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Evaluation of ERG Responsive Proteome in Prostate Cancer

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Abstract

BACKGROUND—Gene fusion between *TMPRSS2* promoter and the *ERG* proto-oncogene is a major genomic alteration found in over half of prostate cancers (CaP), which leads to aberrant androgen dependent ERG expression. Despite extensive analysis for the biological functions of ERG in CaP, there is no systematic evaluation of the ERG responsive proteome (ERP). ERP has the potential to define new biomarkers and therapeutic targets for prostate tumors stratified by ERG expression.

METHODS—Global proteome analysis was performed by using ERG (+) and ERG (–) CaP cells isolated by ERG immunohistochemistry defined laser capture microdissection and by using *TMPRSS2-ERG* positive VCaP cells treated with *ERG* and control siRNA.

RESULTS—We identified 1196 and 2190 unique proteins stratified by ERG status from prostate tumors and VCaP cells, respectively. Comparative analysis of these two proteomes identified 330 concordantly regulated proteins characterizing enrichment of pathways modulating cytoskeletal and actin reorganization, cell migration, protein biosynthesis, and proteasome and ER-associated protein degradation. ERPs unique for ERG (+) tumors reveal enrichment for cell growth and survival pathways while proteasome and redox function pathways were enriched in ERPs unique for ERG (–) tumors. Meta-analysis of ERPs against CaP gene expression data revealed that *Myosin VI* and *Monoamine oxidase A* were positively and negatively correlated to *ERG* expression, respectively.

CONCLUSIONS—This study delineates the global proteome for prostate tumors stratified by ERG expression status. The ERP data confirm the functions of ERG in inhibiting cell differentiation and activating cell growth, and identify potentially novel biomarkers and therapeutic targets.

INTRODUCTION

Carcinoma of prostate is the most frequently diagnosed non-skin cancer in the United States with an estimated 238,590 newly diagnosed cases and 29,720 deaths in 2013 (1). Rapidly increasing understanding of the molecular basis of CaP is providing new insights into the

etiology and improved prognosis of the disease (2-4). Prevalent gene rearrangements in CaP involve the fusion promoter region of AR regulated genes (predominantly, serine 2 trans-membrane protease: *TMPRSS2*) and protein coding sequence of an *ETS* related gene (primarily *ERG*). While *TMPRSS2-ERG* is detected in 40%-65% of patients, *SLC45A3* and *NDRG1* serve as fusion partners for approximately 10% of the tumors with ERG rearrangements (5-7)

Despite the high prevalence of *TMPRSS2-ERG* gene fusions detected in CaPs of Western populations, the frequency is lower in African Americans (31%-43%) compared to Caucasian Americans (50-66%), and it is even lower in Asian populations (5-24.4%) (8-10). We have recently reported that ERG frequency is strikingly less in the index tumors of African American patients (28.6%) compared to Caucasian Americans (63.3%), suggesting that the ERG based stratification of CaP may help distinguish the biologic differences of CaP between the ethnic groups (10). Studies comparing ERG (+) and ERG (-) CaP have also suggested the expression of genes unique to ERG (+) or ERG (-) tumors (11,12).

Multiple studies on the ERG regulated transcriptome have investigated the function of ERG in the context of prostate epithelial cells and its effect on tumor cell invasion or prostate epithelial differentiation (13-16). However, the underlying mechanisms of ERG function remain to be better elucidated. Although there have been considerable efforts to characterize the CaP proteome (17-21), a systematic evaluation of **ERG responsive proteome (ERP)** has not been carried out. Since ERG oncoprotein is a nuclear transcription factor, it is neither an optimal biomarker nor an ideal cancer therapeutic target. The evaluation of ERG Responsive Proteins (ERPs) may identify surrogate biomarkers from secreted or cell surface proteins or druggable targets such as growth factor receptors or kinases in the ERG network. Furthermore, differential expression of proteins in ERG (+) and ERG (-) CaP may delineate the biochemical differences and identify potential biomarkers and therapeutic targets of specific for these two tumor types.

Until recently, the lack of reliable ERG antibodies has restricted the analysis of ERG aberrations in CaP specimens to fluorescence in situ hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR) assays (22,23). We have adopted a novel approach to study the ERG modulated proteome by identifying tumor cells positive or negative for ERG protein expression using ERG-MAb-based immunohistochemistry (IHC) staining of prostate tumor specimens (24), followed by the isolation of cells using laser capture microdissection (LCM) (25). Using *ERG* siRNA, we also inhibited the expression of the ERG protein in VCaP cells, which enabled us to compare ERG responsive proteome in the presence or absence of ERG.

The application of sensitive and quantitative methods in shotgun proteomics has significantly improved the resolution proteomic of analysis. In this study, we used a unique platform based on capillary isotachopheresis (CITP) and capillary zone electrophoresis (CZE) coupled with electrospray ionization (ESI) linear ion trap tandem mass spectrometry (MS/MS). The combined CITP/CZE-nano-ESI-MS/MS system has been demonstrated to be at least one to two orders of magnitude more sensitive than that found in conventional electrophoresis and column-chromatography based proteome technology, covering a much

wider concentration range, necessary for increasing the range of protein profiling (26). This improvement is achieved by the selective analyte enrichment through electrokinetic stacking of CITP, and the excellent resolving power of CZE (27), which results in diluting the major components while concentrating the trace compounds. The on-column transition from CITP to CZE also minimizes additional band broadening with superior analyte resolution.

We defined the differentially expressed ERP both from prostate tumor specimens and from VCaP cell line and revealed a total of 330 overlapping proteins that concordantly respond to ERG expression. Literature-based evaluation for functionally interacting signaling pathways revealed networks regulating multiple cellular functions including AR signaling, protein synthesis and trafficking, and cell growth and migration. By sorting for ERPs that were detected at higher MS ratios in ERG (+) and ERG (–) tumors relative to benign tissues, we sought to distinguish these tumors based on their specific signal transduction pathway signatures. ERPs unique for ERG (+) and ERG (–) tumors were examined for potential surrogate biomarkers or therapeutic targets based on their cellular localization or enzymatic activity. Consistent with previous reports, we observed the effect of ERG on stimulating cell growth and inhibiting cell differentiation. This is evident in ERG silenced VCaP cells, where we observed increased expression of markers of prostate luminal epithelial differentiation and regulators of cell polarity concomitant with reduced expression of EGFR signaling pathway proteins. Furthermore, to identify correlation to ERG expression at the level of both protein and mRNA expressions, ERPs were compared against the CPDR 80-GeneChip/40-patient tumor vs. benign gene expression dataset (5). *Myosin VI* and *MAOA* were found to be positively and inversely correlated to *ERG* expression, respectively. Combined detection of ERG, Myosin VI and MAOA to distinguish ERG (+) and Myosin VI (+) tumors from ERG (–) and MAOA (+) tumors may facilitate the diagnosis and stratification of CaP patients.

MATERIALS AND METHODS

Cell culture and ERG siRNA knock-down

Human prostate tumor cell lines, VCaP, LNCaP, CWR22Rv1, DU145, PC-3, RWPE-1 and RWPE-2 were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained as recommended. The LNCaP subline, C4-2B was purchased from Urocor (Oklahoma, OK) and cultured as recommended. LAPC-4 cells were kindly provided by Dr. Charles L. Sawyers. RC170N cells were established in our laboratory and cultured in Keratinocyte serum-free medium, supplemented with bovine pituitary extract and recombinant epidermal growth factor (Life Technologies, Inc., Carlsbad, CA). (28). ERG (5'-CGACAUCCUUCUCUCACAUAU-3') and non-targeting (NT; D-001206-13-20) small interference RNA (siRNA) oligo duplexes were from Thermo Scientific (Lafayette, CO) (13). VCaP cells were seeded in 10 cm tissue culture dishes at 2×10^6 cells per dish in DMEM (ATCC, Rockville, MD), supplemented with 10% charcoal:dextran stripped fetal bovine serum (cFBS; Gemini Bioproducts, West Sacramento, CA) and propagated for three days. Cells were transfected with 25 or 50 nM of *ERG* or non-targeting (NT) siRNAs using Lipofectamine 2000 (Life Technologies, Inc., Carlsbad, CA) (29). Twelve hours after transfection, VCaP cells were treated with 0.1 nM of the synthetic androgen analogue

R1881. A near complete ERG knock-down was achieved by growing the cells for four days following transfection, which was confirmed by immunoblot analysis of the cell lysates.

