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Biochemical and Functional characterization of the LEDGF/p75-MeCP2 Interaction in Tumor Cells

Lai Sum Leoh
Loma Linda University

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Biochemical and Functional Characterization of the LEDGF/p75-MeCP2 Interaction in Tumor Cells

by

Lai Sum Leoh

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

_____, Chairperson

Carlos A. Casiano, Associate Professor of Microbiology, Molecular Genetics, Biochemistry, and Medicine

_____

Penelope Duerksen Hughes, Professor of Biochemistry, Associate Dean for Basic Science and Translational Research

_____

Thomas A. Linkhart, Professor of Biochemistry and Microbiology, Research Professor of Pediatrics

_____

Donna D. Strong, Professor of Biochemistry and Microbiology, Research Professor of Medicine

_____

Nathan R. Wall, Assistant Professor of Biochemistry and Microbiology

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I would like to dedicate this dissertation and all my work to my parents. I would not have made it without you. I love you.

CONTENTS

FIGURES

Figures Page

ABBREVIATIONS

AA	African American
AD	Atopic Dermatitis
ADH	Alcohol Dehydrogenase
ALDH	Aldehyde Dehydrogenase
AML	Acute Myeloid Leukemia
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
ANA	Antinuclear Autoantibodies
AOP2	Antioxidant Protein 2
ASK	Cdc7 activator of S-phase kinase
ATCC	American Type Culture Collection
AR	Androgen Receptor
bLZ	Basic Leucine Zipper
BPH	Benign Prostatic Hyperplasia
CFL	Cofilin
ChiP	Chromatin Immunoprecipitation
CLU	Clusterin
CML	Chronic Myeloid Leukemia
CR	Charged Region
CREB1	cAMP responsive element binding protein 1
CRPC	Castration-resistant Metastatic Prostate Cancer
CTT	C-terminal Tail

DCF	2’,7’-dicholorofluorescein
DCFH-DA	2’,7’-dichlorodihydrofluorescein diacetate
DFS 70	Dense Fine Speckle Protein 70 kDa
DMSO	Dimethyl Sulfoxide
DRE	Digital Rectal Exam
DTT	Dithiothreitol
DTX	Docetaxel
EGTA	Ethylene Glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
ENCODE	Encyclopedia of DNA Elements
ERp57	ER luminal glycoprotein specific thiol oxidoreductase
FADD	Fas-Associated protein with Death Domain
GFP	Green Fluorescent Protein
GST	Glutathione S Transferase
GWAS	Genome-wide Association Studies
H_2O_2	Hydrogen Peroxide
HDAC	Histone Deacetylase Complex
HDGF	Hepatoma-Derived Growth Factor
HGPIN	High-grade prostatic intraepithelial neoplasia
HIV-IN	Human Immunodeficiency Virus Integrase
HMG	High Mobility Group
HMT	Histone Methyltransferase
HPC1	Hereditary Prostate Cancer Locus-1

HRP	Horseradish Peroxidase
HRP-2	HDGF-Related Protein 2
HSE	Heat Shock Element
HSF1	Heat Shock Transcriptional Factor 1
Hsp27	Heat Shock Protein 27
HTH	Helix Turn Helix
IBD	Integrase Binding Domain
IGFBP5	Insulin Growth Factor Binding Protein 5
IN	Integrase
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazoly-carbocyanine Iodide
LEC	Lens Epithelial Cell
LEDGF/p75	Lens Epithelium Derived Growth Factor/p75
LTR	Long Terminal Repeats
MBD	Methyl CpG Binding Domain
MDR1	Multidrug Resistance Gene
MeCP2	Methyl CpG DNA Binding Protein 2
MLL	Mixed-Lineage Leukemia
MMP	Mitochondrial Membrane Potential
MSR1	Macrophage-scavenger Receptor 1s
MTF1	Metal-regulatory Transcription Factor 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NLS	Nuclear Localization Signal

NM	Nanometers
NMDA	N-methyl-d-aspartate
NMR	Nuclear Magnetic Resonance
NSCLC	Non-small Cell Lung Cancer
NUP98	Nucleoprotein 98
PON1	Paraoxonase 1
PBS	Phosphate Buffered Saline
PCa	Prostate Cancer
PIA	Proliferative Inflammatory Atrophy
PIN	Prostate Intraepithelial Neoplasia
PMSF	Phenylmethanesulphonylfluoride
PogZ	PogoTtransposable Element derived Protein with Zinc Finger
Prdx 6	Peroxiredoxin 6
PSA	Prostate-specific Antigen
PSIP	PC4 and SFRS1 Interacting Protein
PTEN	Phosphatase and Tensin Homologue
PVDF	Polyvinyl Difluoride
PWWP	Proline-Tryptophan-Tryptophan-Proline
RNASEL	Ribonuclease L
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
SEER	Surveillance Epidemiology and End Results

SEREX	Serologic Identification of Recombinant Expressed Proteins
SMYD1	SET and MYND domain containing 1
SOX	Sry-related HMG box
SRD	Supercoiled DNA-recognition domain
STRE	Stress Related Element
STS	Staurosporine
SUMO	Small Ubiquitin-like Modifier
TBHP	Tert-butyl Hydrogen Peroxide
TCP75	Transcription Co-Activator p75
TIE	TGF-β1 Inhibitory Element
TMA	Tissue Microarray
TRAIL	Tumor Necrosis Factor –related Apoptosis Inducing Ligand
TRD	Transcription Repression Domain
VEGF-C	Vascular Endothelial Growth Factor C
VKH	Vogt-Koyanagi-Harada
WB	Western Blot
WHO	World Health Organization
Z-VAD.fmk	Benzylocarbonyl Val-Ala-Asp-fluoromethyl Ketone

ABSTRACT OF THE DISSERTATION

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by

Lai Sum Leoh

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
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Dr. Carlos A. Casiano, Chairperson

The lens epithelial derived growth factor p75 (LEDGF/p75) is a novel pro-survival and stress-inducible transcription co-activator that protects mammalian cells from various environmental stresses such as oxidative stress, heat shock, and serum starvation. This emerging cancer-related protein is highly expressed in prostate tumors and other tumor types and promotes resistance to chemotherapy in cancer cells. LEDGF/p75 is also involved in acquired immunodeficiency syndrome (AIDS) since it interacts with HIV-1 integrase to facilitate the integration and replication of the HIV virus in human cells. In addition, LEDGF/p75 has been shown to interact with MLL (mixed lineage leukemia)/menin transcription complex in leukemia cells to facilitate the transcription of cancer-associated genes and leukemic transformation. In order to understand the mechanisms by which LEDGF/p75 contributes to cancer development, we explored its interactions with other transcription factors and the influence of these interactions on its transcriptional activity. Using complementary molecular, biochemical, and cellular approaches we discovered that the amino-terminal region of LEDGF/p75 interacts with the transcription regulator and methylation associated protein MeCP2 in prostate cancer cells and other cancer cell types. We observed that both proteins regulate

the expression of the heat shock protein 27 gene by transactivating its promoter region. We propose that the interaction between LEDGF/p75 and MeCP2 modulates the expression of cancer-associated genes in response to environmental stressors. These findings provide a plausible mechanism that can be targeted for the treatment of advanced prostate cancer, which is the second leading cause of cancer deaths in the United States, with a disproportional burden among African American men.

CHAPTER ONE

INTRODUCTION

The Prostate

The prostate is a gland that surrounds the urethra at the base of the bladder and produces secretory proteins to the seminal fluid. It is not required for viability or basal levels of fertility; thus, its primary significance is its relevance for human disease, as treatment strategies can disrupt normal urinary, bowel and sexual functions [1]. The prostate contains three defined regions (Figure 1) [2-5]: the peripheral zone, the periurethral transition zone, the central zone, together with an anterior fibromuscular stroma [6]. Benign prostatic hyperplasia (BPH) arises from the transition zone, while prostate carcinomas are mostly found in the peripheral zone [1].

Prostate Cancer

Prostate cancer is generally multifocal, since primary tumors often contain multiple independent histologic foci of cancer that are often genetically distinct [7-10]. In contrast, despite the phenotypic heterogeneity of metastatic prostate cancer [11], molecular and cytogenetic analyses show that multiple metastases in the same patient are monoclonal [12-13]. These findings suggest that metastatic prostate cancer may arise from the selective advantage of individual clones during cancer progression. Alternatively, this could be a result of therapeutic interventions such as androgen

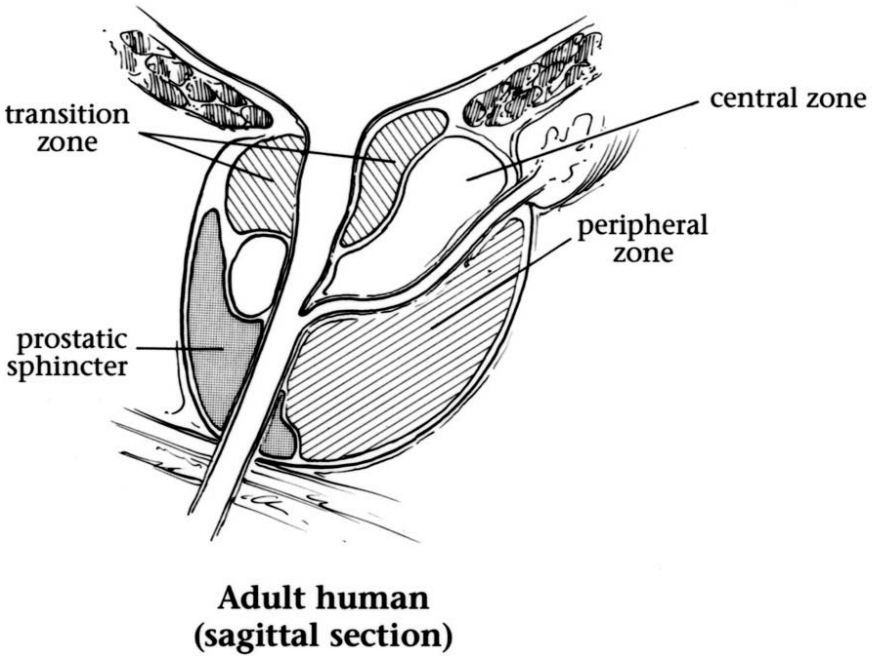


Figure 1. Schematic illustration of the anatomy of the human prostate by Abate-Shen et al. [1]. In the illustration the three main regions of the prostate can be observed the peripheral, transition and central zone.

deprivation and chemotherapy, which may differentially target cells of varying malignant potential. Although human prostate cancer displays significant phenotypic heterogeneity, the majority of prostate cancers are adenocarcinomas.

