Published in final edited form as:

Prostate. 2012 May 1; 72(6): 597–611. doi:10.1002/pros.21463.

Pathway Specific Gene Expression Profiling Reveals Oxidative Stress Genes Potentially Regulated by Transcription Co-Activator LEDGF/p75 in Prostate Cancer Cells

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Abstract

BACKGROUND—Lens epithelium-derived growth factor p75 (LEDGF/p75) is a stress survival transcription co-activator and autoantigen that is overexpressed in tumors, including prostate cancer (PCa). This oncoprotein promotes resistance to cell death induced by oxidative stress and chemotherapy by mechanisms that remain unclear. To get insights into these mechanisms we identified candidate target stress genes of LEDGF/p75 using pathway-specific gene expression profiling in PCa cells.

METHODS—A “Human oxidative stress and antioxidant defense” qPCR array was used to identify genes exhibiting significant expression changes in response to knockdown or overexpression of LEDGF/p75 in PC-3 cells. Validation of array results was performed by additional qPCR and immunoblotting.

RESULTS—Cytoglobin (CYGB), Phosphoinositide-binding protein PIP3-E/IPCEF-1, superoxidase dismutase 3 (SOD3), thyroid peroxidase (TPO), and albumin (ALB) exhibited significant transcript down- and up-regulation in response to LEDGF/p75 knockdown and overexpression, respectively. CYGB gene was selected for further validation based on its emerging role as a stress oncoprotein in human malignancies. In light of previous reports indicating that LEDGF/p75 regulates peroxiredoxin 6 (PRDX6), and that PRDXs exhibit differential expression in PCa, we also examined the relationship between these proteins in PCa cells. Our validation data revealed that changes in LEDGF/p75 transcript and protein expression in PCa cells closely paralleled those of CYGB, but not those of the PRDXs.

CONCLUSIONS—Our study identifies CYGB and other genes as stress genes potentially regulated by LEDGF/p75 in PCa cells, and provides a rationale for investigating their role in PCa and in promoting resistance to chemotherapy- and oxidative stress-induced cell death.

Keywords

LEDGF/p75; prostate cancer; oxidative stress; gene profiling; peroxiredoxin; cytoglobin

INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed cancer in men and the second leading cause of male cancer deaths in the U.S.A [1]. This growing public health challenge is aggravated by the documented disparities in the incidence and mortality of PCa affecting African-American (AA) men [2]. The identification of reliable markers that are predictive of PCa and influence tumor survival mechanisms is essential for the development of more effective therapeutic interventions for advanced stage PCa, and the elimination of health disparities associated with this disease.

Emerging evidence suggests that an augmented state of cellular oxidative stress contributes to the etiology and progression of PCa by inducing the upregulation of stress and redox proteins that protect cells against oxidative damage and promote tumor progression [3-6].

Lens epithelium-derived growth factor of 75 kDa (LEDGF/p75), also known as dense fine speckled autoantigen of 70 kD (DFS70), transcription coactivator p75 (TCP75), and PC4 and SFRS1 interacting protein (PSIP), is a stress-regulated survival protein that protects mammalian cells against oxidative stress and other environmental stressors [7-12]. LEDGF/p75 has been implicated in autoimmunity, inflammation, neuroprotection, HIV-1 replication, and cancer [13-23]. Our group demonstrated previously that LEDGF/p75 is an autoantigen in PCa that is overexpressed in prostate tumor cells and tissues [22]. Recent studies by our group demonstrated that this protein is also upregulated in other major human cancers [Basu and colleagues, unpublished data]. The overexpression of LEDGF/p75 in PCa cells and other tumor cell types attenuates lysosomal membrane permeabilization in the presence of chemotherapeutic drugs, thus promoting chemoresistance [23-26].

The mechanisms by which LEDGF/p75 promotes resistance to cell death induced by oxidative stress and chemotherapy remain largely unknown, although it has been proposed that this protein transactivates stress, antioxidant, and cancer-associated genes via interaction with heat shock elements (HSE) and stress response elements (STRE) in promoter regions [27-29]. Specific genes known to be transactivated by LEDGF/p75 are heat shock protein 27 (Hsp27), peroxiredoxin 6 (PRDX6), involucrin, alcohol dehydrogenase, aldehyde dehydrogenase, α B-crystallin, gamma-glutamylcysteine synthetase, vascular endothelial growth factor C (VEGF-C), and interleukin 6 (IL-6) [27-35]. There is also evidence that LEDGF/p75 interacts with specific transcription factors to facilitate the transactivation of stress and cancer-related genes. For instance, LEDGF/p75 binds to and tethers the MYC-binding protein JPO2 to chromatin, suggesting that it plays a role in the MYC-regulatory network [36,37]. LEDGF/p75 also interacts with the menin/mixed lineage leukemia (MLL) transcription complex to facilitate the expression of cancer associated target genes and leukemic transformation [38]. Previous studies by our group demonstrated that the transcriptional and stress-survival activities of LEDGF/p75 are regulated by alternative splicing and caspase-mediated disruption of its N- and C-terminal domains [15,30].

This study was undertaken to identify candidate target genes regulated by LEDGF/p75 using stress gene expression profiling analysis in PC-3 prostate tumor cells depleted of this protein or overexpressing it. Since LEDGF/p75 transcriptionally activates PRDX6 in COS-7 and lens epithelial cells [33], and members of the PRDX family are upregulated in PCa [6], we also investigated its influence on the expression of the PRDXs in PCa cells.

MATERIALS AND METHODS

Prostate Cell Lines and Antibodies

RWPE-1, RWPE-2, DU 145, PC-3, LNCaP, MDA PCa 2b, and 22Rv1 cell lines were purchased from American Type Culture Collection. BRF-41T and BRF-55T were purchased from AthenaES. PrEC and PrSC were obtained from Clonetics, Lonza. Cell lines were cultured as recommended by the suppliers in a humidified incubator with 5% CO₂ at 37°C. The following antibodies were used: Mouse monoclonals anti-β-actin (Sigma-Aldrich), and anti-CYGB (1:200, Abnova); rabbit polyclonals anti-PRDX1 (1:1,000, Abcam), anti-PRDX2 (1:10,000, Abcam), anti-PRDX3 (1:5,000, Abcam), anti-PRDX4 (1:1,000, Abcam), anti-PRDX5 (1:1,000, Santa Cruz Biotechnology), anti-PRDX6 (1:3,000, Abcam), anti-LEDGF/p75 (1:1,000, Novus Biologicals); and horseradish peroxidase (HRP)-labeled secondary IgG antibodies (Zymed).

