in PCa migration.

We next investigated the mechanism through which p66Shc regulates the migratory activity of PCa cells. p66Shc has been demonstrated to promote PCa growth via ROS generation, therefore the effect of ROS on AS PCa cell migration was first explored [26]. Figure 3A–B shows hydrogen peroxide can promote the migratory activity of LNCaP-AS and MDA-AS

cell lines in a dose-dependent manner, which is mitigated upon antioxidant NAC treatment. To validate that p66Shc promotes PCa migration through its ability to induce ROS generation, the effect of NAC on the migration of p66Shc subclone cells was examined. NAC can reduce intracellular ROS levels of p66Shc subclones [32], and upon NAC treatment, increased migratory activity of subclones was fully mitigated (Fig. 3C). Further, upon transfection with redox-deficient DN p66Shc W134F cDNA, both LNCaP-AI and p66Shc subclone cell migration and cellular ROS is reduced in a dose-dependent manner (Fig. 3D–E). Together the data demonstrates the mechanism of p66Shc-induced PCa migration is reliant on p66Shc's oxidase activity and generation of ROS.

To identify the functional proteins involved in p66Shc-mediated migration, a global protein microarray was used to initially identify potential down-stream targets of p66Shc (Fig. 4A). The proteins chosen for further validation by immunoblot analysis are based on the findings of previous studies as well as their known association with cell migration. Briefly, p66Shc has been demonstrated to promote phosphorylation and activation of ErbB-2 at Y1221/2 through oxidation/inactivation of cPacP, a phosphoprotein tyrosine phosphatase [7,29–30,38]. This allows for activation of ErbB-2 downstream targets ERK and AKT/mTOR kinases by phosphorylation [7,26]. Microarray data revealed PYK2, the dominant adhesion protein in CR PCa adenocarcinoma, possessed increased activation in subclones compared to V1 cells; thus PYK2 was chosen for further evaluation [31]. Moreover, while not included in the microarray, FOXM1 and its down-stream target CDC25B are up-regulated by ERK and AKT and shown to promote the metastatic phenotype in multiple carcinomas, though little is known of its role in PCa [49]. Additionally, Rac1 is a well-established regulator of cell migration and lamellipodia formation. Rac1 has been shown to be regulated by p66Shc in mouse embryonic fibroblasts as well as esophageal and breast carcinomas, however knowledge of their interaction in PCa is limited [11,53,54]. Importantly, all of these proteins are confirmed to be activated by p66Shc in Figures 4B and C. To further validate p66Shc-regulation of each protein of interest, immunoblot analysis was conducted on LNCaP-AI and mixed subclone cells transfected with p66Shc shRNA and p66Shc oxidase-deficient mutant DN W134F cDNA. Figures 5A–D show all proteins were confirmed to be down-regulated in a dose dependent manner upon p66Shc knock-down and transfection of DN p66Shc W134F cDNA, with the exception of PACP, a negative growth regulator [38], which was increased as expected. Thus, this data demonstrates these molecules are activated by p66Shc-mediated ROS generation.

The involvement of each protein in PCa cell migration was then investigated to determine its functional role in p66Shc-induced migration. As shown in Figure 6A and B, small molecule inhibitors were initially used to screen for the effects of each protein on the migration of p66Shc subclones and results were normalized to their respective 24-hour growth inhibition. While the FOXM1 inhibitor FDI-6 did not significantly inhibit migration over 24 hours, 48-hour pretreatment (72 hours total treatment) with small-molecule inhibitor FDI-6 significantly reduced the migration, but not growth, of subclones compared to V1 control cells. Additionally, while inhibitors of AKT and PYK2 were potent suppressors of cell migration, they had no significant difference in the selective effect on p66Shc subclone cells over V1 cells. Alternatively, inhibition of ERK and Rac1 resulted in selective inhibition of subclone cell migration over V1 cells, with Rac1 inhibition being most potent. Furthermore,