Statistics

Prostate cancer (PCa) is the most common male cancer and the second leading cause of male cancer associated deaths in the United States. It is estimated that over 217,730 men will be diagnosed with and 32,050 men will die from PCa in 2010 [14]. Based on rates from 2003-2005, one in six men will be diagnosed with PCa during their lifetime [15] and a 3.4% chance to die because of this disease [16-17]. The number of men with PCa increases dramatically as the population of males over the age of 50 grows worldwide. Therefore, understanding the causes that increase the risk of PCa has become an important epidemiological concern. Epidemiological studies have lead to the identification of various risk factors that can increase susceptibility to PCa.

PCa Risk Factors

Risk factors that promote prostate carcinogenesis include family history, race, oxidative stress and aging, and diet. These factors will be discussed below.

Family History

Hereditary factors account for a small percent (~10%) of PCa [1]. The first putative hereditary prostate cancer locus, HPC1, was localized to chromosome 1q24–q25 [18]. Genome-wide linkage analysis of 1,233 prostate cancer pedigrees from the International

Consortium for Prostate Cancer Genetics using novel sumLINK and sumLOD analyses confirmed significant linkage evidence at chromosome 22q12 and at twelve other regions [19]. The major contributing prostate cancer susceptibility locus found in southern and western Europe is PCAP, predisposing for PCa, which maps to 1q42–q43 [20-21], while CAPB, 1p36, is reported in families with a history of both prostate and brain cancers [22]. Other prostate cancer susceptibility loci includes HPC2 at 17p11, Xq27-28 (HPCX) [23-25], hereditary prostate cancer locus 3HPC20 20q13 [26], Ribonuclease L (RNASEL), macrophage-scavenger receptor 1 (MSR1), CHEK2, vitamin D receptor and paraoxonase 1 (PON1) [18, 27-31]. In addition, mutations in BRCA gene was also found to be strongly associated with rapidly progressing lethal prostate cancer [32]. VEGF and HSP70-hom polymorphisms were also reported to be significantly associated with PCa susceptibility and prognosis [33].

A series of sequence variants located along chromosome 8q24 have been associated with an increased risk of PCa [34-36], as reported by more recent genome-wide association studies (GWAS) [37-39]. Colon, breast and ovarian [40] risks are also associated with mutations of 8q24. In the first stage of the UK GWAS, 20 tagSNPs out of 53 significant tagSNPs were on chromosome 8q, and 6 were on chromosome 17q (8,12), consistent with previous GWAS [37, 41]. In addition, seven new susceptibility regions on human chromosomes 3, 6, 7, 10, 11, 19 and X were linked to a risk of developing PCa [42]. Under multiplicative risk model, and in combination with previously reported SNPs on 8q and 17q, these loci explain 16% of the familial risk of the disease [43]. Recently, seven new prostate cancer susceptibility loci were identified on chromosomes 2, 4, 8, 11 and 22 through a genome-wide association study in which 43,671 SNPs in 3,650 PCa

cases and 3,940 controls were genotyped [44]. Carriers of five high-risk alleles in the androgen receptor (AR), Cytochrome P450 (CYP17), and Steroid-5-alpha-Reductase type 2 (SRD5A2) genes were reported to have a two-fold excess risk to develop PCa [45]. Disappointingly, however, other population-based studies did not identify many of the loci identified in GWAS, including analyses of groups with high-risk for prostate cancer, such as African-Americans [46].

Race

Disparities in the incidence and mortality of PCa patients with different racial backgrounds have lead to the observation that African American (AA) men have one of the highest reported incidence rates in the world, with distinctly higher mortality compared to White American (WA) men [47]. AA men develop the disease 1.6 times more frequently with a mortality rate 2.5 times greater than their WA men counterparts of the same age [48-50]. In addition, SEER (Surveillance Epidemiology and End Results) incidence statistics reported that PCA incidences per 100,000 men were 146.3 in WA and 231.9 in AA men, compared to 82.3 in Asian/ Pacific Islander (A/PI) (these rates are based on cases diagnosed in 2002-2006 from 17 SEER geographic areas) [51]. Death rates per 100,000 men were 23.6 in WA, 56.3 in AA, and 10.6 in A/PI. AA men are younger at the time of diagnosis, have tumors that are higher in stage and grade, and have a shorter survival rate when compared to WA men [52]. Fowler et al. compared the outcomes of 396 WA men and 524 AA men with PCa diagnosed between 1982 and 1992 and reported that localized PCa was more lethal in AA men than in WA men [53].

An analysis of 48 articles that provided data that enabled an estimated hazard ratio and standard error to be derived allowed comparison of AA and WA men in terms of overall survival, PCa survival or biochemical recurrence. One out of every 125 AA men with PCa will die of the disease within 10 years due to biological factors of their ethnicity, having adjusted for age, clinical presentation and socioeconomic status [54]. Similarly, a pooled all-cause mortality hazard ratio comparing AA to WA men following a diagnosis of PCa was calculated based on summary statistics from 17 studies. Of the 14 cancers compared, blacks were at a significantly higher risk of cancer-specific death only for cancer of the breast, uterus, or bladder [55]. Interestingly, Cooney *et al.* reported that African-American families contributed disproportionately to the observation of linkage to hereditary prostate cancer gene HPC1 [56].

Others propose that the reasons for racial differences in PCa incidence and mortality are multifactorial, including socioeconomic status, access to health care, genetic susceptibility and diet [57]. However, three recent studies with large cohorts indicated that after correction for socioeconomic status, disparities in PCa incidence still persist among AA, suggesting that biological factors might be involved [58-61].

Wallace et al. [62] compared the expression profile of primary prostate tumors from 33 AA men and 36 WA men by using microarray analyses and identified two genes that were upregulated in AA men when compared to WA men: *PSPHL* and *CRYBB2* [62], which were reported in eye diseases but had unknown function in the prostate. Further studies will be needed to confirm their importance in PCa disparities. It has also been reported that AA men have higher levels of androgen metabolites as compared to WA men, such as higher circulating levels of testosterone [63-64]. In addition, prostate-

specific antigen (PSA) values at diagnosis are greater, and high grade prostatic intraepithelial neoplasia (HGPIN) expression is more common in AA men compared to WA men [53], [65-66]. Insulin-like growth factor one (IGF-1) and IGF binding protein 3 (IGFBP-3) levels are higher in WA compared to AA men [67].

The role of somatic genetic mutations, including loss or amplification or specific genes in prostate tumors, has been correlated with the natural history of PCa progression and therefore clinical prognosis [68-69]. Inherited mutations in genes involved in regulation of somatic DNA damage or repair and metabolism of steroid hormones that induce the growth of PCa may be associated with disease prognosis if they are involved in metabolic events that lead to tumor progression [70].

Oxidative Stress, Inflammation and Aging

Oxidative stress results from the imbalance of reactive oxygen species (ROS) and detoxifying enzymes that control cellular levels of ROS, which leads to cumulative damage to lipids, proteins, and DNA. Evidence linking oxidative stress and PCa initiation include correlative studies showing that major antioxidant enzymes are reduced in human PIN and PCa, together with a coincidental increase in the oxidized DNA adduct 8-oxy-7,8,dihydro-2′-deoxyguanosine (8-oxy-dG) [71]. APE/Ref1, a multifunctional enzyme involved in redox control of key enzymes and base excision repair, is up-regulated in PCa, while polymorphisms in the *APE* gene are associated with increased PCa risk [72-73]. *Nkx3.1* loss of function leads to deregulated expression of oxidative damage response genes and increased levels of 8-oxy-dG, correlating with the onset of PIN [74], while its gain of function protects against DNA damage in PCa cell lines [75].

The prevalence of PCa increases by age group, with 3% for men aged 60-64, 10% for men aged 70-74, 15% for men aged 80-84, and 14% for men over 85 years old [16, 76]. Autopsy studies revealed PCa incidence up to 60% in men aged 80 and above [77-78]. These numbers are expected to increase with an estimated 20% of the population at 65 years or older in year 2030, compared to 13% today [79]. Increasing evidence has indicated that oxidative stress is associated with aging and cancer through an imbalance in cellular prooxidant-antioxidant status [80-82].

Young healthy individuals are equipped with adequate antioxidant defense mechanism to protect against free radicals through ROS detoxification enzymes such as superoxide dismutases (SODs), glutathione peroxidase (GPx), glutathione-S-transferases (GST), other constitutive and inducible antioxidants, DNA repair enzymes, and other cellular mechanisms of genomic surveillance, such as cell cycle checkpoint control systems [83]. In aging individuals, a decrease in detoxification enzyme activities has been observed [82], leading to progressive accumulation of DNA adducts, increase in DNA strand-break frequency and point mutations, telomere attrition, and alterations in methylation pattern [84-86] [82, 87-88]. Stimulation of DNA damage can either arrest or induce transcription, signal transduction pathways, replication errors, genomic instability, abnormal function of proteins involved in cellular growth, responses to cellular stress, and inflammation [89-90], all of which are associated with carcinogenesis [91-93].

In addition, telomere erosion-induced replicative senescence and oxidative stress, oncogene activation or DNA damage-induced premature-senescence are also linked to aging [94-95]. The development of an abundant, highly disorganized and fragmented collagen matrix in the prostate is a result of aging, and promotes oxidative stress [96].

This leads to the increased expression of stress response proteins such as Apoliprotein D, as well as the increase in inflammatory infiltrates. Together with redox-induced biochemical alterations, modulation of redox sensitive transcriptional activators leads to changes in biological functions of proteins, leading to tumor initiation and malignant transformation [97-98].