Prostate tissues and laser capture-microdissection

Under an IRB approved protocol (Protocol No. 20405-28), prostate tumor cells and benign cells (distant to tumor focus) from the same tissue section were isolated by LCM from whole-mounted FFPE prostate sections of five patients that were matched for age (50-65 yrs), race (Caucasian American), and tumor cell differentiation (well to moderate), Gleason grade (3+3 or 3+4) and nuclear grade (grade II). Whole-mounted prostate tissue sections of 8 μ m thickness placed on uncharged glass slides were analyzed for malignant and benign cells by hematoxylin and eosin staining and for ERG oncoprotein expression status by IHC with the CPDR anti-ERG monoclonal antibody, ERG-MAb, 9FY (24). Two ERG (+) and three ERG (–) specimens were selected. Approximately 100,000 tumor cells and an equivalent number of matching benign cells were isolated using the Arcturus PixCell II system on LCM caps from each of the sections. The caps were placed into micro-centrifuge tubes with 50 μ l of ultra-pure water, immediately frozen on dry ice and stored in -80°C until proteomic analysis.

Proteomic Analysis of ERG Responsive Proteome

ERG (+) tumor cells were pooled together from two specimens; ERG (–) tumor cells, from three specimens; and benign cell, from five specimen. The workflow for proteomic analysis is outlined in Figure 1A. Proteins extracted from the cell pellets were denatured, reduced and alkylated before trypsinization. Digested peptides were desalted, purified and lyophilized. Peptides were then stacked, resolved and fractionated using capillary isotachopheresis (CITP) and capillary zone electrophoresis (CZE)-based multidimensional separations (26). Peptides fractions were analyzed by nano-reversed-phase liquid chromatography and eluants were monitored by a linear ion-trap mass spectrometer equipped with an electrospray ionization interface. Raw LTQ data were converted to peak list files, which were searched against the UniProt sequence library (www.uniprot.org). A 1% false discovery rate (FDR) for total peptide identifications, which correlates with the maximum sensitivity versus specificity, was chosen as a cutoff. Only proteins identified with at least 2 peptides and 1 unique peptide were included in the final list of identified proteins. The complete description of these procedures is described in detail in the supplementary materials.

Gene Ontology annotation and comparison of datasets

The classification and clustering of *proteins* dataset were performed using ProteinCenter, v3.2 (Thermo Scientific, West Palm Beach, FL). The differentially expressed proteins detected from the NT siRNA and ERG siRNA experiments were analyzed using the Genomatix (Ann Arbor, MI) *GeneRanker* and *Genomatix Pathway System (GePS)* programs. The over-representation of different biological terms (literature association-based or curated canonical pathways) within the input protein list were ranked by their p-values by *GeneRanker*. Functional interaction networks were generated from these ranked lists based on co-citations within the same sentence in PubMed abstracts linked by a function word.

The interaction of ERPs was represented by a network layout that emphasizes high co-citations connectivity and interactions.

Western blot and immunofluorescence assays

VCaP cells were lysed in mammalian protein extraction reagent (M-PER) (Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Sigma, St Louis, MO). Cell lysates equivalent to 20 µg of protein were separated on 4-12% Bis-Tris Gel (Life Technologies, Inc., Carlsbad, CA) and transferred to PVDF membrane. Membranes were incubated overnight at 4°C with primary antibodies and washed before treated with goat anti-Mouse IRDye 800CW or goat anti-Rabbit IRDye 680CW secondary antibodies (Li-Cor Biosciences, Lincoln, NE) at 25°C. Bands were visualized and signal intensities of the bands were quantitatively measured using the Odyssey infra-red imaging scanner (Li-Cor Biosciences).

VCaP cells were seeded onto poly-L-lysine coated coverglass (BD Bioscience; San Jose, CA) in 10% CSS two days prior to siRNA transfection. Cells were induced with 0.1 nM R1881 one day after transfection and incubated for 48 hours. Cells were fixed with PBS buffered 4% paraformaldehyde before permeabilization in 1x PBS with 0.1% Triton X-100. Prior to incubation in primary antibody, cells were blocked in 1% normal horse serum (Vector Laboratories; Burlingame, CA) in PBS. Cells were incubated with a species specific secondary antibody (Alexa-Fluor-594 goat anti-mouse, Alexa-Fluor-488 goat anti-rabbit; Life Technologies, Inc., Carlsbad, CA), and with DAPI (4',6-Diamidino-2-Phenylindole) as a nuclear counterstain.

The antibodies used in for immunoblot and immunofluorescence analysis were acquired from the following sources: ERG-MAb (9FY) from Biocare Medical, Concord, CA ; GAPDH (sc-25778) from Santa Cruz Biotechnology, (Santa Cruz, CA); SHC (610082) from BD BioSciences (San Jose, CA); PAP (2906-1) and ERG (EPR3864(2)) from Epitomics (Burlingame, CA); PSA (A056201-2) and SLC45A3/Prostein (Clone 5E10, M3615) from Dako (Carpinteria, CA); p44/ERK1 (#4372), α-tubulin (11H10, #2125), Cool1/βpix/ ARHGEF7 (#4515) and Myosin VI (#9200) from Cell Signaling Technology (Beverly, MA); RAC1 (ARCO3) from Cytoskeleton (Denver, CO); MSMB (TA501072) from Origene (Rockville, MD); Myosin VI (ab126751) and MAOA (EPR7101; ab11096) from Abcam (Cambridge, MA).

Comparison of ERG Responsive Proteome to Gene Expression Datasets

The CPDR 40 patient/80-gene-chip gene expression dataset (GSE32448) was acquired on Affymetrix Human Genome U133 Plus 2.0 arrays using RNA derived from LCM isolated prostate tumors and matching benign tissue specimens. The ERG expression status of the specimens, which were equally represented by moderately-differentiated tumors and poorly-differentiated tumors, were confirmed by IHC and FISH as 14 ERG (–) and 26 ERG (+). Gene expression data of probesets that match ERPs from ERG (+) and ERG (–) tumors were fitted by linear regression model fitting using the *lmfit* function in the Limma package (30) within the R program. Probesets with most significant correlation or inverse correlation to ERG expression were ranked by *eBayes* according to the Bayes test statistics in the order

differential expression. Statistical significance of a data set was computed using two-tailed t-tests after excluding outliers defined by data-points that are greater than 2.5 standard deviations. Genes and proteins were then compared for concordance in similar up- or down-regulation of tumor vs. normal gene expression ratios to relative MS ratio in ERG (+) and ERG (–) tumors.

RESULTS

Isolation of ERG Responsive Proteins (ERPs)

The strategy to analyze the ERPs in ERG (+) and ERG (–) CaP cells is outlined in **Figure 1A**. Proteins were isolated from pooled ERG (+) or ERG (–) tumor cells and benign cells from whole-mounted sections of five prostatectomy specimens from patients matched for pathologic stage, age and race (**Figure 1B**). Proteins were also isolated from NT siRNA and *ERG* siRNA treated VCaP cells (**Figure 1C**). Trypsin digested proteins were fractionated by using a CITP/CZE- based multidimensional separations. Peptide fragments were detected with nano-electrospray ionization linear ion trap-tandem mass spectrometry (nano-ESI-MS/MS). The near complete silencing of ERG protein expression was confirmed by immunoblot analysis of *ERG* siRNA treated VCaP cell lysates (**Figure 1C**) using ERG-MAb (9FY), which detects ERG protein of 52kDa in VCaP cells (24).

Analysis of the differential expression of ERPs between LCM derived ERG (+) vs. ERG (–) prostate tumor cells

The analysis of ERG responsive proteins isolated from LCM derived ERG (+) and ERG (–) prostate tumor cells and from matched benign cells detected, at 5% false discovery rate (FDR) threshold for total peptide identifications, a combined global proteome of **6171** proteins (**Supplementary Figure 1A, Supplementary Table 1A**), of which a total of **4,684** were ERPs (**Supplementary Figure 1B, Supplementary Table 1B**). At stringent threshold for total peptide identifications of 1% FDR, a total of **1,196** ERPs were detected, of which **518** and **500** were unique to ERG (+) and ERG (–) tumor cells, respectively (**Figure 2A**).

Analysis of the differential expression of ERPs between *ERG* siRNA vs. NT siRNA transfected VCaP cells

VCaP cells transfected with control NT siRNA oligos showed a robust expression of ERG protein and ERG expression was successfully depleted in the *ERG* siRNA transfected VCaP cells (**Figure 1C**). At 5% FDR threshold for total peptide identifications, a total number of 11,416 proteins detected in NT siRNA and *ERG* siRNA treated VCaP cells (**Supplementary Figure 1C, Supplementary Table 1C**). At stringent threshold for total peptide identifications of 1% FDR, a combined ERG responsive global proteome of **2,190** proteins was detected. This proteome consisted of **562** proteins detected exclusively in control NT siRNA transfected VCaP cells, **59** proteins exclusively in ERG silenced VCaP cells, and **1,569** differentially expressed proteins common in both NT siRNA and *ERG* siRNA transfected cells (**Figure 2B**).