while AKT and PYK2 inhibitors were significantly more potent than Rac1 inhibitor for suppression of V1 cell migration, there was no significant difference between these three inhibitors on p66Shc subclone cell migration. Due to Rac1's combined potency and selectivity, it was chosen for further validation by cDNA transfection. Shown in Figure 6C, upon transfection of V1 and p66Shc subclones cells with DN Rac1 T17N cDNA, cell migration was inhibited by about 30% and 70%, respectively, demonstrating p66Shc is reliant on Rac1 activation to induce PCa cell migration. Conversely, upon transfection of parental LNCaP-AS cells with constitutively-active Rac1 G12V cDNA, cell migration was increased by about 45%. Notably, there was no significant difference in migration between control V1 and parental LNCaP-AS cells, demonstrating transfection with vector alone has no phenotypic effect. Together, the data reveals while p66Shc-induced activation of multiple proteins contribute to increased PCa cell migration, Rac1 activation plays a mechanistic role.

It is established that the primary mechanism by which Rac1 promotes cell migration is through facilitating the formation of lamellipodia [11]. Therefore, lamellipodia formation was investigated and observed via confocal microscopy where F-actin, which is enriched in cell lamellipodia, was stained with rhodamine phalloidin. As shown in Figure 7A, the relative size of lamellipodia compared to total cell area increased as LNCaP cells progressed from AS to AI, correlating with observed increase in p66Shc protein level and migration (Fig. 1B). Similarly, the size of lamellipodia was increased in p66Shc subclones compared to V1 cells, again correlating with an increase in cell migration (Fig. 2B). Furthermore, upon transfection of p66Shc subclones with DN Rac1 T17N cDNA, lamellipodia area was reduced by more than double that of V1 cells (Fig. 7B), which closely correlates with observed decrease in migration (Fig. 6C). Collectively, the data demonstrates Rac1 activation is critical to the mechanism of p66Shc-induced lamellipodia formation and migration.

5. Conclusion

In summary, our results clearly show that p66Shc increases PCa migratory activity through generation of ROS for activation or inhibition of ROS-sensitive proteins. As Figure 8 summarizes, p66Shc can translocate to the mitochondria where it oxidizes cytochrome C, decoupling the electron transport chain and generating ROS. The increase in cellular ROS leads to inactivation of cPacP, preventing de-phosphorylation of ErbB-2 and promoting activation of ErbB-2 downstream migration-associated proteins including: ERK, AKT, mTOR, FOXM1, PYK2, and Rac1. Nevertheless, there are additional mechanisms through which p66Shc may activate Rac1, independent of ErbB-2. Interestingly, p66Shc has been reported to activate Rac1 through cytosolic interaction with Son of Sevenless 1 (SOS1) protein [55]. ROS has also been shown to directly mediate Rac1 GDP-GTP nucleotide exchange and thus is another possible mechanism of p66Shc-mediated Rac1 induction [56]. The exact mechanism of p66Shc-Rac1 interaction regulating PCa migration requires further investigation. We will also determine the role and mechanism of several other proteins in p66Shc-mediated PCa cell migration and validate clinical relevance.

To conclude, this work highlights p66Shc's potential as a PCa biomarker for identification of aggressive migratory phenotype. Importantly, because p66Shc possesses enzymatic

activity, it has the potential to be a potent therapeutic target for suppression of tumor proliferation and migration. Moreover, the combined inhibition of p66Shc and its downstream target Rac1 may provide an even more effective method to combat CR PCa migration. Understanding p66Shc's involvement in advanced PCa progression can help determine its potential as a therapeutic target, and elucidating its mechanism of intracellular signaling will enable us to design novel effective treatments for aggressive CR PCa.

Acknowledgments

We thank Ms. Janice A. Taylor and Mr. James R. Talaska of the Advanced Microscopy Core Facility at the University of Nebraska Medical Center for providing assistance with confocal microscopy data as well as Ms. Fen-Fen Lin for procurement of preliminary data. We also thank Dr. Melissa Teoh-Fitzgerald's laboratory for providing rapamycin and Dr. Steve Caplan's laboratory for assistance with confocal microscopy protocols and materials.