Increased ROS levels in cancer cells correlate with tumor aggressiveness and poor prognosis [99-100]. One of the major sources of ROS in cancer cells is activated oncogene-induced upregulation of ROS-producing enzymes as in the case of K-ras transcriptionally activating endothelial NADPH oxidase NOX1 [101]. Other proto-oncogenes that induce ROS include SRC [102], c-MYC [103], and overexpression of receptor tyrosine kinases [104]. Loss of functional p53 also contributes to redox imbalance and elevated ROS levels. Furthermore, mitochondrial DNA (mtDNA) mutations associated with increased ROS levels due to increased leakage of electrons have been reported in both solid tumors and leukemia [105-107]. Extrinsic factors such as inflammatory cytokines (eg: TNFα), nutrient imbalance and hypoxia also result in dysregulation of ROS production [108-109].

ROS facilitates cancer cells proliferation through the activation of downstream targets such as the Keap1/Nrf2 pathway, which upregulates various cytoprotective genes and facilitates cancer progression [110]. The AKT/mTOR pathway ultimately activates nuclear factor κB (NF-κB) to induce pro-survival signals, and ROS are required for NF-κB activation in HeLa cells in response to inflammatory cytokines [111]. The alternative NF-κB pathway component RelB protects PCa cells from the detrimental effects of ionizing radiation, in part, by stimulating expression of the mitochondria-localized

antioxidant enzyme manganese superoxide dismutase (MnSOD) [112]. Cancer cells may acquire adaptive mechanisms, including the activation of ROS-scavenging systems such as glutathione (GSH), as well as the inhibition of apoptosis [113], which may lead to malignant transformation, metastasis and chemo-resistance. Recently, docosahexaenoic acid (DHA, Omega-3) have been shown to selectively induce human PCa cell sensitivity to oxidative stress through modulation of NF-κB [114].

Diet

A variety of dietary factors have been implicated in the development of PCa. One of these factors is higher consumption of polyunsaturated fat. Four case control studies found a positive correlation between higher intake of polyunsaturated fat and higher risk of PCa [115-118]. It is speculated that a higher fat intake leads to alterations in the hormonal profile, increases in oxidative stress, and generation of fat metabolites that act as protein or DNA-reactive intermediates, thereby increasing the risk for PCa [119-121]. A review reported a statistically significant protective effect in diets high in fruit and vegetable consumption in 128 out of 156 studies [122-123], suggesting that a high intake of fruits and vegetables might be a valid tool for cancer prevention.

Other dietary factors implicated with PCa risk are vitamins A, C, D and, E, selenium and lycopene. Studies suggest that there is a correlation between lower levels of these vitamins and higher risk of PCa [117, 124-128]. This was confirmed in randomized double-blind trials showing that a diet supplemented with selenium reduced the risk of PCa [129-130]. Indole-3-carbinol (I3C), a phytochemical from cruciferous vegetables, and its major active metabolite 3,3'-diindolylmethane (DIM), stimulate BRCA1 in breast

and PCa cells and has been shown to protect cells against oxidative stress mediated by hydrogen peroxide (H_2O_2) and γ-radiation [131-132].

However, different results were obtained from the Prostate Cancer Prevention Trial, which examined nutritional risk factors for PCa among 9,559 participants. High-grade cancer was associated with high intake of polyunsaturated fats, but protective effects of dietary and nutrition supplements were not shown (United States and Canada, 1994-2003) [133].

Traditionally, diets comsumed by African Americans are higher in fat intake and lower in fruit and vegetable consumption in comparison to other ethnic groups [134] such as the Japanese, who consume a relatively low fat diet with high intake of soy products, and have lower PCa risk [135]. However, it has been observed that as the fat content of Japanese diet increases towards Western levels, their incidence of PCa has also increased [136], [137-138]. Physical inactivity and higher fat and meat intake in Western countries has been proposed to contribute to the increase in cancer risk [139]. Obesity results in an increase in serum concentrations of estrogen, testosterone, insulin, insulin-like growth factor 1 (IGF-I), which have all been linked to PCa, and leptin, which has been associated with high-grade PCa [140-143]. An increased level of inflammation is also observed, as seen in increased levels of interleukin 6 (IL-6) and tumor necrosis factor-α [144]. Chronic inflammation has been reported to be associated with proliferative inflammatory lesions that may lead to prostate tumors [145-147].

Although some studies linked obesity to modestly reduced incidence of low-grade disease [148]; others found no association between obesity and PCa. However, obese men have been linked to slightly decreased PSA levels [149-153] and enlarged prostates

[154-155], which leads to delayed detection of PCa and worse clinical outcome. Obese AA men with low PSA levels have been observed to have more aggressive prostate tumors than non-AA men [156]. In many trials, obesity increases the risk of more aggressive PCa, by a modulating effect of adiponectin, insulin or IGF-I, and may decrease either the occurrence or the likelihood of diagnosis of less-aggressive tumors [122, 157].

Prostate Cancer Detection

Common tests used to screen for PCa include digital rectal exam (DRE) and PSA. Despite the poor accuracy of DRE (59%) [158], it is used routinely for PCa screening because of its ability to detect cancer in some men with normal PSA levels with small and well differentiated tumors [159]. However, early cancers are rarely palpable, and clinically important cancers are usually located in distant regions of glands evasive to digital palpation [160].

Although the levels of PSA are higher in men with PCa, they are also elevated in other conditions such as benign prostatic hyperplasia (BPH), prostatitis, or even induced by medications or medical procedures, leading to many unnecessary biopsies on patients [161]. The inadequacies found in PSA testing underscore the need for new PCa biomarkers. Most recent biomarkers include Prostate cancer gene 3 (PCA3) and CD14. The Progensa™ (Gen-Probe Inc., San Diego, CA, USA) PCA3 urine test, is a quantitative test that detects PCA3 mRNA levels, which are overexpressed in more than 95% of primary prostate tumors [162]. Urinary CD14 is highly specific (>81%) and allows the distinction of BPH from cancer with high specificity (84-100%) when

combined with urinary prostate-specific antigen [163]. Future diagnostic tools with combinations of multiple biomarkers will optimize the detection and characterization of PCa.

Progression of Prostate Cancer

The progression from a normal prostate to PCa is illustrated in Figure 2. PCa progression starts with a prostatic lesion called proliferative inflammation atrophy (PIA), proceeding towards prostatic intraepithelial neoplasia (PIN), leading to PCa and ending in metastasis [164].

PIA

PIA usually occurs in the periphery of the prostate, consisting of focal areas of epithelial atrophy that fail to differentiate into columnar secretory cells [164-167]. PIA has been associated with chronic inflammation, arising as a consequence of the regenerative proliferation of prostate epithelial cells in response to injury caused by inflammatory oxidants [164]. Some characteristics in the development of PIA from a normal prostate are the infiltration of lymphocytes, macrophages, and neutrophils, which might be caused by repeated infections, dietary factors, and/or the onset of autoimmunity [145]. These phagocytes release reactive oxygen and nitrogen species causing DNA damage, cell injury, and cell death, thus triggering the onset of epithelial cell regeneration [145]. Regions of PIA are also often located in proximity with PIN and adenocarcinoma, and have been proposed to represent a precursor lesion for PCa [164, 168].

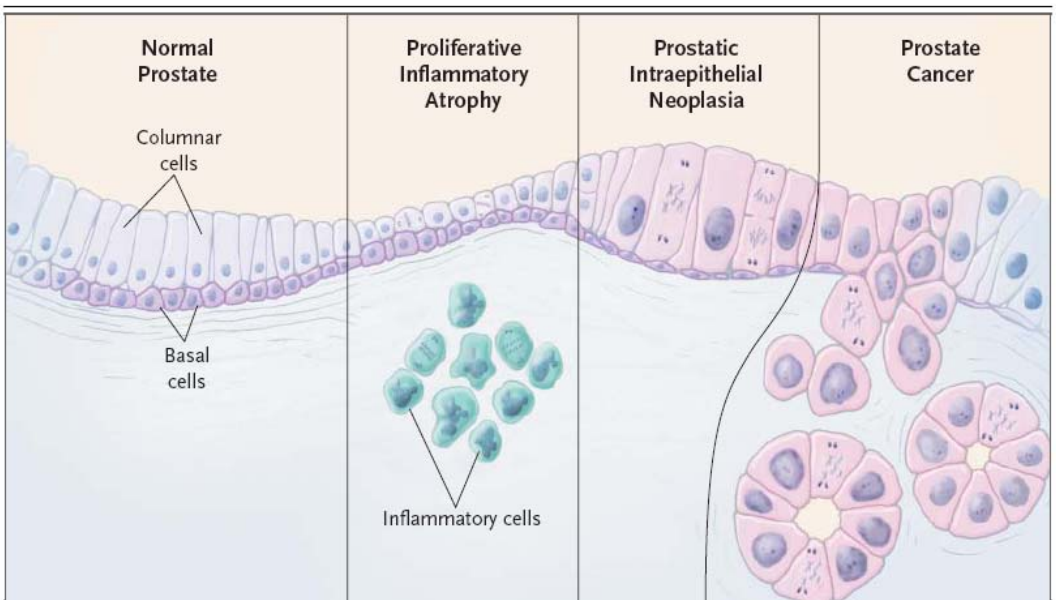


Figure 2. Schematic drawing of PCa progression by Nelson et al. [166]. The normal prostate has differentiated basal and columnar cells. As it progresses to proliferative inflammatory atrophy (PIA) focal areas of epithelial atrophy develop and, cells fail to differentiate into columnar secretory cells. In prostatic intraepithelial neoplasia (PIN) lesions disruption of the basal cell layer can be seen as well as nuclear abnormalities. When it progresses to PCa the basal cell layer is lost as well as the distinct glandular architecture. Cells appear dedifferentiated.

PIN

PIN is widely accepted as a precursor for prostate cancer, supported by the finding of PIN lesions in the peripheral zone, where PCa develops [169], although this relationship has not been demonstrated conclusively [168]. PIN is histologically characterized by the appearance of luminal epithelial hyperplasia, loss of basal cells, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia, and nuclear atypia [170] (Figure 2). PIN lesions precede those of the carcinoma by ten years [171], and possess chromosomal abnormalities that resemble those found in early stages of PCa [172-175]. The architectural and cytological features of PIN also resemble those found in PCa [176]. In addition, markers of differentiation found in early PCa are found in PIN lesions [172, 177]. The main differences between them are that PIN lesions have intact basement membrane and do not produce high levels of PSA compared to the initial stages of PCa [172, 176].