RNA Interference-Mediated Knockdown of LEDGF/p75

Transient knockdown of LEDGF/p75 in PC-3 cells was carried out using the Amaxa Nucleofection method (Amaxa, Lonza) according to the manufacturer's protocol. Briefly, 1×10^6 cells per sample were transfected with 380 nM small inhibitory RNAs (siRNA) using Amaxa solution-V and program T-13. LEDGF/p75 siRNA (siLEDGF/p75) and the scrambled siRNA duplex (siSD, negative control) were designed as described by Maertens et al. [39] and synthesized by Integrated DNA Technologies (IDT). The siLEDGF/p75 sequence corresponded to nucleotides 1,340–1,360 (5'-AGACAGCAUGAGGAAGCGAdTdT-3') with respect to the first nucleotide of the start codon of the LEDGF/p75 open reading frame. The sequence for siSD was 5'-GCGCGCUUUGUAGGAUUCGdTdT-3'. In parallel, transfection without a siRNA duplex was performed as a mock control using only the nucleofection solution and these cells are referred to as "Nuc" transfected. Total RNA and proteins were extracted from siLEDGF/p75, siSD and "Nuc" trans-fected PC-3 cells 5 days post-transfection.

PC-3 cells with stable knockdown of LEDGF/p75 were a generous gift from Dr. Eric M. Poeschla (Mayo Clinic, Rochester, MN). These cells were generated using intensified lentiviral vector-based RNA interference as reported previously [40]. Short hairpin (sh) RNA1340 was used to stably knockdown LEDGF/p75 while shSCR served as the non-interfering shRNA control. PC-3 cells were transfected with vectors harboring shSCR control or shRNA1340.

Generation of PC-3 Cells With Stable Overexpression of LEDGF/p75

PC-3 cells stably overexpressing LEDGF/p75 were generated by transduction of the cells using retroviral vectors encoding the full-length LEDGF/p75. Briefly, PC-3 cells were seeded in a 96 well-plate at 30,000 cells per well. Next day, the RPMI medium was removed and was replaced by medium containing serial dilutions of the LEDGF/p75-vector or empty vector. Transduced cells were selected with blasticidin. LEDGF/p75 expression in selected clones was assessed by immunoblotting and immunofluorescence microscopy.

Docetaxel Resistant PC-3 and DU145 Cells

Docetaxel (DTX) resistant PC-3 (PC-3-DR) and DU 145 (DU 145-DR) cell lines were generous gifts from Dr. Julie Djeu (Moffit Cancer Center, Tampa, FL). These cell lines were developed by culturing PC-3 and DU 145 cells in the presence of DTX in a dose-escalation manner [41]. Cells that survived the initial culture in 1 nM DTX were passaged four times prior to increasing the concentration of DTX to 5.5 nM and subsequently to 11 nM. Resistant cells were maintained continuously in 11 nM DTX.

Pathway Specific Gene Expression Profiling

For stress gene profiling analysis, RNA was extracted from PC-3 cells with LEDGF/p75 knockdown or overexpression, and corresponding controls, using the RNeasy plus mini kit (Qiagen). RNA (0.5 µg) was reverse transcribed into cDNA using the RT² first strand kit (SA Biosciences, Qiagen). The “Human Oxidative Stress and Antioxidant Defense” real time profiler qPCR-array (SA Biosciences, Qiagen) was used to quantify changes in gene expression in PC-3 cells with transient or stable knockdown, or overexpression of LEDGF/p75. This qPCR-array contains 84 genes involved in cellular stress response and redox control and includes all the six members of PRDX family. QPCR was performed on the MyiQ real-time PCR detection system (Bio-Rad) using RT² Real Time™ SYBR Green/fluorescein PCR master-mix (SA Biosciences, Qiagen) according to the manufacturer’s protocol. Briefly, a total volume of 25 µl of PCR mixture, which included 12.5 µl of master mix, 11.5 µl of double distilled water, and 1 µl of cDNA was loaded into each of the 96 wells of the qPCR-array. PCR amplification was conducted at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The mRNA expression for each gene was normalized using the average expression of five housekeeping genes: β -2-microglobulin (β 2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin (ACTB). Gene profiling analyses were performed a minimum of three times in independent experiments. The $\Delta\Delta$ Ct method was used for data analysis. Fold changes were calculated for each gene as the difference in gene expression between LEDGF/p75-depleted or -overexpressing cells and their corresponding controls using the RT Profiler qPCR-array data analysis software.

Independent qPCR Gene Validation

For confirmation of LEDGF/p75 depletion or overexpression, and in house independent validation of differentially regulated genes, total RNA was extracted from PC-3 cells with LEDGF/p75 knockdown or overexpression, and corresponding controls, using the RNeasy plus mini kit (Qiagen). RNA was also extracted from PC-3-DR and DU 145-DR cells using the same kit. RNA (0.5 µg) was reverse transcribed into cDNA using iScript cDNA synthesis kit (Biorad). QPCR was performed on the MyiQ real-time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s recommendations. Primer sequences for gene validation were designed using the Primer3 software and were commercially synthesized by IDT (Table I). Target mRNA values were normalized using GAPDH mRNA and data were expressed relative to normalized values of corresponding controls. Samples were analyzed in three independent experiments in triplicates.

Immunoblotting Analysis

Procedures were carried out essentially as described previously [22]. Briefly, total proteins from prostate cells were separated by SDS-PAGE (NuPAGE 4–12%, Invitrogen) followed by transfer to polyvinyl difluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% dry milk solution in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) for 1 hr and probed with primary antibodies. After several washes with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 30 min and then washed again with TBS-T. Protein bands were detected by enhanced chemiluminescence (Amersham).

Statistical Analysis

The Student's t-test (two-tailed) was used to evaluate the significance of changes in mRNA expression between control and experimental groups. Probability values $P < 0.05$ were considered statistically significant.