Funding

This work was supported in part by the National Institute of Health [R01 CA88184, U01 CA185148], Department of Defense [PC121645 and PC141559], the University of Nebraska Medical Center Bridge Fund, University of Nebraska Food for Health Collaboration Initiative Seed Grant from Nebraska EPSCoR, and UNMC Graduate Studies Fellowship, the Purdue Pharma Scholars Award, Epply Cancer Biology Training Program [T32CA009476], the Ministry of Science and Technology, Taiwan [MOST 106-2320-B-182A-001] and Kaohsiung Chang Gung Memorial Hospital, Taiwan [CMRPG8G0341] and Ben J. Lipps Research Fellowship Award from the ASN Foundation for Kidney Research. Support for the UNMC Advanced Microscopy Core Facility was provided by the Nebraska Research Initiative, the Fred and Pamela Buffett Cancer Center Support Grant [P30CA036727], and an Institutional Development Award (IDeA) from the NIGMS of the NIH [P30GM106397].

References

1. Siegel R, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin.* 2017; 67:7–30. DOI: 10.3227/caac.21387 [PubMed: 28055103]
2. Seruga B, Ocana A, Tannock IF. Drug resistance in metastatic castration-resistant prostate cancer. *Nature Reviews Clin Onc.* 2012; 8:12–23. DOI: 10.1038/nrclinonc.2010.136
3. Rajendran M, Thomas P, Zhang L, Veeramani S, Lin MF. P66Shc- a longevity redox protein in human prostate cancer progression and metastasis. *Cancer Met Rev.* 2010; 29:207–222. DOI: 10.1007/s10555-010-9213-8
4. Alam SM, Rajendran M, Ouyang S, Veeramani S, Zhang L, Lin MF. A novel role of Shc adaptor proteins in steroid hormone-regulated cancers. *E. R. Cancer.* 2009; 16:1–16. DOI: 10.1677/ERC-08-0179
5. Kumar A, Kumar S, Rajendran M, Alam SM, Lin FF, Cheng PW, et al. Steroids Up-Regulate p66Shc Longevity Protein in Growth regulation by Inhibiting Its Ubiquitination. *PLoS ONE.* 2011; doi: 10.1371/journal.pone.0015942
6. Lee MS, Igawa T, Chen SJ, VanBemmel D, Lin JS, Lin FF, et al. p66Shc protein is upregulated by steroid hormones in hormone-sensitive cancer cells and in primary prostate carcinomas. *Int J Cancer.* 2004; 108:672–678. DOI: 10.1002/ijc.11621 [PubMed: 14696093]
7. Veeramani S, Chou YW, Lin FC, Muniyan S, Lin FF, Kumar S, et al. Reactive Oxygen Species induced by p66Shc longevity protein mediate non-genomic androgen activation via tyrosine phosphorylation signaling to enhance tumorigenicity of prostate cancer. *F R Biol and Med.* 2012; 53:95–108. DOI: 10.1016/j.freeradbiomed.2012.03.024
8. Le S, Connors TF, Maroney AC. c-Jun N-terminal kinase specifically phosphorylates p66ShcA at serine 36 in response to ultraviolet irradiation. *J Biol Chem.* 2001; 276:48332–6. [PubMed: 11602589]
9. Okada N, Wada K, Goldsmith BA, Koizumi S. The 66-kDa Shc isoform is a negative regulator of the epidermal growth factor-stimulated mitogen-activated protein kinase pathway. *J Biol Chem.* 1997; 272:28042–9. [PubMed: 9346957]

10. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contusi C, et al. Electron Transfer between Cytochrome c and p66Shc Generates Reactive Oxygen Species that Trigger Mitochondrial Apoptosis. *Cell*. 2005; 122:221–233. [PubMed: 16051147]
11. Kirmani D, Bhat HF, Bashir M, Zargar MA, Khanday FA. P66Shc-rac1 pathways-mediated ROS production and cell migration is downregulated by ascorbic acid. *J Recept Signal Transduct Res*. 2013; 33:107–113. DOI: 10.3109/10799893.2013.770527
12. Bhat HF, Baba RA, Adams ME, Khanday FA. Role of SNTA1 in Rac1 activation, modulation of ROS generation, and migratory potential of human breast cancer cells. *Br J Cancer*. 2014; 110(3): 706–14. DOI: 10.1038/bjc.2013.723 [PubMed: 24434436]
13. Kobayashi K, Inoue T, Shimizu Y, Terda N, Maeno A, Kajita Y, et al. Activation of Rac1 is closely related to androgen-independent cell proliferation of prostate cancer cells both in vitro and in vivo. *Mol Endocrinol*. 2010; 280:31003–10. DOI: 10.1210/me.2009-0326
14. Dhar-Mascareno M, Chen J, Zhang RH, Carcamo JM, Golde DW. Granulocytes-macrophage colony-stimulating factor signals for increased glucose transport via phosphatidylinositol 3-kinase- and hydrogen peroxide-dependent mechanisms. *J Biol Chem*. 2003; 178:11107–14.
15. Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signaling. *Nat Rev Mol Cell Biol*. 2014; 15:411–21. DOI: 10.1038/nrm3801 [PubMed: 24854789]
16. Costa A, Scholer-Dahirel A, Mechta-Grigoriou F. The role of reactive oxygen species and metabolism on cancer cells and their microenvironment. *Sem In Cancer Biol*. 2014; 25:23–32. doi: 0.1016/j.semcancer.2013.12.007.
17. Wang Z, Castrensana MR, Newman WH. Reactive oxygen and NF-kappaB in VEGF-induced migration of human vascular smooth muscle cells. *Biochem Biophys Res Commun*. 2001; 285(3): 669–74.
18. Lo SK, Janakidevi K, Lai L, Malik AB. Hydrogen peroxide-induced increase in endothelial adhesiveness is dependent on ICAM-1 activation. *Am J Physiol*. 1993; 264(1):406–12.
19. Lee KL, Petkovich PM, Heersche JN. The effects of sodium butyrate on the retinoic acid-induced changes in 1,25-dihydroxyvitamin D3 receptors in tumorigenic and nontumorigenic bone derived cell lines. *Endo*. 1988; 122(6):2399, 406.
20. Nishio E, Watanabe Y. The involvement of reactive oxygen species and arachidonic acid in alpha 1-adrenoceptor-induced smooth muscle cell proliferation and migration. *BR J Pharmacol*. 1997; 121(4):665–70. [PubMed: 9208132]
21. Frijhoff J, Dagnell M, Augsten M, Beltrami E, Giorgio M, Ostman A. The mitochondrial reactive oxygen species regulator p66Shc controls PDGF-induced signaling and migration through protein tyrosine phosphatase oxidation. *Free Rad Biol And Med*. 2014; 68:268–277. DOI: 10.1016/j.freeradbiomed.2013.12.022 [PubMed: 24378437]
22. Tsirmoula S, Lamprou M, Hatzia Apostolou M, Kieffer N, Papadimitriou E. Pleiotrophin-induced endothelial cell migration is regulated by xanthine oxidase-mediated generation of reactive oxygen species. *Microvas Res*. 2015; 98:74–81. DOI: 10.1016/j.mvr.2015.01.001
23. Park YJ, Kim TY, Lee SH, Kim H, Kim SW, Shong M. p66Shc expression in proliferating thyroid cells is regulated by thyrotropin receptor signaling. *Endocrinology*. 2005; 146:2473–2480. [PubMed: 15705774]
24. Xie Y, Hung MC. p66Shc isoform down-regulated and not required for HER-2/neu signaling pathway in human breast cancer cell lines with HER-2/neu overexpression. *Biochem Biophys Res Commun*. 1996; 221:140–5. [PubMed: 8660324]
25. Grossman SR, Lyle S, Resnick MB, Sabo E, Lis RT, Rosinha E, et al. p66Shc tumor levels show a strong prognostic correlation with disease outcome in stage II A colon cancer. *Clin Cancer Res*. 2007; 13:5798–5804. DOI: 10.1158/1078-0432.CCR-07-0073
26. Veeramani A, Igawa T, Yuan TC, Lin FF, Lee MS, Lin JS, Johansson SL, Lin MF. Expression of p66Shc protein correlates with proliferation of human prostate cancer cells. *Oncogene*. 2005; 24:7203–7212. DOI: 10.1007/s10555-010-9213-8 [PubMed: 16170380]
27. Igawa T, Lin FF, Lee MS, Karan D, Batra SK, Lin MF. Establishment and characterization of androgen-independent human prostate cancer LNCaP cell model. *Prostate*. 2002; 50(4):222–35. [PubMed: 11870800]