The technical reproducibility of the methods applied for proteomic analyses was verified by performing two independent runs through sequential fractionations by CITP and CZE

coupled with LC MS/MS using tryptic digests from VCaP cells that were transfected with NT siRNA and expressing ERG. The reproducibility of the methods used was confirmed by the detection of 80% proteins that were common to two independent runs (**Supplementary Figure 1D, Supplementary Table 1 D**).

The comparative distribution of the ERG responsive proteins from both NT siRNA and *ERG* siRNA treated VCaP cells, according to Gene Ontology (GO) instances of defined physiochemical characteristics, including molecular functions, biological processes and cellular compartments, are shown in **Supplementary Figure 2**. The overall results showed a broad similarity in the range and distribution of proteins from both cells transfected with NT siRNA and with *ERG* siRNA in the different sub-categories of the GO instances, suggesting robust coverage in the isolation and detection of cellular proteome by the methods employed.

Comparison of ERG Responsive Proteome and ERG Responsive Transcriptome in VCaP cells

We have previously evaluated the transcriptome of VCaP cells in response to ERG knock-down by siRNA using GeneChip microarray analysis (13). Normalized gene expression data from 48 h post transfection were denoted as NT siRNA/*ERG* siRNA ratios. Comparison of the present set of **2,190** ERPs from VCaP cells (**Figure 2B**) against probe-sets representing **1,052** distinct genes revealed **250** genes and proteins with concordance response to ERG expression. This represents **23.8%** (250/1,052) of the ERG responsive genes from the gene-chip experiments and **11.4%** (250/2,190) of the ERG responsive proteins (**Supplementary figure 3**).

High stringency analysis of ERG Responsive Proteomes of LCM isolated tumor cells and VCaP cells show a strong concordance in regulation by ERG

We compared the proteome of ERPs detected in the LCM isolated prostate tumor specimens (**Figure 2A**) and in VCaP cells (**Figure 2B**) to determine the extent of correlation between these two sets of proteome. This evaluation showed an overlap of **489** ERPs, of which **330** ERPs show concordance in their response to up- or down-regulation of ERG protein levels (**Figure 3A**). The 330 proteins account for **15.1%** (330/2,190) of ERPs in VCaP cells and **27.6%** (330/1,196) of ERPs in LCM isolated ERG (+) and ERG (–) tumors. The differential levels of detection in ERG (+) vs. ERG (–) tumors and NT siRNA vs. *ERG* siRNA VCaP cells are shown in **Figure 3B**.

Signal transduction pathways of LCM isolated tumor cells and VCaP cells

To evaluate the overall impact of down-stream targets that respond to ERG expression, ERPs from ERG (+) vs. ERG (–) prostate tumor specimens and from VCaP cells were further analyzed using *GeneRanker* and *GePS*. ERG responsive proteome networks derived from **1,196** ERPs isolated from tumors and **2,190** ERPs from VCaP cells, as revealed by *GePS* analysis tool are shown in **Figure 4A** and **4B**, respectively. Proteins that were detected at positive and negative MS ratios for ERG (+) vs. ERG (–) tumor and NT siRNA vs. *ERG* siRNA in VCaP cells are shown as red and green nodes, respectively. By inference, the red and green nodes represent potential up-and down-regulation by ERG. These

networks reflect the impact of ERG expression on protein biosynthesis, chaperone and redox functions, protein trafficking, AR signaling, cell survival and apoptosis, DNA replication, cell cycle control, cell polarity and cell migration. For example, in both ERG (+) vs. ERG (–) tumors and in NT siRNA vs. *ERG* siRNA treated VCaP cells Proliferating Cell Nuclear Antigen (PCNA) is upregulated, in contrast to prostate specific antigen (PSA /KLK3), which is downregulated.

To highlight the conservation of function in prostate tumors and in the cell culture model, the 330 overlapping ERPs with concordant response to ERG expression in both tumor specimens and cell culture model were analyzed by using *GeneRanker* and *GePS* software. The set of 330 overlapping ERPs show, as listed according to p-value rankings in **Table 1** and mapped in the resulting network in **Figure 4C**, an enrichment of pathways regulating cytoskeletal and actin reorganization, as represented by the CDC42-RAC1, the P21 activated protein kinase (PAK) and the actin filaments Y-branching pathways.

ERG knock-down induces the expression of prostate differentiation markers associated with its secretory function and impacts the epidermal growth factor receptor (EGFR) signaling pathway

In our earlier publication, we have noted that ERG interferes with prostate epithelial differentiation by inhibiting a number of genes including *KLK3*, *SLC45A3* (*Prostein*), *C15ORF21* (*Dresden prostate carcinoma 2 protein (D-PCa-2)*) and *MSMB* (β -microseminoprotein/*PSP94*) (13). In the current study, we detected a consistent expression pattern of these proteins in relation to ERG expression in both the LCM isolated tumor cells and VCaP cells with the ERG knock-down. The protein expression and sub-cellular localization of several ERG responsive downstream targets, were validated in VCaP cells following *ERG* siRNA treatment. In response to ERG knock-down the expression of cytoplasmic SLC45A3 and prostatic acid phosphatase (PAP/ACPP) were dramatically upregulated (**Figure 5A and B**), but MSMB expression showed a more subtle increase (**Figure 5C**), consistent with the results of ERG responsive transcriptome. The upregulated expressions of Prostein, PSA, and PAP/ACPP in response to the *ERG* siRNA in VCaP cells were also validated by immunoblot analysis (**Figure 5D**).

Markers of cell growth and proliferations from the epidermal growth factor receptor (EGFR) signaling pathway, such as the Src homology 2 domain containing transforming protein 1 (SHC1) and mitogen-activated protein kinase (p44/ERK1) (31), show higher levels of expression when ERG is expressed in the cell, but becomes down-regulated when *ERG* expression is silenced by siRNA (**Figure 6A-B**). In contrast, the expression of regulators of cell polarity and apical junction assembly, such as Rho-GTPase, RAC1 (32) and Rho guanine nucleotide exchange factor 7 (ARHGEF7/p85 Coo1/ β Pix) (33) is elevated in response to ERG knock-down (**Figure 6C-D**), which confirms the inhibition of prostate epithelial differentiation by ERG. The downregulation of SHC1 and p44/ERK1 and upregulation of ARHGEF7 were also validated by immunoblot assays (**Figure 6E**).

Signal transduction pathways signatures defined by ERG (+) and ERG (–) tumors

The identification of ERPs that are exclusive to or overexpressed in either ERG (+) or ERG (–) tumors could further reveal functional roles of ERG in prostate tumor initiation and progression. Proteins that are correlated with ERG expression could serve as surrogate biomarkers and/or therapeutic targets in ERG (+) tumors. In contrast, proteins that are overexpressed in tumors lacking ERG could be used as biomarkers that define a separate category of tumors. The relative abundance of a protein in ERG (+) vs. ERG (–) tumors, or in ERG (+) and ERG (–) tumors vs. benign tissues was determined based on its relative MS ratio. Since the same protein could be detected in these separate analyses, we sorted the proteome data again to identify proteins that were detected at higher ratios in ERG (+) or in ERG (–) tumors, relative to benign tissues. **589** proteins were detected at higher ratios in ERG (+) tumors, of which **204** were unique for ERG (+) tumors. Conversely, **504** of the **781** proteins detected at higher ratios in ERG (–) tumors, were exclusively for ERG (–) tumors (**Figure 7**).

To further identify the individual profiles that define the proteome from ERG (+) and ERG (–) prostate tumors, ERPs from each set were analyzed for pathway enrichment and associated literature-based networks using *GeneRanker* and *GePS*. Analysis of ERPs from ERG (+) tumors revealed, as listed according to p-value rankings in **Table 2A**, enrichment for pathways that regulate cell shape and motility (PAK pathway), remodel cytoskeletal structure (CDC42 pathway), promote cell survival (AKT pathway), and enhance protein synthesis and cell growth (AKT-MTOR pathway). The nodes that connect these pathways include MTOR and GSK3B (**Figure 8A**). ERPs from ERG (+) tumors which are localized to the plasma membrane or released into the extracellular compartments include: P21 protein activated kinase 1 (PAK1); synaptotagmin1 (SYN1), a regulator of exocytosis; components of the clathrin-mediated endocytosis, epidermal growth factor receptor pathway substrate 15 (EPS15) and dynamin 1 (DMN1); S100 calcium binding protein A13 (S100A13) and glutathione peroxidase 3 (GPX3) (**Figure 8A**).