RESULTS

Verification of LEDGF/p75 Knockdown and Overexpression in PC-3 Cells

Confirmation of transient and stable knockdown, as well as stable overexpression of LEDGF/p75, was performed by qPCR and immunoblotting (Fig. 1). QPCR in PC-3 cells with transient knockdown showed that LEDGF/p75 expression was depleted to 30.3% of its siSD control levels (Fig. 1A). PC-3 cells with stable LEDGF/p75 knockdown exhibited a 31.6% residual LEDGF/p75 transcript expression relative to the shSCR control (Fig. 1B). PC-3 cells stably overexpressing LEDGF/p75 showed 211% net transcript increase over empty vector control (Fig. 1C). Immunoblotting analysis confirmed the loss and gain of LEDGF/p75 protein expression in the knockdown and overexpressing cells, respectively (Fig. 1A-C, lower panels). Three biological RNA replicates of each sample were selected for subsequent oxidative stress-focused gene expression profiling analysis.

Changes in Oxidative Stress Gene Expression in Response to Knockdown or Overexpression of LEDGF/p75

In light of the documented role of LEDGF/p75 as a transcription co-activator that transcriptionally upregulates stress response and antioxidant genes, we examined the expression of 84 oxidative stress-related genes in PC-3 cells with transient and stable LEDGF/p75 knockdown (Table II). Thirteen genes were differentially regulated ($P < 0.05$, fold change ≥ 2) in cells with transient knockdown relative to siSD control (Fig. 2A, Table II). Of these genes, nine were downregulated and four were upregulated. The number of differentially regulated ($P < 0.05$, fold change ≥ 2) genes in transiently LEDGF/p75-depleted PC-3 cells increased to 23 when compared with the nucleofection (Nuc) control cells (Table II). Of these, only four were upregulated and the rest showed significant downregulation. Eleven genes showed differential regulation in LEDGF/p75-knockdown cells when compared to both siSD and Nuc controls (Table II).

In PC-3 cells with stable LEDGF/p75 knockdown, only three genes exhibited significant downregulation ($P < 0.05$, fold change ≥ 2) relative to shSCR control cells (Fig. 2B). These were angiopoietin-like 7 (ANGPTL7), dual specificity phosphatase 1 (DUSP1), and neutrophil cytosolic factor 2 (NCF2). However, neither of these genes were significantly downregulated in cells with transient depletion of LEDGF/p75, nor significantly upregulated in cells overexpressing this protein (Table II).

The relative expression of the same 84 stress and antioxidant genes was also evaluated in PC-3 cells with stable LEDGF/p75 overexpression (Table II). Fourteen genes were significantly upregulated ($P < 0.05$, fold change ≥ 2) in cells overexpressing LEDGF/p75 relative to vector control cells (Fig. 2C). We did not detect any genes that were significantly downregulated beyond a 2-fold level in the overexpressing cells.

We observed that several genes exhibited significant expression changes in response to both knockdown and overexpression of LEDGF/p75, but the fold-change values did not reach the 2-fold change cut-off in at least one of these two categories (Table II). These genes included CCS (copper chaperone for superoxide dismutase), FOXM1 (forkhead box M1), GSTZ1 (glutathione transferase zeta 1), PDLIM1 (PDZ and LIM domain 1), SGK2 (serum/glucocorticoid regulated kinase 2), and TTN (titin).

Selection and Validation of Candidate Target Genes of LEDGF/p75 in PC-3 Cells

Common genes that were both significantly down-regulated when LEDGF/p75 was transiently knocked down, and upregulated when LEDGF/p75 was stably overexpressed were selected for further validation (Table III). There were five such genes: Albumin (ALB), cytoglobin (CYGB), phosphoinositide-binding protein (PIP3-E, commonly known as IPCEF-1), superoxide dismutase 3 (SOD3), and thyroid peroxidase (TPO). All these five genes exhibited a ≥ 2 -fold down-regulation in transient knockdown and upregulation in overexpressing cells when compared to corresponding controls. Transcript expression of these genes was validated by additional qPCR (Table III) using our own primers, listed in Table I. The direction and magnitude of the fold changes were consistent with those detected in the qPCR-array.

From the above-mentioned five genes, we chose CYGB for further validation based on recent publications implicating it as tumor suppressor protein that is upregulated in hypoxic regions of tumors, and that protects tumor cells from oxidative stress-induced cell death [42-45]. Immunoblotting was performed to validate expression changes at the protein level. CYGB protein expression decreased in PC-3 cells with transient knockdown of LEDGF/p75, whereas it was elevated in overexpressing cells (Fig. 3), indicating that changes in mRNA levels were translated into changes at the protein level.

Analysis of LEDGF/p75, CYGB, and PRDX Expression in Prostate Cell Lines

Our recent observation that members of the PRDX family are upregulated in PCa [6], and the report that PRDX6 is a target gene of LEDGF/p75 in Cos-7 and lens epithelial cells [33], led us to investigate if there were changes in the transcript or protein expression of PRDXs in PC-3 cells in response to knockdown and overexpression of LEDGF/p75, as assessed by qPCR-array and immunoblotting. The results did not reveal significant decrease in the transcript or protein levels of any member of the PRDX family in the transiently or stably LEDGF/p75-depleted PC-3 cells (Fig. 4A,B). Although none of the PRDXs displayed a ≥ 2 -fold transcript increase in LEDGF/p75 over-expressing PC-3 cells, these cells consistently showed in immunoblots slightly higher protein levels of PRDX3 and PRDX6 relative to vector controls (Fig. 4C). However, quantification of the blots showed that these increases were not significant (data not shown).