28. Dillard PR, Lin MF, Khan SA. Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. *Mol And Cell Endo*. 2008; 295:115–20.
29. Lin MF, Lee MS, Zhou XW, Andressen JC, Meng TC, Johansson SL, et al. Decreased expression of cellular prostatic acid phosphatase increases tumorigenicity of human prostate cancer cells. *J of Urol*. 2001; 166:1943–50. doi:0022-5347/01/1665-1943/0. [PubMed: 11586265]
30. Muniyan S, Chen SJ, Lin FF, Wang Z, Metha PP, Batra SK, et al. ErbB-2 signaling plays a critical role in regulating androgen-sensitive and castration-resistant receptor-positive prostate cancer cells. *Cell Signal*. 2015; 27(11):2261–71. DOI: 10.1016/j.cellsig.2015.08.002 [PubMed: 26257301]
31. Yuan TC, Chen SJ, Zietlow L, Heck J, Lin FF, Lin MF. ErbB-2 via PYK2 regulates the adhesive ability of androgen receptor-positive human prostate cancer cells. *Oncogene*. 2007; 26:7552–9. DOI: 10.1038/sj.onc.1210570 [PubMed: 17563746]
32. Veeramani S, Yuan TC, Lin FF, Lin MF. Mitochondrial redox signaling by p66Shc is involved in regulating androgenic growth stimulation of human prostate cancer cells. *Oncogene*. 2008; 27:5057–68. DOI: 10.1038/onc.2008.143 [PubMed: 18504439]
33. Bhat HF, Baba RA, Adams ME, Khanday FA. Role of SNTA1 in Rac1 activation, modulation of ROS generation, and migratory potential of human breast cancer cells. *British J of Cancer*. 2014; 110:706–714. DOI: 10.1038/bjc.2013.723
34. Bhat SS, Anand D, Khanday FA. p66Shc as a switch in brining about contrasting responses in cell growth: implications on cell proliferation and apoptosis. *Mol Cancer*. 2015; 8:14–76. DOI: 10.1186/s12943-015-0354-9
35. Meng TC, Lin MF. Tyrosine Phosphorylation of c-ErbB-2 is regulated by the cellular form of prostatic acid phosphatase in human prostate cancer cells. *J of Biol Chem*. 1988; 273:22096–104.
36. Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liand CZ, et al. PC3 is a cell line characteristic of prostatic small cell carcinoma. *Prostate*. 2011; 71:1668–1679. DOI: 10.1002/pros.21383 [PubMed: 21432867]
37. Ingersoll MA, Miller DR, Martinez O, Wakefield BC, Hsieh KC, Simha MV, et al. Statin derivatives as therapeutic agents for castration-resistant prostate cancer. *Cancer Letters*. 2016; 383:94–105. DOI: 10.1016/j.canlet.2016.09.008 [PubMed: 27687622]
38. Chuang TD, Chen SJ, Lin FF, Veeramani S, Kumar S, Batra SK, et al. Human prostatic acid phosphatase, an authentic tyrosine phosphatase, dephosphorylates ErbB-2 and regulates prostate cancer cell growth. *J Biol Chem*. 2010; 285:23598–2360. DOI: 10.1074/jbc.M109.098301 [PubMed: 20498373]
39. Ingersoll MA, Lyons AS, Muniyan A, D’Cunha N, Robinson T, Hoelting K, et al. Novel imidazopyridine derivatives possess anti-tumor effect on human castration-resistant prostate cancer cells. *PLoS ONE*. 2015; 10:e0131811. doi: 10.1371/journal.pone.0131811 [PubMed: 26121643]
40. Kirui JK, Xie Y, Wolff DW, Jiang H, Abel PW, Tu Y. Gbetagamma signaling promotes breast cancer cell migration and invasion. *J Pharmacol Exp Ther*. 2010; 333(2):393–403. DOI: 10.1124/jpet.109.164814 [PubMed: 20110378]
41. Henderson V, Smith B, Burton LJ, Randle D, Morris M, Oder-Marrah VA. Snail promotes migration through PI3K/AKT-dependent Rac1 activation as well as PI3K/AKT-independent pathways during prostate cancer progression. *Cell Adhesion & Migration*. 2015; 9:255–264. DOI: 10.1080/19336918.2015.1013383 [PubMed: 26207671]
42. Ren W, Liu Y, Wan S, Fei C, Wang W, Chen Y, et al. BMP9 inhibits proliferation and metastasis of HER2-positive SK-BR-2 breast cancer cells through ERK1/2 and PI3K/AKT pathways. *PLoS ONE*. 2014; 9(5):e96816. doi: 10.1371/journal.pone.0096816 [PubMed: 24805814]
43. Xue D, Zhou C, Lu H, Xu R, Xu X, He X. LncRNA GAS5 inhibits proliferation and progression of prostate cancer by targeting miR-103 through AKT/mTOR signaling pathway. *Tumor Biol*. 2016; 37:16187–16197. DOI: 10.1007/s13277-016-5429-8
44. Wang Y, Yao B, Wang Y, Zhang M, Fu M, Gao H, et al. Increased FoxM1 expression is a target for metformin in the suppression of EMT in prostate cancer. *Int J of Molec Med*. 2013; 33:1514–1522. DOI: 10.3892/ijmm.2014.1707