Prostate Carcinogenesis

Normal prostate epithelial cells have a relatively low rate of proliferation. PIN and localized PCa show a seven to ten fold increase in the rate of proliferation [178]. Also, localized PCa is characterized by a continued proliferation of genetically unstable luminal cells and accumulation of genomic changes [145], which are observed in the loss of regions of chromosomes 10q and 13q. The loss of chromosome 10q has been found in approximately 50-80% of PCa, specifically loci 10q23.1 and 10q24-q25 [174, 179-186]. Loss of Phosphatase and tensin homolog (PTEN) gene results in activation of protein kinase B (PKB), leading to decreased sensitivity to cell death [1, 187-188], Evidence implicating PTEN as a key event in prostate carcinogenesis include observations that

PTEN is mutated in four PCa cell lines and PTEN heterozygous mutant mice developed prostatic epithelial hyperplasia and dysplasia [189-192]. Loss of parts of chromosome 13q, including a region for the retinoblastoma (Rb) gene, occurs in 50% of prostate tumors [193-195]. Loss of Rb protein expression has been observed in localized as well as in more advance stages of PCa [183, 196-197].

Epigenetic perturbations are also important contributing factors in prostate carcinogenesis, and may provide useful biomarkers for disease progression [198-200]. DNA methylation mediated gene silencing has been reported in genes involved in signal transduction, hormonal response, cell cycle control, and oxidative damage response, such as glutathione-S-Transferase Pi (*GSTP)1*. Global changes in chromatin modification correlates with cancer progression [201-202], as seen in trimethylation of lysine residue 27 of histone H3 (H3K27-me3), mediated by the histone methyltransferase enzyme Ezh2, a key oncogenic driver of advanced disease and metastasis [203]. Increased levels in prostate cancer are also associated with repression of tumor suppressor genes such as Disabled homolog 2-interacting protein (DAB2IP), a member of the Ras GTPase family [204].

Treatment of PCa

Localized PCa is the most commonly diagnosed stage in patients. The main treatment for this stage is either radiation therapy or prostatectomy [205] combined with hormonal therapy depending on risk status [27]. Unfortunately, relapse often occurs with aberrant prostate tumor growth. A rapid rising in the serum PSA levels is usually the first sign of failure to treatment is [27]. These patients are then subjected to androgen ablation

therapy through either chemical or surgical castration, which generally kills androgen reliant prostate cells but may promote vasomotor flushing, loss of libido, erectile dysfunction, gynaecomastia, weight gain, osteoporosis and loss of muscle mass [205]. However, the majority of such treatments ultimately result in the recurrence of highly aggressive and metastatic androgen independent PCa known as hormone refractory prostate cancer (HRPC) or castration-resistant metastatic prostate cancer (CRPC) [1, 206]. The arise of HRPC is made possible by variable combinations of clonal selection, adaptive upregulation of anti-apoptotic genes, ligand-independent activation of the androgen receptor, and alternative growth factor pathways [207-215]. Chemotherapy agents used in the treatment of HRPC fall into three categories: 1) DNA intercalating agents, 2) alkylating compounds, and 3) microtubule stabilizing agents [205]. Current treatment of HRPC using the microtubule stabilizing drug docetaxel has shown a three month increase in the overall survival in patients with HRPC [216-217].

Chemoresistance in PCa has been associated with the overexpression of survival proteins or downregulation of pro-apoptotic proteins. Survival proteins such as Bcl-2, Bcl-XL, members of the inhibitor of apoptosis protein (IAP) family, and heat shock proteins (hsp), as well as the kinase Pim-1 and the tumor suppressor p27, may result in aggressively growing tumors and contribute to resistance to chemotherapeutic agents [218]. Since most advanced tumors are insensitive to chemotherapy, the resistance provided by these survival proteins becomes a major problem at the clinical level. For this reason, new molecules that influence these survival pathways, administered in combination with cytotoxic drugs have become the preferred strategy in clinical studies to overcome intrinsic drug resistance [218]. We will discuss below various survival

proteins that are being studied mainly in PCa and that contribute to chemoresistance, expecially to taxanes.

PCa Survival Proteins

Bcl-2

Bcl-2 is a proto-oncogene localized on the mitochondrial outer membrane that prevents apoptosis induced by different stimuli [219] by suppressing the release of cytochrome c from the mitochondria [220]. It is the primary member of the Bcl-2 family of proteins which regulates the assembly of pro-caspase/apoptosome complex [221]. All members of the Bcl-2 family contain one of four conserved Bcl-2 homology domains (BH1-BH4). Members of this family are divided in 3 categories: anti-apoptotic, multidomain pro-apoptotic, and BH3-only pro-apoptotic members [222]. In PCa, Bcl-2 overexpression is associated with progression to an androgen-independent form [223]. Increased expression of Bcl-2 induces resistance to androgen ablation therapy, while its inhibition delays progression to androgen-independence and sensitizes PCa cells to therapy [224-225]. PC3 cells treated with G3139 (antisense oligodeoxynucleotides for bcl-2) followed by docetaxel (DTX) treatment resulted in a higher apoptotic rate than treatment with DTX alone [226]. Furthermore, Bcl-2 was overexpressed in the DTX-resistant gastric cancer cell line BGC-823 but not in its non-resistant counterpart [227]. In the androgen-responsive prostate cancer line, LNCaP, overexpression of Bcl-2 permits continued growth *in vitro* and tumor formation *in vivo* despite androgen deprivation [228].

Bcl-xL

Bcl-xL is a member of the Bcl-2 family with anti-apoptotic properties. Like Bcl-2, Bcl-xL regulates the mitochondrial membrane potential and can block the release of cytochrome c and apoptosis inducing factor (AIF) into the cytoplasm [229-230]. Krajewski et al. observed that 100% of prostate adenocarcinoma cases stained positively for Bcl-xL, with correlation between stain intensity and increasing Gleason score [231]. A study combining antisense oligos against Bcl-xL and DTX (as well as other cytotoxic drugs) in the PCa cell lines PC3 and LnCaP [232] showed that inhibition of Bcl-xL sensitized these cells to DTX-induced death [232]. Recently, Gleave's group showed that inhibition of both Bcl-2 and Bcl-xL with bispecific antisense oligos sensitized LnCaP cells to DTX-induced cell death [233].

In another study, mRNA expression levels of all six antiapoptotic Bcl-2 subfamily members was investigated in 68 human cancer cell lines. Mcl-1 represents the anti-apoptotic Bcl-2 subfamily member with the highest mRNA levels in the lung, prostate, breast, ovarian, renal, and glioma cancer cell lines. Prominent expression of Bcl-2 seems to be limited to leukemia cell lines and not in solid tumor cell lines [234]. A subsequent immunohistochemistry (IHC) investigation of 64 adenocarcinomas of the prostate found that 25, 100, and 81% of the tumor samples exhibited observable levels of Bcl-2, Bcl-x_L, and Mcl-1, respectively [235]. Furthermore, pre-clinical studies have shown that tumors highly expressing Mcl-1 are typically resistant to compounds that selectively target Bcl-2 and Bcl-x_L [236-237]

The Inhibitor of Apoptosis Proteins

The inhibitor of apoptosis proteins (IAPs) comprise a family of proteins with one or more baculovirus IAP repeat (BIR) domain repeats. To date, only eight members have been identified: c-IAP1, c-IAP2, NAIP, Survivin, XIAP, Bruce, ILP-2, and Livin [221]. IAPs can block caspase 9 and caspase 3/7 activity leading to cell survival, and can be negatively regulated by Smac, Omi, and XAF-1 [238]. Krajewska et al. were the first to show overexpression of several IAP members (cIAP1, cIAP2, XIAP, and survivin) in PCa tissues [239]. In addition, increased levels of these IAPs were observed during disease progression in a transgenic mouse model of PCa [239]. Survivin is associated with cell proliferation and has a role in 1a,25-dihydroxyvitamin d(3) induced cell growth inhibition in PCa [240]. A recent study show survivin was not detected in cytoplasm of epithelial cells but exhibited increasing expression correlating to Gleason score [241].

Survivin

Survivin has been shown to mediate resistance to paclitaxel therapy in PCa cells. Adenoviral inhibition of survivin lead to sensitization to cell death induced by paclitaxel in PC3, DU145 and LnCaP cells [242]. Small inhibitory RNA (siRNA) -mediated down-regulation of survivin in PC3 cells showed lower tumor formation in nude mice xenografts in vivo [243]. In addition, survivin antisense oligonucleotide (ASO) potently downregulated survivin expression in human cancer cells derived from lung, colon, pancreas, liver, breast, prostate, ovary, cervix, skin, and brain. Inhibition of survivin expression induced caspase-3-dependent apoptosis, cell cycle arrest in the G2-M phase, and multinucleated cells and sensitized tumor cells to chemotherapeutic-induced

apoptosis. Antisense survivin (LY2181308) is being evaluated in a Phase II clinical trial in combination with DTX for the treatment of PCa [244]. Intratumoral injection of p53 and anti-survivin inhibited the growth and survival of tumor xenografts in a nude mouse model [245]. Stimulation with IGF-1 led to increased survivin expression in PCa cells, which is abolished by the mTOR (mammalian target of rapamycin) inhibitor, rapamycin. Rapamycin, alone or in combination with suboptimal concentrations of taxol, was shown to reduce survivin protein levels, and decrease viability of PCa cells [246].

PTEN silences the expression of survivin independent of p53, via direct occupancy of the survivin promoter by FOXO1 and FOXO3a transcription factors. Expression of survivin and PTEN was found to be inversely correlated in cancer patients. [247-249]. Other pivotal tumor suppressors, including p53 [250-251], Adenomatous polyposis coli (APC) [252], or sirtuin (silent mating type information regulation 2 homolog) 1 **(**SIRT1) [253] have been shown to acutely silence the survivin gene via different mechanisms.