These observations led us to further explore the association between the expression of LEDGF/p75, CYGB, PRDX3, and PRDX6 in other PCa cells. Immunoblot analysis of the protein expression of these four proteins in a panel of 11 prostate cell lines revealed high expression of all these proteins in transformed or tumor-derived cells but relatively low expression in primary prostate cell lines (PrEC or PrSC) (Fig. 5A). The protein levels of LEDGF/p75 paralleled those of CYGB in most of the cell lines. The most notable exception was the MDA-PCa-2b cell line, derived from a bone metastasis in an African American patient, in which the levels of LEDGF/p75 were lower than those of CYGB. The protein expression levels of LEDGF/p75 did not closely parallel those of PRDX3 and PRDX6 in all the cell lines. For example, LEDGF/p75 and PRDX6 protein levels paralleled each other in the PrEC, BRF-55T, RWPE-1, RWPE-2, DU 145, PC-3, and LNCaP; however, LEDGF/p75 expression was much lower than that of PRDX6 in the PrSC and MDA-PCa-2b cell lines but relatively higher in the BRF-41T and 22Rv1 cell lines. There was little overlap between the protein expression levels of LEDGF/p75 and PRDX3 in the panel of cell lines.

We took advantage of the availability of a DTX-resistant PC-3 cell line (PC-3-DR) to further compare the expression levels of these proteins. Interestingly, this cell line exhibited a 226% net increase of LEDGF/p75 transcript over the parental DTX-sensitive PC-3 cells (Fig. 5B). This is consistent with the previous observation from our group that LEDGF/p75,

when overexpressed in PC-3 cells, promotes resistance to DTX-induced cell death [25]. Immunoblotting analysis revealed that LEDGF/p75, CYGB, and PRDX6, but not PRDX3, exhibited elevated expression in PC-3-DR cells as compared to parental PC-3 cells (Fig. 5C). To determine if the elevated expression of CYGB and PRDX6 in PC-3-DR was dependent on LEDGF/p75, we transiently knocked down LEDGF/p75 in this cell line and examined its effects on the protein expression of these proteins. Immunoblotting analysis revealed that while CYGB protein was impaired in the LEDGF/p75-depleted PC-3-DR cells, LEDGF/p75 knockdown in PC-3-DR cells had no effect on the expression of PRDX3 and PRDX6 (Fig. 5D). As additional confirmation, we transiently knocked down LEDGF/p75 in PC-3 cells with stable overexpression of the protein. In this case the protein levels of CYGB, but not those of PRDX3 and PRDX6, were also diminished in the LEDGF/p75-depleted cells (Fig. 5E).

To investigate if similar changes occurred in another cell line, we evaluated the mRNA and protein levels of LEDGF/p75 and CYGB in a DTX-resistant DU 145 cell line (DU 145-DR) relative to parental DU 145 cells. This resistant cell line demonstrated 207.5% and 185.1% increases in the LEDGF/p75 and CYGB mRNA levels, respectively (Fig. 6A). Immunoblotting analysis confirmed overexpression of both proteins in the DU 145-DR cells (Fig. 6B), as compared to the parental cell line. To determine if the elevated expression of CYGB was dependent on LEDGF/p75, we transiently depleted LEDGF/p75 in DU 145-DR cells. QPCR analysis showed that depletion of LEDGF/p75 mRNA to 26.5% of its siSD control levels led to a 44% reduction in CYGB mRNA levels (Fig. 6C). These findings were confirmed at the protein level by immunoblotting, although the decrease in CYGB protein was not as drastic as the one observed in PC-3-DR cells depleted of LEDGF/p75 (Fig. 6D).

Taken together, these results suggested that LEDGF/p75 regulates the expression of CYGB in PCa cells. On the other hand, PRDX6, previously reported as regulated by LEDGF/p75 in COS-7 cells and lens epithelial cells [33], does not appear to be directly regulated by this transcription co-activator in PC-3 cells.

DISCUSSION

We reported previously that LEDGF/p75 is highly expressed in PCa cells and clinical tissues and promotes resistance to DTX-induced caspase-independent lysosomal cell death [22,25]. LEDGF/p75 has also been shown to induce chemoresistance in other tumor cell types [23,24,26]; however, the molecular mechanisms by which it promotes chemoresistance in cancer cells remain elusive. The identification of its interacting partners and target genes is essential for understanding these mechanisms. We report here changes in the expression of multiple stress genes in response to knockdown and overexpression of LEDGF/p75 in PC-3 PCa cells. These candidate target genes of LEDGF/p75 were identified using a pathway-focused “oxidative stress and antioxidant defense” qPCR-array. This approach is cost-effective and relatively easy-to-use, and combines the multigene profiling capabilities of microarray with real-time PCR performance. Out of a total of 84 genes in this stress microarray, five genes associated with oxidative stress responses (ALB, CYGB, PIP3-E, SOD3, and TPO) were considered as regulated by LEDGF/p75 because they were significantly ($P < 0.05$, fold change ≥ 2) downregulated in PC-3 cells transiently depleted of this protein and upregulated in LEDGF/p75 overexpressing cells.

It should be noted that stable LEDGF/p75 knockdown did not reveal striking changes in stress gene expression because only three genes (ANGPTL7, DUSP1, and NCF2) were significantly downregulated under these conditions. However, none of these genes were downregulated in cells with transient knockdown, or upregulated by stable overexpression of LEDGF/p75, and most likely are not regulated by this protein. In light of the previous

observation by Dugaard et al. [23], that transient siRNA-mediated knockdown of LEDGF/p75 induces cell death in some tumor cell lines, we could speculate that surviving PC-3 cell clones that were selected for stable depletion of this protein may have developed compensatory pathways to adapt to environmental stress. Nevertheless, we cannot rule out the possibility that transient or stable depletion of LEDGF/p75 may increase cellular sensitivity to specific stressors such as chemotherapeutic drugs.

Previous gene microarray profiling studies in LEDGF/p75-deficient cells (stable knockdown or knockout) did not unambiguously identify the regulation of a particular gene pathway [46,47]. However, two groups suggested that this protein regulates the expression of members of the HOX gene family [12,38], which would be consistent with the developmental abnormalities observed in mice with a disrupted *Ledgf/Psip1* gene [12]. It is likely that LEDGF/p75 participates in the regulation of specific genes depending on the microenvironmental context (cell type, stressor type, etc). Transient increases in LEDGF/p75 expression in response to different stressors (e.g., chemotherapeutic drugs, oxidative and thermal stress, radiation, hypoxia, and serum starvation stress), or sustained overexpression of the protein in tumor cells, could induce its binding to specific DNA regions and transcription factors to facilitate the transactivation of genes that promote resistance to a particular stressor.