45. Amicis FD, Lanzino M, Kisslinger A, Cali G, Chieffi P, Ando S, et al. Loss of proline-rich tyrosine kinase 2 function induces spreading and motility of epithelial prostate cells. *J of Cellular Physiol.* 2006; 209:74–80. DOI: 10.1002/jcp.20709 [PubMed: 16783820]
46. Dirat B, Ader I, Golzio M, Massa F, Mettouchi A, Laurent K, et al. Inhibition of the GTPase Rac1 mediates the antimigratory effects of metformin in prostate cancer cells. *Mol Cancer Therapeutics.* 2014; 14(2):586–596. DOI: 10.1158/1535-7163.MCT-14-0102
47. Wang X, Kiyokawa H, Dennewitz MB, Costa RH. The forkhead box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. *Proc Natl Acad Sci USA.* 2002; 99:16881–16886. DOI: 10.1073/pnas.252570299 [PubMed: 12482952]
48. Wang SE, Shin I, Wu FY, Friedman DB, Arteaga CL. Her-2/Neu (ErbB-2) signaling to Rac1-Pak1 is temporally and spatially modulated by transforming growth factor beta. *Cancer Res.* 2006; 66(19):9591–600. DOI: 10.1158/0008-5472.CAN-06-2071 [PubMed: 17018616]
49. Xie Y, Li Y, Kong Y. OPN induces FoxM1 expression and localization through ERK1/2, AKT, and p38 signaling pathway in HEC-1A cells. *Int J Mol Sci.* 2014; 15(12):23345–58. DOI: 10.3390/ijms151223345 [PubMed: 25522167]
50. Fukuda T, Kiuchi K, Takahashi M. Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. *J Biol Chem.* 2002; 24(21):19114–21. DOI: 10.1074/jbc.M200643200
51. Scaccianoce E, Festuccia D, Dondi D, Guerini V, Bologna M, Motta M, et al. Characterization of prostate cancer DU145 cells expressing the recombinant androgen receptor. *Oncol Res.* 2003; 14:101–112. [PubMed: 14649544]
52. Tilley WD, Wilson CM, Marcelli M, McPaul MJ. Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res.* 1990; 50:5382–5386. [PubMed: 2386943]
53. Khanday FA, Yamamori T, Mattagajasingh I, Zhang Z, Bugayenka A, Naqvi A, et al. Rac1 leads to phosphorylation-dependent increase in stability of the p66Shc adaptor protein: Role in Rac1-induced oxidative stress. *Molec Biol Of the Cell.* 2006; 17:122–129. DOI: 10.1091/mbc.E05-06-0570
54. Bashir M, Kirmani D, Bhat HF, Baba RA, Hamza R, Nagash S, et al. P66shc and its downstream Eps8 and Rac1 proteins are upregulated in esophageal cancers. *Cell Commun Signal.* 2010; 8:13.doi: 10.1186/1478-811X-8-13 [PubMed: 20565814]
55. Khanday FA, Santhanam L, Kasuno K, Yamamori T, Naqvi A, DeRicco J, et al. Sos-mediated activation of rac1 by p66shc. *J of Cell Biol.* 2006; 172(6):817–22. DOI: 10.1083/jcb.200506001 [PubMed: 16520382]
56. Heo J, Campbell SL. Mechanism of redox-mediated guanine nucleotide exchange on redox-active Rho GTPases. *J Biol Chem.* 2005; 280:31003–10. DOI: 10.1074/jbc.M504768200 [PubMed: 15994296]