Clusterin

Clusterin (CLU) is a cytoprotective chaperone protein expressed in virtually all tissues and found in all human fluids [254-256]. CLU is upregulated under conditions of cellular stress [257], such as oxidative stress [258], ionizing radation, and heat shock [259]. Its role has been documented in PCa for paclitaxel/docetaxel resistance as well as in renal, breast, and lung tumor cells. Moreover, it is abnormally upregulated in numerous advanced stage and metastatic cancers spanning prostate, renal, bladder, breast, head and neck, colon, cervical, pancreatic, lung carcinomas, melanoma, and lymphoma.

In the prostate, its expression levels have been correlated with pathological grade on biopsy [256, 260]. CLU expression is downregulated in tumor samples in comparison with benign matched tissues [261], as well as in both low- and high-grade PCa, suggesting early event in PCA onset [262-263]. Meta-analysis of available microarray data shows that CLU mRNA is significantly downregulated in PCa tissue compared to normal prostate in 14 out of 15 independent studies[264]. However, its mRNA expression increased in LNCaP tumors after radiation and decreased radiosensitivity of tumors [265]. Contradictory reports regarding location of CLU expression: epithelial versus stromal compartment [266-267], suggests the different subcellular location of different forms of CLU, which may play different roles in PCa.

It is noteworthy that only the cytoplasmic/secretory clusterin form (sCLU), and not the nuclear form, is expressed in aggressive late stage tumors, which is in line with its antiapoptotic function. Most significantly, sCLU expression is documented to lead to broad-based resistance to other unrelated chemotherapeutic agents such as doxorubicin, cisplatin, etoposide, and camphothecin. Resistance to targeted death-inducing molecules, tumor necrosis factor, Fas and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), or histone deacetylase inhibitors can also be mediated by sCLU. The actual mechanisms for sCLU induction are unclear but signal transducer and activator of transcription 1 (STAT1) is required for its constitutive upregulation in docetaxel-resistant tumor cells. In addition, sCLU appears to stabilize Ku70/Bax complexes, sequestering Bax from its ability to induce mitochondrial release of cytochrome c that triggers cell apoptosis [268].

Recently, it was shown that CLU is silenced by promoter methylation in the murine TRAMP-C2 cell line, as well as in the human prostate cancer cell line LNCaP [269]. Treatment of PC3 cells with antisense (AS) CLU oligodeoxynucleotide (ODN) decreased CLU mRNA by >80%. Combined treatment with adenoviral-mediated p53 gene transfer or mitoxantrone completely eradicated PC3 tumors and lymph node metastases from orthotopic PC3 tumors in 60% and 100% of mice respectively [270]. Combination of CLU AS oligonucleotide with DTX, paclitaxel or mitoxantrone enhanced antitumor activity of the drugs *in vitro* and *in vivo* [271],[256]. Phase I study reported that an AS CLU, OGX-011, inhibited CLU expression in prostate cancer tissues by more than 90% [272]. A subsequent Phase II study in Patients with Metastatic Castration-Resistant Prostate Cancer reported that median progression-free survival of patients on OGX-011 and DTX combination treatment was 23.8 months at a median follow up of 35 months, longer than 16.9 months for patients on DTX in combination with dexamethasone and prednisone [273]. Strong preclinical and clinical proof-of-principle data provide rationale for further study of sCLU inhibitors in randomized phase III trials, which are planned to begin in 2010 [274].

PKB plays a critical role in upregulating cytoplasmic/secretory sCLU, which is responsible for DTX resistance. Loss of AKT function resulted in loss of sCLU and was accompanied by chemosensitization to docetaxel and increased cell death via a caspase-3-dependent pathway. AKT mediates sCLU induction via signal transducer and activator of transcription activation, which drive sCLU gene expression [275].

Heat Shock Proteins

Heat shock proteins (HSPs) are molecular chaperones made up of a set of highly conserved proteins whose expression is induced by a wide variety of physiological and environmental insults including chemical and physical stresses [276-277]. HSPs help cells in adapting to changes in their environment, modifying the structure and interactions of other proteins, thus allowing them to survive to lethal conditions [278]. Mammalian HSPs are classified according to their molecular weight: HSP90, HSP70, HSP60 and small HSPs (15–30 kDa) that include HSP27 [257]. HSPs family members are expressed either constitutively or regulated inductively, and are present in different subcellular compartments.

High molecular weight HSPs are ATP-dependent chaperones, and rely on co-chaperones to modulate their conformation and ATP binding. In contrast, small HSPs act in an ATP-independent fashion [279]. Hsp27 and Hsp70 are the most strongly induced during cellular stress [257], their overexpression increases tumorigenic potential of rodent cells in syngenic animals [280-284], while HSP70 depletion led to tumor regression [283, 285].

In stressful conditions, HSPs enhance the ability of cells to cope with increased concentrations of unfolded or denatured proteins. They achieve this by either stabilizing or targeting selected proteins for proteasomal degradation [286]. In addition, HSPs interfere with apoptotic pathways by associating with key effectors of the apoptotic machinery such as cytochrome c [287], apoptotic protease activating factor 1 (Apaf-1) [288], or AIF [289], thereby inhibiting both caspase-dependent and caspase-independent cell death.

HSP27

HSP27 is abundantly expressed in cancer cells and further increased after various death stimuli including hyperthermia, oxidative stress, inhibition of tyrosine kinases, ligation of Fas/Apo-1/CD95 death receptor, radiation or addition of cytotoxic drugs [288]. Large, non-phosphorylated oligomers of HSP27 are powerful ATP-independent chaperones preventing aggregation. They decrease ROS in cells, consequently increasing anti-oxidant defense [290] and neutralizing the toxic effects of oxidized proteins [291], thus protecting from cell death. Hsp27 large oligomers are also involved in its post-mitochondrial anti-apoptotic effect [287]. In contrast, small oligomers stabilize actin microfilmaents [292] and participate in ubiquitination and degradation of selected proteins under stress conditions [286]. Hsp27 interferes with caspase activation upstream of the mitochondria when present in high levels. It prevented cytoskeletal disruption and Bid intracellular redistribution preceding cytochrome c release in L929 fibroblasts treated with cytochalasin and staurosporine [293]. Furthermore, phosphorylated Hsp27 directly interacts with Daxx to prevent cell death [294].

Increased expression of Hsp27 has been observed in hormone refractory prostate cancer (HRPC). Its inhibition in PC3 cells with antisense (AS) oligos led to the sensitization to paclitaxel-induced cell death, decreased PC3 tumor progression [295] growth, and induced apoptosis via caspase-3 activation *in vitro* [296]. Hsp27 knockdown in athymic mice bearing LNCaP tumors significantly delayed LNCaP tumor growth after castration [297]. A second-generation AS oligo, generated using the 2′-*O*-(2-methoxy) ethyl (2′-MOE) backbone, targeting Hsp27 inhibited bladder tumor growth in mice, enhanced sensitivity to paclitaxel, and induced significantly higher levels of apoptosis

compared with xenografts treated with control oligonucleotides [298]. Recently, it was reported that Hsp27 interacts with eIF4E and decreases eIF4E ubiquitination and proteasomal degradation. Overexpression of eIF4E induced resistance to androgen-withdrawal and paclitaxel treatment in LNCaP prostate cells *in vitro* [299]. In addition, IGF-1 induces Hsp27 phosphorylation in a time- and dose-dependent manner in PCa. Hsp27 knockdown destabilizes Bad/14-3-3 complexes and increases the apoptotic rate of cancer cells [300]. Hsp27 is also involved in metastasis through its association with p38 MAPK, stimulating cancer cell adhesion in the presence of dietary fatty acids [301]. All these support the importance of targeting HSPs as one method to curb cancer cell growth.

It should be noted that Hsp27 is a target gene of lens epithelium derived growth factor/p75 (LEDGF/p75) [302], and we cannot rule out the possibility that upregulation of Hsp27 and other stress proteins by LEDGF/p75 may contribute to DTX resistance through the ability of these proteins to reduce ROS and prevent lysosomal destabilization. Studies from our group described in this dissertation have provided evidence that the stress protein LEDGF/p75, in its interaction with MeCP2, might be involved in the resistance of PCa cells against chemotherapy by upregulating heat shock protein 27.

The Biology of LEDGF/p75

LEDGF/p75, also known as transcription co-activator p75 [303], autoantigen of 70 kD associated with dense fine nuclear speckles (DFS70) [304], and its alternate spliced variant LEDGF/p52 were first described in 1998 by Ge and colleagues as enhancers of transcriptional activation found in the nucleus of cells, with LEDGF/p52 being the more potent co-activator [303].

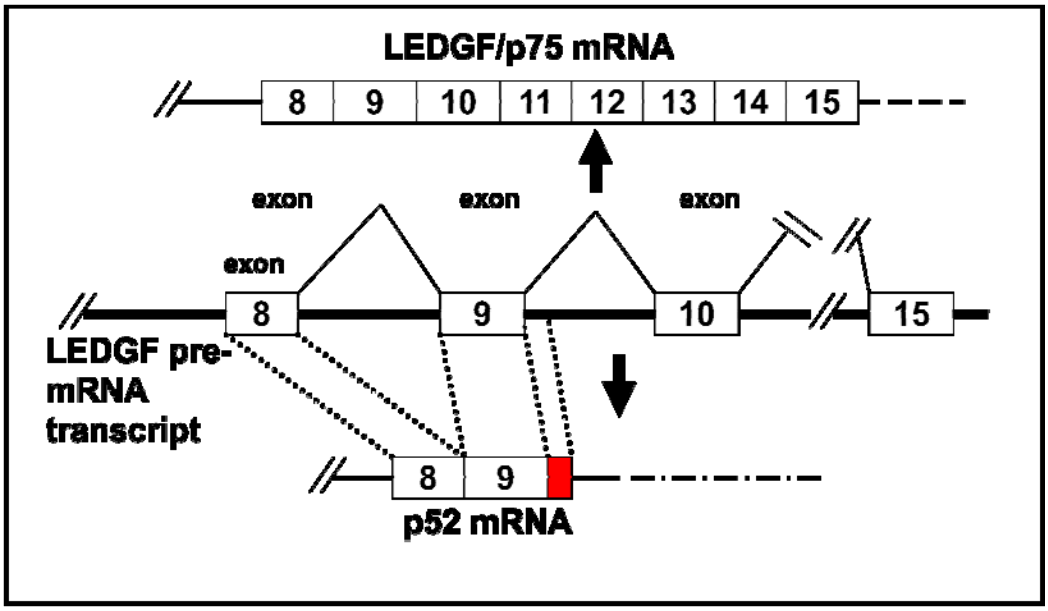
LEDGF/p75 and Autoimmunity

Initial studies by Ochs and colleagues indicated that autoantibodies to LEDGF/p75 were present at significantly high frequencies in patients with atopic dermatitis (AD) [304]. Ayaki et al. [305] confirmed this observation by reporting that some patients with AD who produce autoantibodies to LEDGF/p75 had cataracts, and that these autoantibodies, when concentrated, exhibited cytotoxic activity against lens epithelial cells in monolayer or organ cultures. Autoantibodies to LEDGF have been found in patients with inflammatory conditions associated with eye and skin disorders such as Vogt-Koyanagi-Harada syndrome, sympathetic ophthalmia, Behcet's disease, sarcoidosis, alopecia areata, interstitial cystitis, and atypical retinal degeneration [306-308], as well as diverse organ-based and systemic autoimmune diseases, nonspecific musculoskeletal complaints, and miscellaneous inflammatory conditions [309]. Taken together, these studies suggest that autoimmunity to LEDGF/p75 is associated with inflammatory conditions in which this protein might be upregulated, leading to loss of immune tolerance characterized by an autoantibody response to this protein.