The five genes identified in this study as candidate target genes of LEDGF/p75 in PCa cells play different roles in the cellular response to environmental stress. ALB, for instance, exerts antioxidant effects in its native form, but altered cellular redox status can oxidize this plasma protein causing impairment of its antioxidant capacity [48]. In cancer patients with cachexia ALB levels are diminished by chronic oxidative stress [49]. Very little is known about the role of PIP3-E in the cellular response to stress. This protein, also called IPCEF1, is involved in translocating cytohesins (guanine nucleotide exchange factors) to the plasma membrane and in Rac signaling [50,51]. SOD3 (extracellular SOD) is a major antioxidant enzyme that is located in the extracellular matrix and on the cell surface, where it protects against tissue damage initiated by extracellular reactive oxygen species (ROS). Although its role in PCa is unclear, this protein appears to play a protective role against thyroid cancer and breast cancer progression [52,53]. The main function of TPO is the oxidation of iodide to iodine using hydrogen peroxide for thyroid hormone synthesis, thereby decreasing the potentially damaging effects of ROS [54]. Very little is known about its function in non-thyroid tumor tissues.

Our data suggested that CYGB is regulated by LEDGF/p75 since both its transcript and protein expression were downregulated in response to LEDGF/p75 knockdown and upregulated in response to LEDGF/p75 overexpression in PC-3 and DU 145 cancer cells. CYGB is an attractive candidate to follow as a potential target gene of LEDGF/p75 because it is emerging as a cancer-associated stress protein. CYGB has been implicated as a tumor suppressor gene due to its silencing by promoter hypermethylation and downregulation in various human cancers [42]. However, recent studies provided evidence that this protein is upregulated in tumor cells by hypoxic stress, and its increased expression in hypoxic tumor regions correlates with clinicopathological features of tumor aggressiveness in head and neck cancer [43,44]. Its overexpression in human SH-SY5Y neuroblastoma cells protected against oxidative stress-induced cell death [45]. The role of CYGB in PCa remains to be investigated. Future studies should focus on correlating CYGB expression with that of LEDGF/p75 in clinical prostate tumors, and determining if LEDGF/p75 binds to and transactivates its promoter region under basal and stress (oxidative and hypoxic) conditions.

Recently, our group demonstrated that the six members of the PRDX family are differentially expressed in PCa cells and clinical tumors [6]. Fatma et al. [33] also reported

that PRDX6 is transactivated by LEDGF/p75 in both COS-7 and lens epithelial cells. These observations prompted us to evaluate if PRDX gene expression is influenced by LEDGF/p75 in PC-3 cells. The results from the qPCR-array and the immunoblotting analysis failed to show any significant fold change in PRDX1-6 transcript or protein in response to LEDGF/p75 knockdown or overexpression. Our results suggested that baseline PRDX6 expression in PCa cells is likely under the control of other transcription factors since it is not affected by depletion of LEDGF/p75. However, we cannot rule out that overexpression of LEDGF/p75 in response to environmental stressors could contribute to increased levels of PRDX6 over baseline as a mechanism to counter cellular stress. Alternatively, LEDGF/p75 might regulate PRDX6 in a cell type- or stressor-dependent manner.

A question that arises in studies aimed at identifying candidate target genes of LEDGF/p75 is whether this transcription co-activator directly regulates specific stress and antioxidant genes by binding to STRE in their promoter regions. Although there is evidence to support this notion [27,28,32-34], recent studies by members of this research team demonstrated that LEDGF/p75 binding to chromatin is not necessarily restricted to STRE in the genome, but is predominantly associated with active chromatin markers, such as H3 and H4 acetylation, H3K4 monomethylation, and RNA polymerase II binding [55]. It is likely that under conditions of stress, LEDGF/p75 interacts with specific transcription factors to tether them to active chromatin regions, where they can form complexes with RNA polymerase II to bind to promoter regions of specific stress and antioxidant genes and activate their expression.

In conclusion, this work provides evidence that transient knockdown and stable overexpression of LEDGF/p75 in PC-3 cells result in significant changes in the expression of stress and antioxidant genes such as ALB, CYGB, PIP3-E, SOD3, and TPO. It is likely that overexpression of LEDGF/p75 and its target genes in PCa cells plays a protective role against oxidative stress, chemotherapeutic drugs, and other stressors in the prostate tumor microenvironment. Further studies need to be performed to better understand the interplay between LEDGF/p75 and CYGB in altering cell death and survival in the presence of stress, and their role in PCa progression and therapy resistance. In particular, it would be crucial to establish whether knockdown of these two proteins in chemoresistant cancer cells sensitize against drug-induced cell death. LEDGF/p75 and CYGB might be important components of a mechanism of resistance against chemotherapy and oxidative damage in advanced prostate tumors and may represent novel therapeutic targets.

Acknowledgments

The authors are grateful to Dr. Eric M. Poeschla (Mayo Clinic College of Medicine, Rochester, MN) for kindly providing the PC-3 cells with stable knockdown of LEDGF/p75. They thank Dr. Julie Y Djeu (H. Lee Moffitt Cancer Center, Tampa, FL) for her generous gift of the DTX resistant PCa cells. The authors thank Stephanny Acevedo-Martinez for technical assistance. This work was supported by grant NIH-NCMHD 5P20MD001632 (De Leon M, Casiano CA), KU Leuven Research Council grant OT/09/047 and the Flanders Research Foundation (FWO) grant G.0530.08 (Debyser Z, Gijssbers R).

Grant sponsor: NIH-NCMHD; Grant number: 5P20MD001632; Grant sponsor: KU Leuven Research Council; Grant number: OT/09/047; Grant sponsor: Flanders Research Foundation (FWO); Grant number: G.0530.08.

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Fig. 1.

Verification of LEDGF/p75 depletion and overexpression in PC-3 cells. Real-time PCR (qPCR) and immunoblotting were used to determine the levels of LEDGF/p75 transcript (upper panels) and protein (lower panels), respectively, in PC-3 cells with transient knockdown (**A**), stable knockdown (**B**), and stable overexpression (**C**) of LEDGF/p75. Cells were transfected with siLEDGF/p75, shLEDGF/p75, and retroviral vector harboring full-length LEDGF/p75 to induce transient knockdown, stable knockdown, and stable overexpression, respectively. PC-3 cells with mock transfection (Nuc) or transfection with small interfering scrambled RNA duplex (siSD), short hairpin scrambled RNA (shSCR), and empty vector (Vector) served as corresponding controls. LEDGF/p75 mRNA levels were normalized to GAPDH mRNA levels and expressed relative to normalized corresponding control values respectively. * $P < 0.01$; ** $P < 0.001$. P values were determined with two-tailed Student's t -test.