Highlights

- p66Shc is elevated in prostate androgen-independent cell lines.
- p66Shc, via ROS, promotes castration-resistant prostate cancer migration.
- Through ROS, p66Shc activates ErbB-2, AKT, mTOR, ERK, FOXM1, PYK2, and Rac1.
- p66Shc promotes lamellipodia formation and migration through Rac1 activation.
- p66Shc is a promising biomarker and therapeutic target for advanced prostate cancer.

Fig. 1. p66Shc protein level correlates with the CR phenotype and PCa cell migration
Immunoblotting for Shc and loading control β -actin using (A) androgen-sensitive LNCaP (LN-AS/C-33) and androgen-independent PC3 and DU145, (B) androgen-sensitive (AS) C-33 and androgen-independent (AI) C-81 LNCaP, and (C) AS and AI MDA-PCa2b cell lines. The transwell assay was conducted in which cells were seeded in 24-well plate transwell inserts (5×10^4 cells per well) and allowed to migrate for 24 hours. Migrated cells were fixed and stained before counting. Results presented are mean $\pm$ SE; $n=3 \times 3$. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

Fig. 2. p66Shc promotes PCa cell migration

(A) Immunoblot staining for Shc and loading control β -actin using LNCaP-AS C-33 cells transfected with vector-alone (V1) or p66Shc cDNA to generate stable subclones S32 and S36. The transwell assay was conducted in which cells were seeded in 24-well plate transwell inserts (5×10^4 cells per well) and allowed to migrate for 24 hours. Migrated cells were fixed with methanol and stained with crystal violet before counting. Images at $40\times$ magnification. Cellular ROS levels were analyzed via DCF-DA staining and presented as relative fluorescence units (RFU). (B) V1 or equally mixed population of stable S31, S32, and S36 subclones transiently transfected with 0–6 μ g p66Shc shRNA or vector alone. Immunoblot staining, transwell, and DCF-DA assays were conducted as described in (A).

(C) AI LNCaP C-81 cells transiently transfected with 0–6 μ g p66Shc shRNA or vector alone. Immunoblot staining and transwell assays were conducted as described in (A). Results presented are mean $\pm$ SE; transwell n=3 $\times$ 3, DCF-DA n=3. “r” denotes closeness to regression line between shRNA dosage and RFU or migration levels, respectively. * p <0.05; ** p <0.001; *** p <0.0001.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3. p66Shc promotes PCa cell migration via ROS

(A) Transwell assay in which androgen-sensitive (AS) LNCaP C-33 or MDA-PCa2b cells were seeded in 24-well plate transwell inserts (5×10^4 cells per well), treated with 0–20 μ M hydrogen peroxide in the lower chamber, and allowed to migrate for 24 hours. Migrated cells were fixed and stained before counting. (B) Transwell assay as described in (A) in which AS LNCaP C-33 or MDA-PCa2b cells were treated with 10 μ M hydrogen peroxide, 10mM NAC, or both. (C) Transwell assay as described in (A) using V1 or stable subclones treated with or without 10mM NAC in the lower chamber of the transwell (D,E) Androgen-independent (AI) LNCaP C-81 (D) or V1 and equally mixed population of stable S31, S32, and S36 subclone cells (E) were transiently transfected with 0–6 μ g of dominant-negative redox-deficient p66Shc W134F cDNA or vector alone. Transwell and DCF-DA assays were conducted as described in (A). Results presented are mean $\pm$ SE; transwell $n=3 \times 3$, DCF-DA $n=3$. “ r ” denotes closeness to regression line between cDNA dosage and RFU or migration levels, respectively. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