Pathways that were found to be enriched for ERPs derived from ERG (–) tumors, as listed according to p-value rankings in **Table 2B**, function in the proteolytic degradation of proteins (proteasome pathway), integrin-mediated cell migration (mammalian calpain pathway), MAP kinase pathway activation via G protein coupled receptors (GPCR pathway), actin remodeling and cell migration (CDC42-RAC pathway), and the prevention of oxidative damage of proteins (redox pathway). The nodes that connect these pathways function in the control of cell motility (CDC42 and Calpain II (CAPN2)), proteolysis of extracellular matrix (plasminogen (PLG)), fatty acid metabolism (adiponectin (ADIPOQ)), and signal transduction at the caveolae scaffolding of plasma membrane (caveolin I (Cav1)) (**Figure 8B**).

Association of *Myosin VI (MYO6)* and *Monoamine oxidase A (MAOA)* to ERG mRNA and protein expression

In order to identify biomarkers that are tightly regulated by ERG, both at the level of protein expression and gene expression, we compared ERPs that are detected at higher ratios in ERG (+) or in ERG (–) tumors against the CPDR tumor vs. normal 80-GeneChip gene

expression dataset (GSE32448) of tumor vs. normal gene expression ratios from 14 ERG (–) and 26 ERG (+) cases (5). ERPs were matched against the probesets in this dataset and linear regression model fitting was used to identify genes that most closely correlate to or inversely correlate to ERG expression. By ranking the probesets according to their differential expression, *MYO6* was identified to correlate most closely to the gene expression profile of ERG across 40 patients (p-value = 3.92E-06) (**Table 3A, Figure 9A, B and D**). The probability of differential expression of *MYO6* in ERG (+) and ERG (–) tumors relative to *ERG* was 0.92. There is a 3.6-fold difference between the means of gene expression ratios between ERG (+) vs. ERG (–) cases for *MYO6*. On the contrary, the expression of *MAOA* is noted have the most statistically significant inverse correlation to *ERG* expression (p-value=0.0134) and a high probability to be mutually exclusive to *ERG* (0.90). (**Table 3B, Figure 9C and D**).

Verification of Proteomic and Gene expression Data for Myosin VI and MAOA

The correlation of Myosin VI and inverse correlation of MAOA to ERG expression were further validated in independent *ERG* siRNA experiments using VCaP cells. The silencing of *ERG* in comparison to the control experiment resulted in a reduction of Myosin VI expression by half compared to a two-fold increase of MAOA expression (**Figure 9E**), as measured by quantitative evaluation of the immunoblot intensities. Immunofluorescence assay confirmed the diminished expression of Myosin VI expression and upregulation of MAOA in response to *ERG* siRNA (**Figure 9F**). The response of MAOA expression to ERG protein levels corroborates with the data from mass spectrometric analysis, which show MAOA to be detected exclusively in ERG (–) tumors and at approximately 3-fold higher MS ratios in *ERG* siRNA vs. NT siRNA treated VCaP cells (**Figure 3B**).

In order to find out the status of Myosin VI and MAOA expression in other CaP cells that do not express ERG, we examined the expression of these two proteins in a panel of CaP cell lines (**Figure 9G**). In addition to VCaP cells, Myosin VI was shown to be strongly expressed in LNCaP cells, which lacks ERG expression. Interestingly, the expression of MAOA was shown to be associated with the level of AR expression in AR positive LNCaP, C4-2B and CWR22rv1 cells. We next examined the regulation of Myosin VI and MAOA by androgen using LNCaP cell and VCaP cells grown under starvation conditions and induced with 0.1 nM and 1 nM of R1881 (**Figure 9H**). Myosin VI expression level did not show any detectable response to R1881 in either LNCaP or VCaP cells. However, a two-fold increase for MAOA expression in LNCaP cells was observed after 48 h induction, both at 0.1 nM and at 1 nM, as measured from the immunoblot intensities (**Figure 9H, right panel**). In contrast, MAOA expression is downregulated concomitant with the R1881 induced ERG expression in VCaP cells (**Figure 9H, left panel**), which confirms the inverse correlation gene expression between MAOA and ERG.

Meta-analysis of the association of gene expression between ERG, *MYO6* and MAOA in independent datasets

The association of gene expression between *ERG* with *MYO6* and *MAOA* was further evaluated in two independent CaP gene expression data sets with known *TMPRSS2-ERG* gene fusion status: the study involving the Swedish watchful waiting cohort (GSE16560)

(34) and the Memorial Sloan-Kettering Cancer Center (MSKCC) study using primary and metastatic tumors (GSE21032) (35). ERG fusion status was available in 272 of the 281 cases from the Swedish watchful waiting cohort by FISH analysis. In the MSKCC cohort, ERG fusion status was confirmed for 128 cases by array comparative genomic hybridization (aCGH) data. Patient data were clustered according to ERG fusion status and the ranges for log 2 gene expression for ERG fusion positive and ERG fusion negative were plotted as box and whisker plots. *MYO6* expression was significantly higher in ERG (+) tumors versus ERG (–) tumors in both the Swedish watchful waiting cohort (p-value = 1.71 E-6) and the MSKCC cohort (p-value = 1.40 E-7), confirming that *MYO6* expression is significantly correlated with *TMPRSS2-ERG* fusion or ERG over-expression. However, a similar evaluation of MAOA expression did not show any inverse correlation as observed in this study.

Discussion

The discovery of *ERG* overexpression in prostate tumors and the fusion of genes involving *TMPRSS2* promoter region with the *ERG* coding sequences in more than 50% of CaP has opened avenues for exploration of biomarkers useful for the detection and the stratification of CaP. The PSA assay used in the clinical screening of CPa is known to lack both sensitivity and specificity (36). Therefore there is a need to identify biomarkers that have the potential to not only accurately detect clinically relevant CaP in asymptomatic patients but also able to differentiate indolent from aggressive CaP. Analysis of ERG (+) and ERG (–) tumor specimens is likely to provide additional information about novel biomarkers for potential clinical use.

We analyzed ERG responsive proteome in CaP cells with the aim to understand the function of ERG in the etiology of CaP and to identify biomarkers that are associated with ERG (+) or ERG (–) status of the prostate tumor. A notable feature of this study is the typing of tumors for ERG expression by IHC followed by targeted selection by LCM to overcome the challenges imposed by the presence of ERG (+) and ERG (–) tumor foci in the same prostate. Hence, a straightforward comparative analysis involving ERG (+) and ERG (–) tumors and normal cells is likely to show the potential differences at the proteome level. Unlike other CaP-proteomics studies (18,20,37,38), the current study focused specifically on the detection of proteins that are differentially expressed in relation to ERG oncoprotein status. To address this, we have utilized a unique proteomics platform based on C1TP/CZE multidimensional separation coupled with nano-ESI-MS/MS, which involve minimal front end purification prior to mass spectrometry analysis. The ability to reproducibly detect over 80% of the same peptides in consecutive runs using aliquots of the same protein samples demonstrated the reliability of the techniques used (**Supplementary Figure 1D**).

In addition to normal prostate epithelial cells and ERG stratified tumor cells, we have also analyzed the ERG responsive proteome of VCaP cell line. Such an analysis revealed a distinct pattern of up and downregulation of proteins in response to ERG that was corroborated by concordance to mRNA expression reported by previous gene expression analyses (13). The detection of similar responses in protein and mRNA expression in protein markers of prostate luminal epithelial differentiation and secretory function to ERG siRNA

knock-down further confirmed the reliability of the methods used in this proteomic analysis. Examples of these proteins, which are shown in **Figure 3** and in **Supplementary Figure 3**, include KLK3 (PSA), SLC45A3, TMPRSS2 and prostate-specific membrane antigen-like protein (FOLH1B), semenogelin-2 (SEMG2) and transgelin (TAGLN).

ERP highlights overlapping pathways in ERG (+) and ERG (–) prostate tumors and in VCaP cell line

The characterization of ERG function through analysis of interaction networks based on ERG responsive proteome datasets captured a representation of previously reported ERG target genes (11-13,16). The overlapping signal transduction networks revealed for ERG (+) and ERG (–) prostate tumors and NT siRNA and *ERG* siRNA treated VCaP cells are consistent with the activation of cell growth and cell proliferation and the inhibition of prostate epithelial differentiation by ERG oncoprotein (**Figure 4A and B**).