Fig. 2.

Identification of stress genes potentially regulated by LEDGF/p75. A human oxidative stress and antioxidant defense qPCR-array was used to identify genes exhibiting significant up- or down-regulation of transcript levels in PC-3 cells with transient knockdown (A), stable knockdown (B), and stable overexpression (C) of LEDGF/p75. Messenger RNA levels were normalized using the average expression of five housekeeping genes (β 2M, HPRT1, RPL13A, GAPDH, β -actin) and expressed relative to normalize dmRNA values of respective controls. Gene expression displaying significant differential regulation (fold-change ≥ 2 ; * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$) was represented graphically. P values were determined with two-tailed Student's t -test.

Fig. 3. Analysis of changes in CYGB protein expression in response to knockdown or overexpression of LEDGF/p75. Expression of CYGB protein was evaluated by immunoblotting in PC-3 cells with transient knockdown (A) and stable overexpression of LEDGF/p75 (B), to further validate the qPCR data. Protein loading was assessed with antibody to β -actin.

Fig. 4. Transcript and protein expression changes of PRDXs in PC-3 cells in response to knockdown or overexpression of LEDGF/p75. PRDX expression was evaluated using the qPCR gene microarray (upper panels) and immunoblotting (lower panels) in PC-3 cells with transient knockdown (A), stable knockdown (B), and stable overexpression (C) of LEDGF/p75. Levels of PRDX mRNA were normalized using the average expression of five housekeeping genes in the qPCR microarray, and expressed relative to normalized corresponding control values respectively. Protein loading in immunoblots was assessed with antibody to β -actin. *P* values were determined with two-tailed Student's *t*-test and were not significant for any of the PRDXs.

Fig. 5.

Expression analysis of LEDGF/p75, CYGB, PRDX3 and PRDX6 in a panel of human prostate cell lines. Protein expression was evaluated by immunoblotting in a panel of prostate cell lines (**A**) that included non-tumor (BRF-55T, PrEc, PrSc), a nontumorigenic immortalized normal human prostate epithelial cell line (RWPE-1), a tumorigenic transformed RWPE-1 cell line (RWPE-2), androgen-independent (DU-145, PC-3), and androgen-responsive (LNCaP, MDA-PCa-2b, BRF-41T, and 22RV1) cell lines. LEDGF/p75 transcript overexpression in a docetaxel resistant PC-3 cell line (PC-3-DR) was verified using qPCR (**B**). The expression of these four proteins was also evaluated by immunoblotting in PC-3-DR cells (**C**), PC-3-DR cells with transient LEDGF/p75 knockdown (**D**), and PC-3 cells stably overexpressing LEDGF/p75 with transient knockdown of the protein (**E**). Parental PC-3 cells served as control for (**B**) and (**C**), while siSD transfected-PC-3-DR cells and LEDGF/p75-overexpressing PC-3 cells served as corresponding controls in (**D**) and (**E**), respectively. Protein loading was assessed with antibody to β-actin. * $P < 0.001$. P values were determined with two-tailed Student's t -test.

Fig. 6.

Expression analysis of LEDGF/p75 and CYGB in docetaxel resistant DU 145 cells. LEDGF/p75 and CYGB transcript and protein overexpression in a docetaxel resistant DU 145 cell line (DU 145-DR) were verified using qPCR (**A**) and immunoblotting (**B**), respectively. Transient knockdown of LEDGF/p75 and its effect on CYGB expression were evaluated in DU145-DR cells by qPCR (**C**) and immunoblotting (**D**). Protein loading was assessed with antibody to β-actin. * $P < 0.05$, ** $P < 0.001$. P values were determined with two-tailed Student's t -test.

TABLE I

Sequences of Primers Used for qPCR

Gene	Forward sequence (5' to 3')	Reverse Sequence (5' to 3')
LEDGF/p75	TCAAAGTCAACAGGCAGCAAC	TAGCTGCAGGTCGTCTCTTT
ALB	GAGCTTTTGTAGCAGCTTGG	GGTTCAGGACACGGATAGA
CYGB	CTTCAGCCAGTTCAAAGCACA	AGTACACCGGTTCCACCTTG
PIP3-E	GCCTGAGTGGAA CGTCGTAT	ATGAACTTGCACACAGCAAACG
SOD3	CTGGGTGCAGCTCTCTTTTC	ACATGTCTCGGATCCACTCC
TPO	CCACTGTGTCCAAACGTGTTT	ATGTCCATGGGCTGAAGAAA
GAPDH	CGAGATCCCTCCAAAAATCAA	TTCACACCCATGACGAACAT

TABLE II
Changes in Gene Expression in “Oxidative Stress and Antioxidant Defense” qPCR Gene Microarray in Response to Knockdown or Overexpression of LEDGF/p75 in PC3 Cells