Fig. 4. Identification of p66Shc down-stream targets by molecular profiling

(A) Total cell lysates of V1 or equally mixed population of stable S31, S32, and S36 subclones analyzed via Kinexus KAM-900P protein microarray. Results presented as percent change from control (%CFC) in which red represents an increase and green a decrease in protein levels. (B,C) Immunoblot analysis of total cell lysates from V1 or equally mixed population of stable S31, S32, and S36 subclones used in the protein microarray (B) or V1 and individual stable S31, S32, and S36 subclones (C). Total and phosphorylated or GTP-activated proteins associated with migration were analyzed. β-actin was used as a loading control. n=3.

Fig. 6. Determination of functional molecules in p66Shc-regulated PCa cell migration
(A) Transwell assay using V1 or equally mixed population of stable subclones treated with small-molecule inhibitors (at IC₅₀ in LNCaP cells) in the lower chamber of the transwell for functional migration-associated proteins previously identified. Cells were seeded in 24-well plate transwell inserts (5×10^4 cells per well) and allowed to migrate for 24 hrs. Migrated cells were fixed and stained before counting. Cell migration is normalized to small-molecule 24 hour growth inhibition. (B) Transwell assay using V1 or equally mixed population of stable subclones treated with 5 μ M FOXM1 inhibitor FDI-6 (IC₅₀ in LNCaP cells). Cells were treated with FDI-6 in the lower chamber during the 24 hour transwell or with additional 24 or 48 hour pre-treatment (For a total of 24, 48, and 72 hours of FDI-6 treatment). FDI-6 effect on cell growth of V1 or equally mixed population of stable subclone

cells was determined. Cells were seeded at 3×10^3 cells/cm² and allowed to attach for three days before treated with 5 μ M FDI-6 for 24, 48, and 72 hrs. Cells were trypsinized and live cell number was counted. (C) V1 or equally mixed population of stable subclones were transiently transfected with HA-Tagged dominant-negative Rac1 T17N cDNA or vector alone. AS LNCaP C-33 cells were transiently transfected HA-Tagged constitutively-active Rac1 G12V cDNA or vector alone. Transwell assay was conducted as described in (A). Successful transfection was determined via immunoblot of whole cell lysates for HA-Tag. β -actin was used as a loading control. Results presented are mean $\pm$ SE; n=3 $\times$ 3. * p <0.05; ** p <0.001; *** p <0.0001.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 7. p66Shc promotes lamellipodia formation via Rac1 activation

(A) Androgen-sensitive (AS) C-33 and androgen-independent (AI) C-81 LNCaP, V1, and equally mixed population p66Shc subclone cells were stained with F-actin binding rhodamine phalloidin (Red) to visualize lamellipodia and DAPI (Blue) to detect nuclei. The ratio of lamellipodia to total cell area of 20 randomly selected cells was quantified. (B) V1 and equally mixed population subclone cells were transfected with HA-Tagged DN Rac1 cDNA and stained with F-actin binding rhodamine phalloidin (Red) to visualize lamellipodia, DAPI (Blue) to visualize nuclei, or anti-HA-tag (Green) to visualize dominant-negative Rac1 cDNA transfected cells. The ratio of lamellipodia to total cell area of 20 randomly selected cells was quantified. Results presented are mean $\pm$ SE; $n=20 \times 3$. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

Fig. 8. Proposed mechanism of p66Shc-regulated PCa cell migration

Upon elevation of protein level, p66Shc translocates into the mitochondria where it binds and oxidizes cytochrome C, decoupling the electron transport chain, and generating ROS. Increased cellular ROS oxidizes cellular prostatic acid phosphatase (cPacP), preventing it from dephosphorylating ErbB-2. Phosphorylated ErbB-2 then activates downstream targets PI3K/AKT/mTOR, ERK, FOXM1, PYK2, and Rac1, all of which contribute to PCa cell migration. p66Shc may also activate Rac1 via SOS1 in the cytoplasm or through ROS-mediated Rac1 GDP-GTP nucleotide exchange.