A more concise signal transduction network of literature-based interactions of the ERP was generated from the 330 proteins concordantly regulated by ERG in both tumors and in VCaP cells. The central nodes of this network (**Figure 4C**), represented by vimentin (VIM) and albumin (ALB) highlights the role ERG plays in regulating modulators of glandular prostate epithelial differentiation and secretory function. Vimentin is an intermediate filament protein that is expressed early during cell differentiation, promotes cell invasiveness, is expressed by motile prostate cell lines and positively correlates with poorly differentiated cancers and bone metastases (39). Albumin acts as a carrier protein for steroids, fatty acids, and thyroid hormones and functions to stabilize extracellular fluid volume in body fluids including prostatic fluids (40). This network complements the signal transduction pathways from *GeneRanker* analysis, which show an enrichment of pathways regulating cytoskeletal and actin reorganization, cell migration, protein biosynthesis and proteasome and ER-associated protein degradation pathways (**Table 1**). These pathways underscore the association between structure and function during prostate epithelial differentiation or epithelial-mesenchymal-transition (EMT). During these events, changes in cell shape and polarity occur simultaneously with changes in the expression of protein markers of prostate epithelial differentiation or EMT. For example, the dynamic response to ERG expression by Rho-GTPase CDC42-RAC1 signaling related pathways that regulate actin filament dynamics (**Figure 6C and D**) is accompanied by equally robust alterations to AR function evident from in the dysregulation of PSA, SLC45A3, and PAP/ACPP (**Figure 5**).

ERPs from ERG (+) and ERG (–) tumors reveal distinct protein markers and signature pathways

The disparity in ERG fusion frequency among different ethnic populations points to yet undiscovered genetic alterations that may contribute to the initiation and progression of CaP. We have defined the ERP by ERG expression status to better understand the biological features that distinguish these two classes of tumors. The different profiles of the ERP from ERG (+) and ERG (–) tumors, while highlighting the role ERG plays in regulating diverse cellular functions, may reveal distinctive signatures that could help to stratify ERG (+) from ERG (–) tumors and discover new treatment options. The identification of **204** and **504** ERPs unique for ERG (+) and ERG (–) tumors, respectively, represent a distinct and

informative subset of the ERPs (**Figure 7**). The connection of PAK, CDC42 and AKT pathways enriched in ERG (+) tumor derived ERPs by MTOR and GSK3B, underscore the impact that ERG overexpression may have on the PI3K/AKT/mTOR, the PI3K/AKT/GSK3B or Wnt signaling pathways (**Table 2A, Figure 8A**). The pathways enriched in ERPs from ERG (–) tumors regulate functions that include CDC42-RAC and calpain modulated cell motility and proteasome and redox functions. These pathways were shown to be connected by CDC42, CAV1, SOD2, ADIPOQ and MAPK3 (**Table 2B, Figure 8B**). These links reveal that in addition to changes in cell differentiation and migration, cell survival and apoptosis, the absence of ERG in these tumors also affect changes in endocytosis and protein trafficking, redox and proteasome functions, as well as fatty acid metabolism.

The PI3K/AKT/mTOR and the MAP kinase pathways have been implicated in CaP tumorigenesis and development of castrate resistant prostate cancer, and targeting these pathways to treat CaP using small molecule inhibitors is an active area of investigation (41,42). Several of the ERG (+) and ERG (–) specific ERPs, which are identified to be secreted in body fluids or found localized to the plasma membrane warrant more detailed studies to evaluate their potential as diagnostic protein biomarkers or as targets for treatment for CaP.

Correlation of Myosin VI and Monoamine oxidase A with ERG gene and protein expression

The combined analysis of proteomic and genomic data for proteins positively correlated and inversely correlated to ERG expression identified *Myosin VI* and *MAOA* as potential protein and gene expression biomarkers for ERG (+) and ERG (–) tumors. Myosin VI is one of the unconventional myosins, actin-based molecular motors involved in intracellular vesicle and organelle transport. Although Myosin VI has previously been reported to be over-expressed in CaP (43), this is the first report of a correlation with ERG expression in CaP. The localization of Myosin VI on endosomes and the trans-Golgi network, suggest a function in regulating protein secretion (44,45). Myosin VI has been implicated in autophagy by promoting autophagosome maturation and driving fusion with lysosomes (46). The correlation of Myosin VI gene and protein to the expression of ERG suggests possible transcriptional modulation of Myosin VI by ERG.

MAOA is a mitochondrial enzyme expressed in the brain and peripheral tissues that degrades biogenic amines including neurotransmitters serotonin and norepinephrine by oxidative deamination, resulting in the production of hydrogen peroxide (47). In normal prostate glands MAOA is absent or found at very low levels in the luminal secretory epithelial but is elevated in the basal epithelia (48). Increased MAOA expression is also found to be associated with poorly differentiated high grade CaP (49) while a rare polymorphism of the MAOA promoter that confers low expression was associated with reduced CaP risk (50). In this study, both gene and protein expression of MAOA are found to be expressed at higher ratios in ERG (–) tumors compared to ERG (+) tumors. MAOA was detected at almost 3-fold higher MS ratios in ERG silenced VCaP cells compared to NT siRNA control (**Figure 3B**). In the context of prostate epithelium, MAOA is regulated by androgens through promoter-upstream glucocorticoid/androgen response elements (51). We showed that although MAOA is upregulated by R1881 induction in ERG (–) LNCaP cells,

the expression of ERG in VCaP cells appear to interfere with this activation. The inverse correlation of MAOA with ERG suggests that MAOA may define a separate and distinct category of ERG (–) but androgen sensitive tumors. Although the primary function of MAOA is the oxidative deamination of monoamine neurotransmitters, whether the overexpression of MAOA in prostate epithelium leads to the oxidative deamination of prostatic polyamines such as spermine or spermidine, and the release of reactive oxygen species that contribute to tumorigenesis, remains to be shown.

A comparison of *ERG*, *MYO6* and *MAOA* expression to independent gene expression datasets from Sboner *et. al.*, (34) and Taylor *et. al.*, (35) confirmed the significant correlation of *MYO6* but not the inverse correlation of *MAOA* with ERG. This could be attributed the differences in procedures used for mRNA sampling from tissues or in the sensitivity of microarray platforms. Unlike the two larger datasets, which used only tumor mRNA, the 80-GeneChip dataset analyzed in this study used mRNA from both tumor and normal cells. The preparation of the mRNA from LCM isolated cells further reduces heterogeneity or contamination of non-tumor cells.

Potential treatment of CaP based on the stratification for ERG, Myosin VI and MAOA expression

The prevalence *TMPRSS2-ERG* fusion and its function as a driver mutation in the initiation and progression of CaP present a promising therapeutic target. Transcription factors such as ERG were considered “undruggable”, mainly due to its inaccessibility. Nevertheless inhibition of ERG function exemplified by the use of small molecule inhibitors and *TMPRSS2-ERG* fusion junction specific siRNAs have been successfully carried out with varying degree of success (52). The inhibition of the DNA dependent interaction of ERG with poly(ADP-ribose) polymerase (PARP) with PARP inhibitors (53) has advanced rapidly due to the availability of pharmacological inhibitors. The development of combination assays using triple immunostaining cocktails and /or nucleic acid detection panels could help categorize tumors according to their expression of ERG, Myosin VI or MAOA. Parallel advances in the development of specific small molecule inhibitors, used either alone or in a combinatorial approach with other drugs, could be applied to synergistically inhibit ERG (+) or ERG (–) tumors. The small molecule inhibitor, 2,4,6-triiodophenol has been recently shown to reduce the number of Myosin VI-dependent vesicle fusion events at the plasma membrane during constitutive secretion (54) and could be used to inhibit the formation of autophagosomes (46). Since the genetic alterations that define ERG (–) tumors are not well understood, the identification of ERPs overexpressed in ERG (–) tumors or are expressed in inverse correlation to ERG, such as MAOA, could help to uncover the mechanisms responsible for the initiation and progression of ERG (–) CaP. The therapeutic potential of MAOA inhibitors such as clorgyline, which induces differentiation in primary cultures of normal basal epithelial cells and high-grade CaP, is being actively investigated (55). Further developments in this direction could translate to the development of clinical treatments for CaP patients based on the ERG expression status of the tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin*. 2013; 63(1):11–30. [PubMed: 23335087]
2. Dobi, A.; Sreenath, T.; Srivastava, S. Androgen-Dependent Oncogenic Activation of ETS Transcription Factors by Recurrent Gene Fusions in Prostate Cancer: Biological and Clinical Implications.. In: Wang, Z., editor. *Androgen-Responsive Genes in Prostate Cancer*. Springer; New York: 2013. p. 307-328.
3. Irshad S, Abate-Shen C. Modeling prostate cancer in mice: something old, something new, something premalignant, something metastatic. *Cancer Metastasis Rev*. 2013; 32(1-2):109–122. [PubMed: 23114843]
4. Barbieri CE, Bangma CH, Bjartell A, Catto JW, Culig Z, Gronberg H, Luo J, Visakorpi T, Rubin MA. The Mutational Landscape of Prostate Cancer. *Eur Urol*. 2013
5. Petrovics G, Liu A, Shaheduzzaman S, Furusato B, Sun C, Chen Y, Nau M, Ravindranath L, Dobi A, Srikantan V, Sesterhenn IA, McLeod DG, Vahey M, Moul JW, Srivastava S. Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. *Oncogene*. 2005; 24(23):3847–3852. [PubMed: 15750627]
6. Rubin MA, Maher CA, Chinnaiyan AM. Common gene rearrangements in prostate cancer. *J Clin Oncol*. 2011; 29(27):3659–3668. [PubMed: 21859993]
7. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science*. 2005; 310(5748):644–648. [PubMed: 16254181]
8. Mao X, Yu Y, Boyd LK, Ren G, Lin D, Chaplin T, Kudahetti SC, Stankiewicz E, Xue L, Beltran L, Gupta M, Oliver RT, Lemoine NR, Berney DM, Young BD, Lu YJ. Distinct genomic alterations in prostate cancers in Chinese and Western populations suggest alternative pathways of prostate carcinogenesis. *Cancer Res*. 2010; 70(13):5207–5212. [PubMed: 20516122]
9. Magi-Galluzzi C, Tsusuki T, Elson P, Simmerman K, LaFargue C, Esgueva R, Klein E, Rubin MA, Zhou M. TMPRSS2-ERG gene fusion prevalence and class are significantly different in prostate cancer of Caucasian, African-American and Japanese patients. *Prostate*. 2011; 71(5):489–497. [PubMed: 20878952]
10. Rosen P, Pfister D, Young D, Petrovics G, Chen Y, Cullen J, Bohm D, Perner S, Dobi A, McLeod DG, Sesterhenn IA, Srivastava S. Differences in frequency of ERG oncoprotein expression between index tumors of Caucasian and African American patients with prostate cancer. *Urology*. 2012; 80(4):749–753. [PubMed: 22950997]
11. Gasi Tandefelt D, Boormans JL, van der Korput HA, Jenster GW, Trapman J. A 36-gene Signature Predicts Clinical Progression in a Subgroup of ERG-positive Prostate Cancers. *Eur Urol*. 2013
12. Massoner P, Kugler KG, Unterberger K, Kuner R, Mueller LA, Falth M, Schafer G, Seifarth C, Ecker S, Verdorfer I, Graber A, Sultmann H, Klocker H. Characterization of transcriptional changes in ERG rearrangement-positive prostate cancer identifies the regulation of metabolic sensors such as neuropeptide Y. *PLoS One*. 2013; 8(2):e55207. [PubMed: 23390522]

13. Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, Shaheduzzaman S, Tan SH, Vaidyanathan G, Whitman E, Hawksworth DJ, Chen Y, Nau M, Patel V, Vahey M, Gutkind JS, Sreenath T, Petrovics G, Sesterhenn IA, McLeod DG, Srivastava S. TMPRSS2-ERG fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. *Oncogene*. 2008; 27(40):5348–5353. [PubMed: 18542058]
14. Tomlins SA, Laxman B, Varambally S, Cao X, Yu J, Helgeson BE, Cao Q, Prensner JR, Rubin MA, Shah RB, Mehra R, Chinnaiyan AM. Role of the TMPRSS2-ERG gene fusion in prostate cancer. *Neoplasia*. 2008; 10(2):177–188. [PubMed: 18283340]
15. Kunderfranco P, Mello-Grand M, Cangemi R, Pellini S, Mensah A, Albertini V, Malek A, Chiorino G, Catapano CV, Carbone GM. ETS transcription factors control transcription of EZH2 and epigenetic silencing of the tumor suppressor gene Nkx3.1 in prostate cancer. *PLoS One*. 2010; 5(5):e10547. [PubMed: 20479932]
16. Sreenath T, Dobi A, Petrovics G, Srivastava S. Oncogenic activation of ERG: A predominant mechanism in prostate cancer. *J Carcinog*. 2011; 10:37. [PubMed: 22279422]
17. Cho JY, Lee M, Ahn JM, Park ES, Cho JH, Lee SJ, Kim BG, Heo SH, Park HJ, Zerbini LF, Hwang D, Libermann TA. Proteomic analysis of a PDEF Ets transcription factor-interacting protein complex. *J Proteome Res*. 2009; 8(3):1327–1337. [PubMed: 19203193]
18. Drake RR, Elschenbroich S, Lopez-Perez O, Kim Y, Ignatchenko V, Ignatchenko A, Nyalwidhe JO, Basu G, Wilkins CE, Gjurich B, Lance RS, Semmes OJ, Medin JA, Kislinger T. In-depth proteomic analyses of direct expressed prostatic secretions. *J Proteome Res*. 2010; 9(5):2109–2116. [PubMed: 20334419]
19. Khan AP, Poisson LM, Bhat VB, Fermin D, Zhao R, Kalyana-Sundaram S, Michailidis G, Nesvizhskii AI, Omenn GS, Chinnaiyan AM, Sreekumar A. Quantitative proteomic profiling of prostate cancer reveals a role for miR-128 in prostate cancer. *Mol Cell Proteomics*. 2010; 9(2):298–312. [PubMed: 19955085]
20. Sardana G, Jung K, Stephan C, Diamandis EP. Proteomic analysis of conditioned media from the PC3, LNCaP, and 22Rv1 prostate cancer cell lines: discovery and validation of candidate prostate cancer biomarkers. *J Proteome Res*. 2008; 7(8):3329–3338. [PubMed: 18578523]
21. Martin DB, Gifford DR, Wright ME, Keller A, Yi E, Goodlett DR, Aebersold R, Nelson PS. Quantitative proteomic analysis of proteins released by neoplastic prostate epithelium. *Cancer Res*. 2004; 64(1):347–355. [PubMed: 14729644]
22. Hu Y, Dobi A, Sreenath T, Cook C, Tadase AY, Ravindranath L, Cullen J, Furusato B, Chen Y, Thangapazham RL, Mohamed A, Sun C, Sesterhenn IA, McLeod DG, Petrovics G, Srivastava S. Delineation of TMPRSS2-ERG Splice Variants in Prostate Cancer. *Clin Cancer Res*. 2008 in press.
23. Perner S, Demichelis F, Beroukhim R, Schmidt FH, Mosquera JM, Setlur S, Tchinda J, Tomlins SA, Hofer MD, Pienta KG, Kuefer R, Vessella R, Sun XW, Meyerson M, Lee C, Sellers WR, Chinnaiyan AM, Rubin MA. TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer. *Cancer Res*. 2006; 66(17):8337–8341. [PubMed: 16951139]
24. Furusato B, Tan SH, Young D, Dobi A, Sun C, Mohamed AA, Thangapazham R, Chen Y, McMaster G, Sreenath T, Petrovics G, McLeod DG, Srivastava S, Sesterhenn IA. ERG oncoprotein expression in prostate cancer: clonal progression of ERG-positive tumor cells and potential for ERG-based stratification. *Prostate Cancer Prostatic Dis*. 2010; 13(3):228–237. [PubMed: 20585344]
25. Johann DJ, Rodriguez-Canales J, Mukherjee S, Prieto DA, Hanson JC, Emmert-Buck M, Blonder J. Approaching solid tumor heterogeneity on a cellular basis by tissue proteomics using laser capture microdissection and biological mass spectrometry. *J Proteome Res*. 2009; 8(5):2310–2318. [PubMed: 19284784]
26. Fang X, Wang W, Yang L, Chandrasekaran K, Kristian T, Balgley BM, Lee CS. Application of capillary isotachopheresis-based multidimensional separations coupled with electrospray ionization-tandem mass spectrometry for characterization of mouse brain mitochondrial proteome. *Electrophoresis*. 2008; 29(10):2215–2223. [PubMed: 18425750]
27. Fang X, Yang L, Wang W, Song T, Lee CS, DeVoe DL, Balgley BM. Comparison of electrokinetics-based multidimensional separations coupled with electrospray ionization-tandem