Gene	Description	Transient KD			Stable KD			Stable OE		
		KD vs. SD		Gene	KD vs. Nuc		Description	KD vs. SCR		OE vs. Vec
		Fold change	P value		Fold change	P		Fold change	P value	Fold change
ALB	Albumin	-2.74	0.002	-3.28	0.001	1.77	0.060	2.89	0.036	
ALOX12	Arachidonate 12-lipoxygenase	-1.39	0.044	-1.53	0.002	-1.83	0.454	1.47	0.018	
ANGPTL7	Angiotensin-like 7	1.71	0.017	1.04	0.820	-2.06	0.000	1.22	0.116	
AOX1	Aldehyde oxidase 1	2.14	0.002	2.12	0.001	-1.58	0.008	-1.43	0.000	
APOE	Apolipoprotein E	2.87	0.000	2.23	0.000	1.21	0.286	2.40	0.185	
ATOX1	ATX1 antioxidant protein 1 homolog	-1.19	0.072	-1.38	0.016	-1.52	0.064	1.19	0.041	
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-1.87	0.002	-1.73	0.001	1.02	0.970	-1.03	0.859	
CAT	Catalase	-1.63	0.059	-1.56	0.041	1.24	0.683	2.36	0.000	
CCL5	Chemokine (C-C motif) ligand 5	1.05	0.806	-2.53	0.338	-1.83	0.047	1.62	0.308	
CCS	Copper chaperone for superoxide dismutase	-1.72	0.010	-2.13	0.000	1.04	0.883	1.63	0.008	
CSDE1	Cold shock domain containing E1,	1.51	0.053	1.22	0.257	-1.05	0.906	1.32	0.036	
CYBA	Cytochrome b-245, alpha polypeptide	-1.78	0.024	-1.38	0.044	-1.58	0.417	2.40	0.185	
CYGB	Cytoglobin	-2.51	0.000	-6.13	0.000	1.51	0.029	7.35	0.001	
DGKK	Diacylglycerol kinase, kappa	-1.39	0.044	-1.53	0.002	-1.58	0.417	2.40	0.185	
DHCR24	24-dehydrocholesterol reductase	-1.02	0.806	-1.20	0.068	-1.03	0.937	-1.32	0.145	
DUOX1	Dual oxidase 1	-1.37	0.053	-2.09	0.000	-1.05	0.876	2.14	0.016	
DUOX2	Dual oxidase 2	-1.37	0.019	-2.20	0.000	-1.05	0.925	2.40	0.185	
DUSP1	Dual specificity phosphatase 1	1.13	0.026	1.04	0.482	-2.03	0.002	-1.51	0.009	
EPHX2	Epoxide hydrolase 2, cytoplasmic	-1.72	0.000	-2.36	0.000	-1.42	0.792	2.01	0.201	
EPX	Eosinophil peroxidase	-1.37	0.115	-2.28	0.001	1.51	0.268	6.11	0.003	
FOXMI	Forkhead box M1	1.74	0.023	2.08	0.006	-1.47	0.159	-1.59	0.010	
GLRX2	Glutaredoxin 2	1.01	0.782	1.04	0.543	-1.02	0.917	1.23	0.010	
GPRI56	G protein-coupled receptor 156	-1.11	0.551	-1.14	0.447	-1.81	0.009	1.13	0.697	
GPXI	Glutathione peroxidase 1	1.07	0.882	1.24	0.533	1.08	0.961	1.20	0.043	

Gene	Description	Transient KD			Stable KD			Stable OE		
		KD vs. SD		Gene	KD vs. SCR		Gene	OE vs. Vec		Gene
		Fold change	P value		Fold change	P value		Fold change	P value	
GPX2	Glutathione peroxidase 2	-1.09	0.598	-1.70	0.021	1.01	0.977	1.69	0.224	
GPX3	Glutathione peroxidase 3 (plasma)	-1.30	0.017	-2.06	0.000	1.18	0.243	1.91	0.018	
GPX4	Glutathione peroxidase 4	1.25	0.099	1.02	0.839	-1.18	0.474	1.64	0.013	
GPX5	Glutathione peroxidase 5	-1.84	0.073	-1.51	0.167	-1.58	0.417	2.40	0.185	
GPX6	Glutathione peroxidase 6 (olfactory)	-1.39	0.044	-1.53	0.002	-1.58	0.417	2.40	0.185	
GPX7	Glutathione peroxidase 7	-1.94	0.003	-2.86	0.005	-1.27	0.642	2.40	0.185	
GSR	Glutathione reductase	-1.04	0.874	-1.16	0.376	-1.21	0.834	1.16	0.563	
GSS	Glutathione synthetase	-1.04	0.827	-1.16	0.518	-1.11	0.754	1.47	0.003	
GSTZ1	Glutathione transferase zeta 1	-2.19	0.000	-2.62	0.000	1.01	0.984	1.82	0.001	
GTF2I	General transcription factor II, i	-1.30	0.040	-1.76	0.000	1.24	0.466	2.27	0.001	
KRT1	Keratin 1 (epidermolytic hyperkeratosis)	-1.39	0.044	-1.89	0.008	-1.54	0.436	2.40	0.185	
LPO	Lactoperoxidase	-1.39	0.044	-1.53	0.002	-1.58	0.417	2.40	0.185	
MBL2	Mannose-binding lectin (protein C) 2	-2.15	0.042	-2.49	0.013	-1.05	0.908	1.92	0.222	
MGST3	Microsomal glutathione S-transferase 3	-1.47	0.021	-1.70	0.000	1.14	0.214	1.22	0.135	
MPO	Myeloperoxidase	-1.60	0.185	-1.31	0.340	-1.58	0.417	2.40	0.185	
MPV17	MpV17 transgene,	-1.13	0.073	-1.61	0.001	1.03	0.954	1.46	0.020	
MSRA	Methionine sulfoxide reductase A	-1.28	0.186	-1.61	0.008	1.58	0.042	1.26	0.031	
MT3	Metallothionein 3	-1.39	0.044	-1.53	0.002	-1.58	0.417	2.40	0.185	
MTL5	Metallothionein-like 5, testis-specific	-1.78	0.001	-2.62	0.000	1.53	0.250	1.14	0.170	
NCF1	Neutrophil cytosolic factor 1	-1.17	0.544	-1.95	0.007	-1.36	0.578	1.65	0.033	
NCF2	Neutrophil cytosolic factor 2	1.80	0.012	1.98	0.003	-2.06	0.009	-1.84	0.001	
NME5	Non-metastatic cells 5,	2.21	0.001	1.66	0.019	-1.36	0.551	2.09	0.199	
NOS2A	Nitric oxide synthase 2A	1.01	0.959	-2.24	0.186	-1.49	0.489	2.40	0.185	
NOX5	NADPH oxidase, EF-hand calcium binding domain 5	1.14	0.452	-1.03	0.809	-1.39	0.287	2.57	0.012	
NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type	1.48	0.019	2.05	0.000	2.72	0.418	1.37	0.512	
OXR1	Oxidation resistance 1	1.09	0.470	-1.33	0.005	1.41	0.122	1.55	0.009	
OXSRI	Oxidative-stress responsive 1	1.27	0.003	-1.01	0.873	-1.11	0.423	1.29	0.114	