LEDGF/p75 Structure and Domain

The LEDGF gene was mapped to chromosome 9p22.2, a region implicated in cancer [310]. The LEDGF gene encodes 15 exons and 14 introns, where LEDGF/p75 is encoded by exons 1-15 (530aa) and LEDGF/p52 by exons 1-9 and part of intron 9 [303] (333aa) (Figure 3a). LEDGF is a nuclear protein that preferentially associates with condensed chromatin areas and remains tightly bound to chromatids during cell division.

A



B

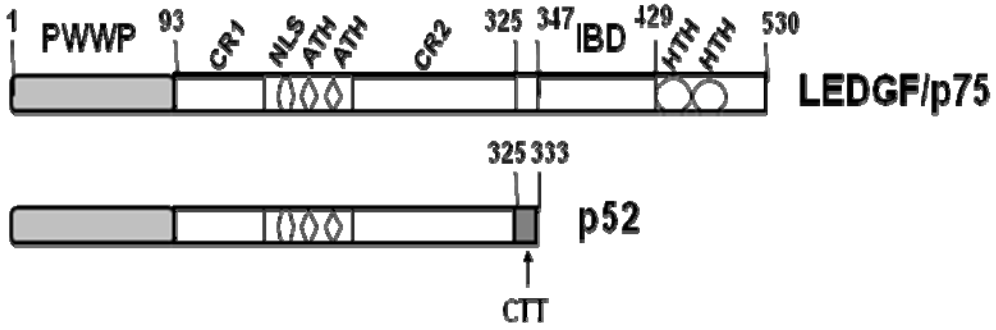


Figure 3. Schematic representation of the domain structure of LEDGF/p75 and LEDGF/p52. A) LEDGF/p75 is composed of exons 1-15. p52 shares the same N-terminal 9 exons, with the addition of intron-derived eight amino acids. B) The shared regions of both proteins contain the PWWP domain, the nuclear localization signal (NLS), the basic leucine zipper (bLZ) domain overlapping the helix-turn-helix (HTH) region, and an AT-hook motif. The LEDGF/p52 C-terminal end contains an intron derived 8 amino acid sequence termed C-terminal tail (CTT) that is not present in LEDGF/p75. The LEDGF/p75 C-terminal region (absent in p52) contains an integrase binding domain (IBD).

Both LEDGF splice variants belong to the hepatoma derived growth factor (HDGF) family, sharing homology in the N-terminal region where a PWWP domain (residues 1-98) is found [311-313]. The name of this domain derives from the presence of the tetrapeptide motif "proline-tryptophan-tryptophan-proline". Most proteins with PWWP domains bind DNA and are putative transcription factors. Secondary structure prediction using PSIPRED program [314] suggested that the PWWP protein is composed of three beta sheets followed by an alpha helix. In addition, both LEDGF proteins share a nuclear localization signal (NLS) at residues 148-156, a Tat like sequence within the NLS region, a basic leucine zipper (bLZ) sequence that overlaps with a helix-turn-helix (HTH) region in residues 154-175, and two AT-hooks in residues 178-183 and 191-197 (Figure 3b) [315-316]. In general, AT-hooks bind to minor groove of AT rich DNA, and are thought to co-regulate transcription by modifying the architecture of DNA to enhance the accessibility of promoters to transcription factors. The tri-partite region, consisting of the NLS, AT-hooks and charged region 1 (CR1), is sufficient for chromatin binding [316], while CR2 facilitates LEDGF/p75 binding to supercoiled DNA [317] . LEDGF variants have little secondary structure, with mostly large random coiled regions which are implicated in DNA and RNA recognition, modulation of protein binding, and control of protein lifetime [318]. Similar to high mobility group (HMGA) proteins, LEDGF variants may be downstream targets that are transcribed or modified specifically to various environmental influences and interact with proteins or DNA in an interactome, influencing a diverse array of normal biological processes including growth, proliferation, differentiation and death [319].

The C-terminus of LEDGF/p75, absent in LEDGF/p52, contains a region (res 339-442) that shares sequence homology with HDGF-related protein 2 (HRP-2) and has been defined as the Human immunodeficiency virus-1 (HIV-1) integrase binding domain (IBD) [320-321]. In addition, this domain overlaps with the epitope recognized by human anti-LEDGF/p75 autoantibodies [322] and is the only ordered region (res 411-438) in the C-terminal domain [306]. This region has also been found to interact with other proteins such as PogZ, JPO2, and menin-MLL (mixed-lineage leukemia) complex [323-326] and tethers them to the chromatin.

A recent report combined different quantitative fluorescence techniques to monitor *in vivo* chromatin interactions of LEDGF/p75 [327]. LEDGF/p75 was observed to have dynamic interactions with immobile protein/chromatin complexes. It moves about in nuclei of living cells in a chromatin scanning/hopping mode typical of transcription factors [328], which most likely is the mechanism used to tether HIV integrase (IN) to random regions. In line with previous reports in HIV and menin/MLL, LEDGF/p75 was observed to mediate chromatin tethering of its cargo [329].

LEDGF/p75 and the Cellular Stress Response

LEDGF/p75 was originally thought to be a lens epithelial cell (LEC) growth factor since its overexpression enhanced the survival and growth rate of mouse LECs, cos7 cells, human fibroblasts, keratinocytes, and retinal cells [330-331]. In addition, deprivation of LECs of this protein with anti-LEDGF/p75 antibodies reduced its nuclear localization and induced cell death [332]. Mice injected with LEDGF protein had better preserved rods and cones and higher levels of Hsp25 and αB-crystallin compared to

vehicle-injected mice [333]. Further analysis of LEDGF/p75 revealed that its function was not that of a growth factor but of a survival protein that protected mammalian cells from thermal, oxidative and serum stress through the transcriptional activation of stress proteins such as heat shock protein 27 (Hsp27), αB-crystallin [330-331, 334-336] antioxidant protein 2/peroxiredoxin 6 (AOP2/Prdx6), alcohol dehydrogenase (ADH) [302, 337-338], involucrin [339], vascular endothelial growth factor (VEGF-c) [340], and interleukin-6 (IL-6) [341].

LEDGF/p75 was reported to recognize stress response elements (STRE) and heat shock elements (HSE) in promoters of stress proteins [302, 342]. However, further studies failed to confirm exclusive binding to these sites [316]. Recently, LEDGF/p75 was demonstrated to primarily bind downstream of active transcription units transcription start site using DamID technology, focusing on the highly annotated ENCODE (encyclopedia of DNA Elements) region. LEDGF/p75 binding was not restricted to STRE or HSE in the genome, and correlated with active chromatin markers and RNA polymerase II binding [343].

Our group has shown that overexpression of LEDGF/p75 protects HepG2 cells from serum deprivation-induced cell death [312]. However, cleavage fragments of LEDGF/p75 generated by caspases appeared to enhance cell death, suggesting that proteolytic cleavage of LEDGF/p75 during apoptosis not only abolishes its survival activity but may accelerate cell death [312]. We also demonstrated that LEDGF/p52 is cleaved by caspases to generate a prominent fragment of 38 kDa termed p38 [344]. Transient overexpression of LEDGF/p52 as well as the p38 fragment displayed classical features of apoptosis in various tumor cell lines [344]. The p38 fragment was found to

inhibit the transcriptional function of LEDGF/p75 necessary for its stress survival activity. These results suggested that while LEDGF/p75 acts as a stress survival protein, its spliced variant p52 has the opposite effect, an inducer of apoptosis that antagonizes the pro-survival function of LEDGF/p75. Consistent with these findings, we demonstrated that human cancer cell lines express high levels of LEDGF/p75 but low levels of p52 [344].

Stable overexpression of LEDGF/p75 in RWPE-2 and PC3 PCa cells conferred protection to the cytotoxic drug docetaxel but not to caspase-dependent apoptosis-inducers, TRAIL and staurosporine (STS), suggesting that LEDGF/p75 might promote resistance preferentially to oxidant-induced caspase-independent cell death associated with lysosomal destabilization [345]. LEDGF/p75 also modulates caspase-independent lysosomal cell death in HeLa and MCF-7 cancer cells, promoting tumorigenic potential of mouse-bearing HeLa tumors [346].

Recent studies by Huang et al. identified other splice variants of p52 that display pro-apoptotic activity in Acute myeloid leukemia (AML) blasts [347]. One of these variants, p52b, is identical to p52 except for an extra 25 amino acid region at the C-terminus due to altered open reading frame. Other low expression variants with gross deletions in exons 5–8 were detected [347]. LEDGF/p75 and p52b antagonized daunorubicin- and cAMP-induced apoptosis in human NB4 AML cell line and HEK 293, whereas LEDGF/p52 splice variants with the deletions had pro-apoptotic effects. Overexpression of full length LEDGF/p75 protected cells against the pro-apoptotic effects of the p52 constructs.