- mass spectrometry for characterization of human salivary proteins. *Anal Chem.* 2007; 79(15): 5785–5792. [PubMed: 17614365]
28. Gu Y, Li H, Miki J, Kim KH, Furusato B, Sesterhenn IA, Chu WS, McLeod DG, Srivastava S, Ewing CM, Isaacs WB, Rhim JS. Phenotypic characterization of telomerase-immortalized primary non-malignant and malignant tumor-derived human prostate epithelial cell lines. *Exp Cell Res.* 2006; 312(6):831–843. [PubMed: 16413016]
 29. Mohamed AA, Tan SH, Sun C, Shaheduzzaman S, Hu Y, Petrovics G, Chen Y, Sesterhenn IA, Li H, Sreenath T, McLeod DG, Dobi A, Srivastava S. ERG oncogene modulates prostaglandin signaling in prostate cancer cells. *Cancer Biol Ther.* 2011; 11(4):410–417. [PubMed: 21178489]
 30. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical applications in genetics and molecular biology.* 2004:3. Article3.
 31. Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Molecular systems biology.* 2005; 2005; 1:0010. [PubMed: 16729045]
 32. Lin D, Edwards AS, Fawcett JP, Mbamalu G, Scott JD, Pawson T. A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat Cell Biol.* 2000; 2(8):540–547. [PubMed: 10934475]
 33. deLeon O, Puglise JM, Liu F, Smits J, ter Beest MB, Zegers MM. Pak1 regulates the orientation of apical polarization and lumen formation by distinct pathways. *PLoS One.* 2012; 7(7):e41039. [PubMed: 22815903]
 34. Sboner A, Demichelis F, Calza S, Pawitan Y, Setlur SR, Hoshida Y, Perner S, Adami HO, Fall K, Mucci LA, Kantoff PW, Stampfer M, Andersson SO, Vahrenhorst E, Johansson JE, Gerstein MB, Golub TR, Rubin MA, Andren O. Molecular sampling of prostate cancer: a dilemma for predicting disease progression. *BMC Med Genomics.* 2010; 3:8. [PubMed: 20233430]
 35. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B, Antipin Y, Mitsiades N, Landers T, Dolgalev I, Major JE, Wilson M, Succi ND, Lash AE, Heguy A, Eastham JA, Scher HI, Reuter VE, Scardino PT, Sander C, Sawyers CL, Gerald WL. Integrative genomic profiling of human prostate cancer. *Cancer Cell.* 2010; 18(1):11–22. [PubMed: 20579941]
 36. Prensner JR, Rubin MA, Wei JT, Chinnaiyan AM. Beyond PSA: the next generation of prostate cancer biomarkers. *Sci Transl Med.* 2012; 4(127):127rv123.
 37. Goo YA, Liu AY, Ryu S, Shaffer SA, Malmstrom L, Page L, Nguyen LT, Doneanu CE, Goodlett DR. Identification of secreted glycoproteins of human prostate and bladder stromal cells by comparative quantitative proteomics. *Prostate.* 2009; 69(1):49–61. [PubMed: 18792917]
 38. Hood BL, Malehorn DE, Conrads TP, Bigbee WL. Serum proteomics using mass spectrometry. *Methods Mol Biol.* 2009; 520:107–128. [PubMed: 19381950]
 39. Lang SH, Hyde C, Reid IN, Hitchcock IS, Hart CA, Bryden AA, Villette JM, Stower MJ, Maitland NJ. Enhanced expression of vimentin in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma. *Prostate.* 2002; 52(4):253–263. [PubMed: 12210485]
 40. Grover PK, Resnick MI. Analysis of prostatic fluid: evidence for the presence of a prospective marker for prostatic cancer. *Prostate.* 1995; 26(1):12–18. [PubMed: 7531324]
 41. Bitting RL, Armstrong AJ. Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. *Endocr Relat Cancer.* 2013; 20(3):R83–99. [PubMed: 23456430]
 42. Grant S. Cotargeting survival signaling pathways in cancer. *J Clin Invest.* 2008; 118(9):3003–3006. [PubMed: 18725993]
 43. Dunn TA, Chen S, Faith DA, Hicks JL, Platz EA, Chen Y, Ewing CM, Sauvageot J, Isaacs WB, De Marzo AM, Luo J. A novel role of myosin VI in human prostate cancer. *Am J Pathol.* 2006; 169(5):1843–1854. [PubMed: 17071605]
 44. Wei S, Dunn TA, Isaacs WB, De Marzo AM, Luo J. GOLPH2 and MYO6: putative prostate cancer markers localized to the Golgi apparatus. *Prostate.* 2008; 68(13):1387–1395. [PubMed: 18543251]
 45. Puri C, Chibalina MV, Arden SD, Kruppa AJ, Kendrick-Jones J, Buss F. Overexpression of myosin VI in prostate cancer cells enhances PSA and VEGF secretion, but has no effect on endocytosis. *Oncogene.* 2010; 29(2):188–200. [PubMed: 19855435]

46. Tumbarello DA, Waxse BJ, Arden SD, Bright NA, Kendrick-Jones J, Buss F. Autophagy receptors link myosin VI to autophagosomes to mediate Tom1-dependent autophagosome maturation and fusion with the lysosome. *Nat Cell Biol.* 2012; 14(10):1024–1035. [PubMed: 23023224]
47. Shih JC, Chen K, Ridd MJ. Monoamine oxidase: from genes to behavior. *Annual review of neuroscience.* 1999; 22:197–217.
48. Zhao H, Flamand V, Peehl DM. Anti-oncogenic and pro-differentiation effects of clorgyline, a monoamine oxidase A inhibitor, on high grade prostate cancer cells. *BMC Med Genomics.* 2009; 2:55. [PubMed: 19691856]
49. True L, Coleman I, Hawley S, Huang CY, Gifford D, Coleman R, Beer TM, Gelmann E, Datta M, Mostaghel E, Knudsen B, Lange P, Vessella R, Lin D, Hood L, Nelson PS. A molecular correlate to the Gleason grading system for prostate adenocarcinoma. *Proc Natl Acad Sci U S A.* 2006; 103(29):10991–10996. [PubMed: 16829574]
50. White TA, Kwon EM, Fu R, Lucas JM, Ostrander EA, Stanford JL, Nelson PS. The monoamine oxidase A gene promoter repeat and prostate cancer risk. *Prostate.* 2012; 72(15):1622–1627. [PubMed: 22473857]
51. Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, Mills IG. New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. *EMBO Rep.* 2007; 8(9):871–878. [PubMed: 17721441]
52. Rahim S, Uren A. Emergence of ETS transcription factors as diagnostic tools and therapeutic targets in prostate cancer. *Am J Transl Res.* 2013; 5(3):254–268. [PubMed: 23634237]
53. Brenner JC, Ateeq B, Li Y, Yocum AK, Cao Q, Asangani IA, Patel S, Wang X, Liang H, Yu J, Palanisamy N, Siddiqui J, Yan W, Cao X, Mehra R, Sabolch A, Basrur V, Lonigro RJ, Yang J, Tomlins SA, Maher CA, Elenitoba-Johnson KS, Hussain M, Navone NM, Pienta KJ, Varambally S, Feng FY, Chinnaiyan AM. Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. *Cancer Cell.* 2011; 19(5):664–678. [PubMed: 21575865]
54. Heissler SM, Selvadurai J, Bond LM, Fedorov R, Kendrick-Jones J, Buss F, Manstein DJ. Kinetic properties and small-molecule inhibition of human myosin-6. *FEBS Lett.* 2012; 586(19):3208–3214. [PubMed: 22884421]
55. Flamand V, Zhao H, Peehl DM. Targeting monoamine oxidase A in advanced prostate cancer. *J Cancer Res Clin Oncol.* 2010; 136(11):1761–1771. [PubMed: 20204405]

Figure 1. Analysis of ERG Responsive Proteome

(A) Outline of the strategy for analysis of ERG responsive proteome from ERG (+), ERG (-), and benign cells isolated by LCM from prostate cancer specimens and from *TMPPSS2-ERG* positive VCaP cells. (B) IHC of representative tumor specimens used for LCM with ERG (-) (a & b) and ERG (+) (c & d) expression, stained with H & E and with ERG MAb. (C) Quality control of VCaP cell lysates used in proteomic analysis. Cell lysates were prepared from 50 nM NT siRNA (lane 1) and *ERG* siRNA (lane 2) transfected VCaP cells and ERG protein was analyzed by immuno-blot analysis with ERG MAb.

Figure 2. Global ERG Responsive Proteome detected with at least 2 unique peptide hits and at 1% FDR from LCM isolated tumors (A) and from NT siRNA vs. ERG siRNA transfected VCaP cells (B).

Figure 4. Functional interaction networks of ERG Responsive Proteome

Literature based functional interaction networks of ERPs from LCM isolated prostate tumor cells (A), NT siRNA /ERG siRNA treated VCaP cells (B), and ERPs concordantly regulated by ERG from A and B. Red and green nodes represent proteins unique for ERG (+) and in ERG (–) cells, in the respective samples. Shades of red and green represent upregulated and downregulated ERPs, respectively. In (C), the left- and right-half of the nodes show response to ERG in the LCM isolated prostate tumor cells and in VCaP cells, respectively. Nodes are shown as polygons if the function is known: kinases as right pointed polygons;

phosphatases, left pointed polygons; receptors, inverted trapezoids; transporters, trapezoid; and cofactors, stars. Nodes are linked by dotted lines if association is by co-citation and by solid lines if association is by expert curation. ($\triangleright$) indicates protein A activates protein B; ($\diamond$), A modulates B; a circle and bar, A inhibits B; a filled arrowhead, gene B has a binding site for A on one of its promoters.