Gene	Description	Transient KD			Stable KD			Stable OE		
		KD vs. SD		P value	KD vs. Nuc		Description	KD vs. SCR		OE vs. Vec
		Fold change	P		Fold change	Gene		Fold change	P value	Fold change
PDLIM1	PDZ and LIM domain 1 (elfin)	-1.52	0.001		-2.20	0.000		-1.71	0.001	1.71
PIP3-E	Phosphoinositide-binding protein PIP3-E	-4.08	0.015		-6.57	0.002		-1.14	0.572	2.24
PNKP	Polynucleotide kinase 3'-phosphatase	1.14	0.338		1.02	0.878		1.07	0.841	2.57
PRDX1	Peroxiredoxin 1	-1.08	0.719		-1.27	0.201		-1.18	0.819	-1.21
PRDX2	Peroxiredoxin 2	1.14	0.459		-1.01	0.951		1.16	0.582	1.76
PRDX3	Peroxiredoxin 3	1.14	0.110		1.16	0.083		-1.28	0.734	-1.47
PRDX4	Peroxiredoxin 4	1.25	0.054		1.14	0.493		-1.36	0.283	1.08
PRDX5	Peroxiredoxin 5	-1.30	0.021		-1.51	0.014		-1.17	0.676	1.75
PRDX6	Peroxiredoxin 6	1.46	0.010		1.47	0.098		-1.44	0.254	1.07
PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	-1.06	0.521		-1.01	0.824		1.04	0.942	1.54
PRG3	Proteoglycan 3	-1.39	0.044		-1.53	0.002		-1.58	0.417	2.40
PRNP	Prion protein (p27-30)	1.07	0.562		-1.03	0.787		-1.30	0.373	1.57
PTGS1	Prostaglandin-endoperoxide synthase 1	-1.37	0.237		-1.10	0.649		-1.59	0.189	2.40
PTGS2	Prostaglandin-endoperoxide synthase 2	2.21	0.001		1.91	0.019		-1.61	0.367	2.68
PXDN	Peroxidasin homolog (Drosophila)	-1.37	0.002		-1.95	0.000		1.12	0.679	2.39
PXDNL	Peroxidasin homolog-like (Drosophila)	-1.42	0.049		-1.92	0.058		-2.01	0.200	2.81
RNF7	Ring finger protein 7	-1.13	0.448		-1.45	0.046		-1.39	0.232	1.18
SCARA3	Scavenger receptor class A, member 3	-1.49	0.053		-1.33	0.072		-1.10	0.561	1.45
SELS	Selenoprotein S	-1.13	0.372		-1.64	0.002		-1.14	0.634	1.93
SEPP1	Selenoprotein P, plasma, 1	-2.56	0.000		-4.18	0.000		1.41	0.576	1.07
SFTPD	Surfactant, pulmonary-associated protein D	-1.21	0.342		1.08	0.574		-1.03	0.944	2.60
SGK2	Serum/glucocorticoid regulated kinase 2	-1.57	0.021		-2.36	0.002		1.33	0.028	2.59
SIRT2	Sirtuin 2	1.09	0.506		-1.38	0.006		1.14	0.290	2.93
SOD1	Superoxide dismutase 1, soluble	1.01	0.860		-1.08	0.302		1.10	0.758	-1.03
SOD2	Superoxide dismutase 2, mitochondrial	-1.11	0.160		-1.31	0.027		-1.33	0.288	1.32
SOD3	Superoxide dismutase 3, extracellular	-3.15	0.002		-5.43	0.000		-1.22	0.929	4.12
SRXN1	Sulfiredoxin 1 homolog (S. cerevisiae)	1.16	0.510		1.20	0.369		-1.49	0.053	1.54

Gene	Description	Transient KD			Stable KD			Stable OE		
		KD vs. SD		Gene	KD vs. SCR		Gene	OE vs. Vec		Gene
		Fold change	P value		Fold change	P value		Fold change	P value	
STK25	Serine/threonine kinase 25	1.07	0.634	-1.33	0.060	-1.11	0.702	1.81	0.035	
TPO	Thyroid peroxidase	-2.31	0.012	-5.33	0.000	1.30	0.662	10.68	0.001	
TTN	Titin	-2.15	0.012	-2.71	0.002	1.12	0.568	1.52	0.025	
TXNDC2	Thioredoxin domain containing 2	-1.69	0.030	-1.29	0.224	-1.63	0.316	2.40	0.185	
TXNRD1	Thioredoxin reductase 1	-1.17	0.248	-1.10	0.453	-1.10	0.820	-1.03	0.958	
TXNRD2	Thioredoxin reductase 2	1.11	0.482	1.02	0.804	-1.42	0.561	-1.12	0.485	

Numbers in bold denote ≥ 2 -fold changes with statistically significant P values ($P < 0.05$). Fold change values represent the average of at least three independent experiments in triplicates. KD, knockdown; SD, scrambled duplex siRNA; SCR, scrambled shRNA; Nuc, nucleofection; OE, overexpression; Vec, empty vector control.

TABLE III
Candidate Target Genes of LEDGF/p75 Selected for Additional Validation by qPCR

Gene	Description	Transient KD vs. SD			Stable OE vs. vec		
		PCR Array		RT-PCR	PCR Array		RT-PCR
		Fold change	P-value	Fold change	Fold change	P-value	Fold change
ALB	Albumin	-2.74	0.002	-1.96	2.89	0.036	3.82
CYGB	Cytoglobin	-2.51	0.000	-2.48	7.35	0.001	3.61
PIP3-E	Phosphoinositide binding protein PIP3-E	-4.08	0.015	-3.30	2.24	0.010	3.06
SOD3	Superoxide Dismutase 3	-3.15	0.002	-4.57	4.12	0.000	2.41
TPO	Thyroid peroxidase	-2.31	0.012	-3.89	10.68	0.001	6.86

Fold change values represent the average of at least three independent experiments in triplicates. Statistical significance was considered at $P < 0.05$. KD, knockdown; SD, scrambled duplex siRNA; OE, overexpression; Vec, empty vector control.