Regulation of LEDGF/p75 Function

The charged domains of LEDGF (comprising of 16% lysines), make it a likely target of small ubiquitin-like modifier (SUMO) modification. SUMOylation sites mapped on LEDGF/p75 and p52 include K75, K250, and K254. Another site, K364, is on the C-terminal end present only on LEDGF/p75 and is different from the other lysine residues in that it is solvent exposed and situated in a typical consensus motif. The cellular localization of LEDGF/p75, as well as its chromatin binding ability, are not affected by SUMOylation [348]. However, mutation of K364R impaired LEDGF/p75 SUMOylation, extended the half life of the protein, and enhanced its transcriptional activity on Hsp27pr [348]. This is in line with previous reports where SUMOylation of transcriptional regulators were found to generally increase their transcriptional repressive activites, as seen in the regulation of genes during interferon response [349-351].

The pro-survival function of LEDGF/p75 appears to be attenuated by transforming growth factor beta (TGF-β1), a known regulator of apoptosis which down-regulates LEDGF/p75 gene transcription and protein expression in human lens epithelial cells [352]. TGF-beta1 also induced down-regulation of LEDGF/p75, Hsp27, and alphaB-crystallin promoter activities. This attenuation was proposed to occur through the TGF-β1 inhibitory element (TIE) on LEDGF/p75 promoter region [353] or through caspase activation, which cleaves LEDGF/p75 [312]. The role of TGF-β1 as a repressor of LEDGF/p75 was confirmed in a mouse cell line derived from a Prdx6 knockout model ($Prdx6^{-/-}$) [354]. This cell line displayed reduced LEDGF/p75 levels, but had increased mRNA and protein levels of TGF-β1, thus confirming its role in LEDGF/p75 repression [354].

Another protein that attenuates LEDGF/p75 survival activity is the anti-apoptotic protein Bcl-2 [355]. Initially, Bcl-2 was found to prevent interleukin-3 (IL-3)-dependent cells from apoptotic death upon withdrawal of the cytokine [356]. Bcl-2 has been shown to protect a variety of cells against apoptosis induced by serum and growth factor depletion, as well as gamma irradiation [357-359]. It also protects mammalian cells from oxidative stress [360-361]. Bcl-2 is involved in the regulation of cell cycle [362], modulation of cell differentiation [363-364], and regulation of gene expression [355, 365-368]. However, Bcl-2 up-regulation does not protect Burkitt's lymphoma and lymphoid cell lines from oxidative stress and oxidative stress-induced apoptosis [369-370], suggesting that its protective effect is cell line dependent. Rabbit LECs (rLECs) transfected with Bcl-2 were found to be more susceptible to H_2O_2-induced apoptosis due to the down-regulation of αB-crystallin through Bcl-2-mediated attenuation of LEDGF/p75 transactivation [355]. This attenuation was achieved through activation of ERK1/2 kinases [371]. Inhibition of the ERK1/2 signaling pathway with pharmacological inhibitors or dominant-negative mutants abolished Bcl-2 modulation of AP-1 and LEDGF/p75.

LEDGF/p75 and Cancer

The presence of serum autoantibodies in patients with PCa was first reported in year 1972 [372]. The characterization of autoantibody responses in PCa has led to the identification and characterization of a number of candidate tumor-associated antigens (TAA). Among these antigens are 5α-reductase, p53, prostasomes, glucose-regulated

protein-78kDa (GRP78), MUC1, PARIS-1, p90 and p62 , and several cancer/testis antigens [373].

Our group reported the presence of autoantibodies to LEDGF/p75 in PCa patients' sera [374]. In that study by Daniels et al., elevated expression of LEDGFp75 was observed in PCa cell lines and tumors but not in normal prostate cells or tissue. The cleavage of overexpressed LEDGF/p75 in dying tumor cells might generate immunogenic forms of the protein that could be potentially immunostimulatory. Since anti-LEDGF/p75 autoantibodies are a common finding among ANA-positive individuals with no obvious symptoms or systemic autoimmune disease, they could be considered fingerprints or sensors of hidden inflammatory conditions associated with increased oxidative stress, which could trigger up-regulation and/or activation of LEDGF/p75, and consequently, loss of immune tolerance to the activated protein.

Up-regulation of LEDGF/p75 has also been reported in breast biopsies [346]. In addition, significant increase in LEDGF/p75 mRNA was reported in bladder cancers but not in colon cancer [346]. Recent studies from our group established that this protein is upregulated (both transcript and protein levels) in several major human cancers, particularly in PCa (Basu et al., unpublished observations). Analysis of LEDGF/p75 transcript expression in Oncomine cancer gene microarray database revealed significant upregulation in 15 out of 17 tumor types, including breast, cervix, head and neck, kidney, skin, and stomach cancer (Basu et al., unpublished observations). Immunohistochemistry (IHC) analysis of LEDGF/p75 protein expression in tissue microarray (TMA)s from over 35 major types of human cancer detected significant overexpression of LEDGF/p75 protein in prostate, colon, liver, thyroid and uterine tumors.

Down-regulation of LEDGF/p75 by Hsp70-2 knockdown was observed in HeLa (cervical cancer), MCF-7 (breast cancer) and U2OS (osteosarcoma) cells. This resulted in reduced cell proliferation, apoptosis-like chromatin condensation, and destabilization of lysosomes [285]. Back-complementation of LEDGF/p75 reversed cell death induced by siRNA knockdown. Addition of pan-caspase inhibitor zVAD-fmk or antiapoptotic protein Bcl-2 failed to inhibit cell death in both LEDGF/p75 deficient HeLa and MCF-7 cells, suggesting involvement of caspase-independent cell death [285].

LEDGF/p75 has also been implicated in leukemia. The protein was found to be overexpressed in blasts from chemotherapy-resistance human AML patients [347]. Using serologic identification of recombinant expressed proteins (SEREX), LEDGF/p75 had been previously identified as an autoantigen in chronic lymphocytic leukemia (CLL) [375]. LEDGF/p75 has been also associated with chromosomal translocations in the t(9;11)(p22;p15) in both adult and pediatric AML and chronic myeloid leukemia (CML) (32-34), resulting in fusion of the C-terminus of LEDGF/p75 with the N-terminus of the nucleoprotein 98 (NUP98). The NUP98 gene is involved in 11p15 translocations in both de novo and therapy related AML as well as T-cell acute lymphoblastic leukemia (ALL) [376]. NUP98 encodes a component of the nuclear pore complex involved in nucleocytoplasmic transport as a docking protein [377]. The N-terminus of NUP98 contains 28 FxFG repeats (where x is usually a small residue such as Ser, Gly or Ala), core sequence motifs known to interact with CREB binding protein (CBP/p300) and act as transactivation domains [378] and has been reported to be rearranged in chromosomal translocations in patients with myelodysplastic syndromes, AML and T-cell acute lymphoblastic leukemia [376, 379-382]. In leukemias expressing NUP98 fusion proteins,

transcriptional regulation is altered, correlating to poor prognosis as in the case of overexpression of HoxA9 in AML patients [383]. Although the role of the NUP98-LEDGF/p75 fusion in AML patients is unknown, it is likely that this fusion might play a role in formation of multimeric complexes or facilitate interaction with other transcription factors or cofactors, thereby enhancing the transcriptional and pro-survival activities of LEDGF/p75 [384].

LEDGF/p75 is also a crucial cofactor required for promoting leukemic transformation or suppressing tumorigenesis in the endocrine lineage (multiple endocrine neoplasia type 1) by interacting with MLL/menin complexes [323] (to be discussed in more detail later).

LEDGF and its Interacting Proteins

HIV-1 IN

LEDGF/p75 is a cellular interaction partner of HIV-1 IN [385-387]. Recombinant LEDGF/p75 added to *in vitro* HIV based integrase assay enhanced recombinant HIV-1 IN strand transfer activity [385], while its knockdown of endogenous LEDGF/p75 abolished nuclear localization of HIV-1 IN together with its association with chromosomes [387-388]. The presence of LEDGF/p75 was also shown in the pre-integration complex (PIC) [389].

Interaction between LEDGF/p75 and HIV-1 IN is through LEDGF/p75's IBD domain (aa 347-429) located on its C-terminus [390-391]. NMR studies show a symmetrical complex containing a pair of IN tetramers and two subunits of LEDGF/p75 [390]. Deletion of both PWWP and AT hook domains depleted chromatin-association of LEDGF/p75, leading to defective HIV-1 replication [390]. Depletion of LEDGF/p75

resulted in a redistribution of HIV-1 integration sites from its characteristic distribution in transcription units (TUs) outside the promoter regions to a more aspecific distribution [392-393]. LEDGF/p75 also protects HIV-1 IN from proteosomal degradation in the cell [388, 394].

Due to LEDGF/p75's function in tethering HIV1-IN to the chromatin, a rigorous search for potential proteins that interfere with its HIV-IN binding has been launched, resulting in the identification of the proteins discussed below.

JPO2

JPO2, also known as RAM2, represses transcription of human monoamine oxidase (MAO) A through binding to three repetitive Sp1 sites in the promoter [395]. JPO2 potentiates c-Myc transforming activity and complements a transformation-defective Myc mutant [396]. JPO2 is closely related to JPO1, a Myc transcriptional target encoded by a Myc responsive gene [397]. It contains a PEST region (peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)) in its N-terminal region (res 29-54), a C-terminal RING-ringer-like zinc-binding motif (res 349-425), a putative leucine zipper (res 213-235), and a putative nuclear localization signal (res 301-318) [395]. Similar to HIV-1 IN, JPO2 is protected from degradation by LEDGF/p75, and is tethered to the chromatin [325]. It competes with IN for LEDGF/p75 binding [324,325]. Over-expression of JPO2 resulted in slight inhibition of HIV-1 replication but activated transcription from the HIV-1 LTR (Long terminal repeats) [325].