Figure 5. ERG knock-down induces the expression of prostate differentiation markers associated with its secretory function
Validation of the upregulated expression prostate differentiation markers, (A) SLC45A3, (B) PAP/ACPP and (C) MSMB in VCaP cells upon ERG siRNA by immunofluorescence assay and by immunoblot analyses (D).

Figure 6. ERG knock-down inhibits genes regulating cell growth and activates genes regulating prostate epithelial differentiation

EGFR pathway proteins (A) SHC1, (B) p44/ERK1 are down-regulated by *ERG* siRNA. The silencing of ERG increased the expression of the Rho-GTPase RAC1 (C), and ARHGEF7 (D). The down-regulation of SHC1 and p44/ERK1 and up-regulation of ARHGEF7 are confirmed by immunoblot analysis.

Figure 7. Identification of ERPs detected at higher ratios in ERG (+) or in ERG (-) cells
204 of 589 ERPs detected more abundantly in ERG (+) relative to ERG (-) tumors or benign tissues are unique to ERG (+) tumors. **504 of 781 ERPs** detected more abundantly in ERG (-) relative to ERG (+) tumors or benign tissues are unique to ERG (-) tumors.

Figure 8. Functional interaction networks of ERPs unique for ERG (+) and ERG (-) tumors
 Red and green nodes represent proteins upregulated in ERG (+) tumors (A) and ERG (-) tumors (B), in the respective samples.

Figure 9. Correlation of gene and protein expression between ERG with Myosin VI and MAOA
Box-plots showing the range of log 2 tumor vs.normal expression ratio from 40-patient gene expression dataset for *ERG* (213541_s_at) (A), *MYO6* (203215_S_at) (B) and *MAOA_204389_at* (C) according to ERG expression status. The line across the box and the blue spot represent the respective median and mean values, respectively. The relative expression of *ERG*, *MYO6* and *MAOA* are normalized by row or Z-score (D, top) or shown as original values (D, bottom). The correlation of *Myosin VI* and *MAOA* expression to ERG expression is validated in ERG siRNA knockdown of VCaP cells and assayed by

immunoblot analysis (E) and immunofluorescence assay (F). Expression of Myosin VI and MAOA were compared in CaP cell lines (G). Induction of VCaP and LNCaP cells with 0.1 nM and 1 nM of R1881 for 12, 24 and 48h following growth in starvation conditions for three days.

Table 1

Concordantly Regulated ERP Pathways Signal transduction pathways of 330 ERPs concordantly Regulated by ERG. Pathways are ranked by p-values that represent enrichment of proteins of a pathway in the sample.

No	Concordantly Regulated ERP Pathways	Pathway ID	P-value	# Genes (observed)	# Genes (expected)	# Genes (total)	List of observed genes
1	ROLE OF PI3K SUBUNIT P85 IN REGULATION OF ACTIN ORGANIZATION AND CELL MIGRATION	BIOCARTA:CDC42 RAC PATHWAY	6.86E-06	6	0.53	16	ACTR2, ARPC2, ARPC1B, PAK1, ARPC1A, ARPC4
3	P21(CDKN1A) ACTIVATED KINASE	PW_PAK_HOMO_SAPIENS	1.98E-05	9	1.54	68	STMN1, ARPC1B, CALD1, FLNA, PAK2, PAK1, MBP, VIM, PAK3
4	Y BRANCHING OF ACTIN FILAMENTS	BIOCARTA:ACTINY PATHWAY	1.16E-04	5	0.53	16	ACTR2, ARPC2, ARPC1B, ARPC1A, ARPC4
5	PROTEASOME COMPLEX	BIOCARTA:PROTEASOME PATHWAY	1.59E-04	7	1.22	37	PSMB2, PSMC1, PSMD7, PSMA5, PSME2, PSMA6, PSMD3
6	CELL DIVISION CYCLE 42	PW_CDC42_HOMO_SAPIENS	3.28E-04	9	2.20	97	DNM2, GNA13, ARHGAP1, INF2, RALA, PAK2, PAK1, VIM, PAK3
7	ER ASSOCIATED DEGRADATION (ERAD)	BIOCARTA:ERAD PATHWAY	2.95E-03	4	1.22	19	MAN2B1, MOGS, CANX, GANAB

Table 2A

Signal Transduction Pathways for 204 ERPs unique for ERG (+) tumors.

No	ERG (+) ERP Pathways	Pathway ID	P-value	# Genes (observed)	# Genes (expected)	# Genes (total)	List of observed genes
1	P21 (CDKN1A) ACTIVATED KINASE	PW_PAK_HOMO_SAPIENS	1.55E-05	8	1.14	68	PAK2, SYN1, PAK1, WASF2, STMN1, PAK4, CALD1, PAK3
2	CELL DIVISION CYCLE 42	NCI-Nature:CDC42	9.36E-04	6	1.17	74	GSK3B, PAK2, PAK1, MTOR, EPS8, PAK4
3	V AKT MURINE THYMOMA VIRAL ONCOGENE HOMOLOG 1	PW_AKT1_HOMO_SAPIENS	2.03E-03	11	3.99	238	PPM1G, GSK3B, PDCD4, DNAJC5, PAK1, HTT, PITPNA, PHLDB1, MTOR, UTRN, SPARC
4	AKT-MTOR	BioCarta:IGF1-MTOR	3.85E-03	6	1.51	90	SYN1, HTT, ITPR1, STMN1, EPS8, RAB11FIP5

Table 2B

Signal transduction pathways 504 ERPs unique for ERG (–) tumors.

No	ERG (–) ERP Pathways	Pathway ID	P-value	# Genes (observed)	# Genes (expected)	# Genes (total)	List of observed genes
1	PROTEASOME COMPLEX	BIOCARTA:PROTEASOME PATHWAY	5.49E-07	11	1.75	37	PSMA3, PSME1, PSMA4, PSMB2, PSMC1, PSMD7, PSMA5, PSMC4, PSME2, PSMD6, PSMB4
2	MCALPAIN AND FRIENDS IN CELL MOTILITY	BIOCARTA:MCALPAIN PATHWAY	7.73E-06	9	1.46	31	PRKAR2B, MAP2K2, CAST, PRKAR1A, CAPN2, ITGA1, PRKAR2A, MAP2K1, MAPK3
3	SIGNALING PATHWAY FROM G-PROTEIN FAMILIES	BIOCARTA:GPCR PATHWAY	2.15E-05	8	1.28	27	PRKAR2B, GNAI1, PRKAR1A, PPP3CA, ASAH1, PRKAR2A, MAP2K1, MAPK3
4	ROLE OF PI3K SUBUNIT P85 IN REGULATION OF ACTIN ORGANIZATION AND CELL MIGRATION	BIOCARTA:CDC42 RAC PATHWAY	5.47E-05	6	0.76	16	ACTR2, ARPC2, ARPC1B, CDC42, ARPC1A, ARPC4
5	REDOX	PW_REDOX_HOMO_SAPIENS	8.55E-05	14	4.28	126	HSPA4, H6PD, CSNK2A1, AKR1B1, MPST, AKR1A1, NAMPT, TXNDC17, ADIPOQ, PSME2, SOD2, TXNRD1, PRDX4, GPX1

Table 3A
Probe sets for *MYO6* revealed gene expression profiles that are most significantly correlated to ERG Expression.

Symbol	Probesets	Rank coeff=2, ERG (+), Lg2 scale	B-Statistic	Probability gene is Differentially Expressed	T-test	Med ERG (+) -Med ERG (-)	Ave ERG (+) -Ave ERG (-)
<i>ERG</i>	213541_s_at	1	12.19	1	3.21E-11	4.11	3.51
<i>MYO6</i>	203215_s_at	3	2.49	0.92	3.92E-06	1.76	1.66
<i>CSDA</i>	201160_s_at	21	-2.78	0.06	2.16E-03	0.99	1.29
<i>UTRN</i>	225093_at	25	-3.02	0.05	7.45E-03	0.81	1.03

Table 3B
Probe sets for *MAOA* revealed gene expression profiles that are most significantly inversely correlated to *ERG* Expression.

Symbol	Probesets	Rank coeff=1 (ERG-) Lg2 scale	B-Statistic	Probability gene is Differentially Expressed	T-test	Med ERG (+) -Med ERG (-)	Ave ERG (+) -Ave ERG (-)
<i>MAOA</i>	204389_at	3	2.16	0.9	1.34E-02	-0.446	-0.372
<i>IMMT</i>	242361_at	45	-2.92	0.05	1.04E-02	-0.344	-0.467
<i>NEDD4L</i>	241396_at	83	-3.65	0.03	1.41E-02	-0.338	-0.321
<i>ERG</i>	213541_s_at	2049	-5.93	0	3.21E-11	4.109	3.510