Menin/MLL complex

Menin is a tumor suppressor implicated in cancer pathogenesis and transcriptional regulation as an integral component of the MLL histone methyltransferase (HMT) complex. LEDGF/p75 binding to the MLL complex is mediated by menin, which tethers the complex onto the chromatin, leading to transcription and leukemic transformation [323]. Menin is a product of the MEN1 gene, and its functions are cell type, cell cycle, and interaction dependent [398]. Its loss of function results in multiple endocrine neoplasia type 1 (MEN1) [399], a syndrome characterized by a triad for parathyroid, enteropancreadtic endocrine, and anterior pituitary gland tumors. Double knock-out of *Men1* in the mouse is embryonic lethal [400], and no homozygous mutation has ever been detected in humans. In addition, it participates in dynamic regulation of pancreatic cell proliferation in response to normal physiologic demands during pregnancy [401].

Like LEDGF/p75, menin regulates transcription of heat shock proteins in fruit flies [402], and associates with RNA pol II [403] and Fanconi anemia group D2 protein (FANCD2), a protein involved in repair of DNA damage [404]. Notably, Menin, c-myc and Ski-interacting protein (SKIP) act together to promote HIV-1 Tat transactivation [405], suggesting that LEDGF/p75 interacts in a complex that works together for multiple functions, either in virus replication, or stress-induced transactivation. Along this line, LEDGF/p75 interactome would need other proteins to tightly regulate their transcription modulation fucntion.

PogZ

Using yeast-two hybrid screening, PogZ (pogo transposable element derived protein with zinc finger) was identified to interact with the C-terminus of LEDGF/p75 [324].

PogZ was previously identified as a potential interaction partner of the transcription factor Sp1 in a yeast-two-hybrid screen [406]. However, its cellular function was unknown. *In silico* analysis revealed that PogZ contains a six zinc-finger array in its N-terminus, with a characteristic catalytic site composed of two or three aspartic acid and/or glutamic acid residues with a specific spatial arrangement to allow coordination of Mg^{2+} cations (DDE (Asp, Asp,Glu motif)) domain) and a helix-turn-helix domain in its C-terminal end. Its DDE domain is essential for DNA strand cleavage, transfer and ligation [407]. PSI-BLAST algorithm uncovered pogZ sequence homology with Tigger Derived (TIGD) transposases, a domesticated transposase related to the DNA-transposases. PogZ does not restrict HIV-1 replication, and was efficiently displaced by HIV-1 integrase in competition assays [324].

Cdc7:ASK

Phosphorylation of cell division cycle 7 bound to activator of S phase kinase (Cdc7:ASK) is essential for interaction with its LEDGF/p75 [408]. Besides stimulating Cdc7:ASK kinase activity *in vitro*, LEDGF/p75 also enhances phosphorylation of MCM2 at Ser 53, the major target of Cdc7 phosphorylation [408]. Cdc7 is involved in meiotic recombination[409] and in replication-dependent DNA repair [410]. ASK expression is cell-cycle dependent [411], while Cdc7 protein levels remain stable throughout the cell cycle [412], with oscillating kinase activity depending on the abundance of its regulatory subunit [411]. Together with S-phase cyclin-dependent kinase, Cdc7 activates individual pre-replication complexes (pre-RCs) assembled at replication origins during G1. The Mini Chromosome Maintenance (MCM) complex is the major target of Cdc7 activity in

mammalian cells [413]. Chromatin-bound Mcm2 is phosphorylated by Cdc7 at the G1-S transition, and is required for initiation of DNA replication [414]. Furthermore, Cdc7 stimulates additional replication factors (Cdc45 and GINS (Go, Ichi, Ni and San) complex) associated with formation of an active helicase [415].

The association of LEDGF/p75 with JPO2, menin/MLL complex, CDC7-ASK suggests that LEDGF/p75 functions as a general adaptor between chromatin and proteins or nucleic-acid protein complexes that must be brought into the proximity of chromatin to exert their function (transcription, viral integration, etc.). It is possible that bridging proteins like JPO2 may facilitate the assembly of LEDGF/p75 chromatin association complexes.

MeCP2

Our efforts to identify interacting transcription factors of LEDGF/p75 led to the detection of methyl CpG binding protein 2 (MeCP2) (discussed in detail in Chapter two). MeCP2 is important for PCa growth and can overecome growth arrest induced by androgen receptor antagonist [416-417]. Furthermore, MeCP2 mRNA levels correlated with estrogen-receptor status in breast cancer specimens [418]. MeCP2 was the first member of its family discovered by Alan Bird's group in their effort to identify proteins bound to methylated DNA [419]. Its methyl CpG binding domain (MBD) is shared with its other family members MBD1-4 [420]. The MBD forms a wedge-shaped structure composed of a β-sheet superimposed over an α-helix and loop, which allows for selective recognition of methylated CpG dinucleotides [421]. It represses transcription through association with histone deacetylase complexes (HDACs) or through chromatin

compaction at its TRD domain (Transcription repression domain) [420]. Defective MeCP2 causes Rett syndrome, a rare X-linked neurodevelopmental disorder [422]. Since most studies on MeCP2 have been performed in neurons, it is in our interest to study the interaction of MeCP2 with LEDGF/p75 in PCa.

Purpose and Significance of this Dissertation

The purpose of this dissertation was to study the interactions of both LEDGF/p75 and p52 with other transcription factors to examine their modulation of genes under oxidative stress environments. The upregulation of the Hsp27 promoter by LEDGF/p75 led to the hypothesis that **LEDGF/p75 interacts with transcription factors to activate stress and antioxidant genes in order to protect cancer cells from stress-induced cell death**

To gain insight into the mechanism by which LEDGF/p75 confers this resistance we pursued the following aims:

1. To investigate the interaction of LEDGF/p75 and p52 with MeCP2.
2. To explore modulation of stress proteins by the LEDGF-MeCP2 interaction.
3. To examine if this interaction protects PCa cells from stress-induced death.

Our studies showed that LEDGF/p75 and p52 interacts with MeCP2 both *in vitro* and in cellular assays. In addition, we showed that MeCP2 increased transactivation of the Hsp27 promoter (Hsp27pr). Furthermore, we showed novel binding of both proteins to the Hsp27pr region. Depletion of LEDGF/p75 resulted in robust upregulation of Hsp27pr in the presence of MeCP2 in U2OS cells. On the other hand, synergistic effects of both proteins were seen with thiol-disulfide oxidoreductase of the endoplasmic

reticulum promoter (ERp57pr) and IGFBP5pr in PC3 PCa cells. This suggests that LEDGF and MeCP2 interaction may have different functional consequences that are promoter and cell type specific.

In other experiments, LEDGF/p75 overexpression was shown to protect PCa cells against tert-butyl hydrogen peroxide (TBHP)-induced cell death. However, LEDGF/p75 did not protect PCa cells against classical inducers of apoptosis such as TRAIL and staurosporine (STS), suggesting that this protein promotes resistance to inducers of non-apoptotic cell death. The role of MeCP2 in PCa chemoresistance remains to be determined in future work.

The studies described in this dissertation will provide insights into the interactions between LEDGF/p75 and other transcription factors. These studies are highly significant because they will contribute to a better understanding of protein-protein interactions in stress gene transcription regulation within the context of chemoresistance. We anticipate that this will lead to the development of innovative molecular targeting strategies that would act synergistically with traditional chemotherapeutic agents in PCa.

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CHAPTER TWO

THE STRESS TRANSCRIPTIONAL COACTIVATOR LEDGF/P75 INTERACTS WITH THE METHYL CPG BINDING PROTEIN MECP2 AND INFLUENCES ITS TRANSCRIPTIONAL ACITIVTY IN CANCER CELLS

Lai Sum Leoh[1], Bart van Heertum[2], Jan De Rijck[3], Maria Filippova[4], Frauke Christ[5], Shannalee R. Martinez[6], Sandy Tungteakkhun[7], Valeri Filippov[8], Marino De Leon[9], Zeger Debyser[10], and Carlos A. Casiano[11]

1. Designed and carried out most of the experiments and wrote the initial drafts of the manuscript.

2. Contributed to the studies on interaction of LEDGF and MeCP2 and experimental design and data interpretation.

3. Supervised sub-cloning experiments, protein binding assays, contributed to experimental design and data interpretation.

4. Supervised most of the experiments, contributed to experimental design and data interpretation and critical revision of manuscript.

5. Supervised protein purification and AlphaScreen® assays, contributed to experimental design and data interpretation.

6. Contributed to the western blots of luciferase reporter assays.

7. Contributed to experimental designs and data interpretation.

8. Supervised ChIP assays, contributed to experimental design and data interpretation.

9. Provided funding for this research.

10. Provided valuable reagents, contributed to the experimental design and critical revision of this manuscript.

11. Contributed to the conception and design of this entire study and the final editing of the manuscript, and provided funding for this research.

The Stress Transcriptional Coactivator LEDGF/p75 Interacts with the Methyl CpG Binding Protein MeCP2 and Influences Its Transcriptional Activity in Cancer Cells

Lai Sum Leoh[1], Bart van Heertum[2], Jan De Rijck[2], Maria Filippova[1], Frauke Christ[2], Shannalee R. Martinez[1], Sandy Tungteakkhun[1], Valeri Filippov[1], Marino De Leon[1], Zeger Debyser[2], and Carlos A. Casiano[1,3]

[1]Center for Health Disparities and Molecular Medicine and Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, California; [2]Laboratory for Molecular Virology and Gene Therapy, Division of Molecular Medicine, Katholieke Universiteit Leuven, Leuven, Belgium; [3]and Department of Medicine, Loma Linda University School of Medicine, Loma Linda, California.

Running title: Interaction between LEDGF/p75 and MeCP2

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Correspondence: Carlos A. Casiano, Center for Health Disparities and Molecular Medicine, Loma Linda University School of Medicine, Loma Linda, CA 92350. Phone: (909)-558-1000 x 42759. Fax: (909)-558-0196. Email: ccasiano@llu.edu

Abstract

The lens epithelium derived growth factor p75 (LEDGF/p75) is a transcription co-activator that promotes resistance to oxidative stress- and chemotherapy-induced cell death. LEDGF/p75 is also known as the dense fine speckles autoantigen of 70 kD (DFS70), and has been implicated in cancer, inflammation, autoimmunity, and HIV-AIDS. To gain insights into the mechanisms by which LEDGF/p75 protects cancer cells against stress, we initiated an analysis of its interactions with other transcription factors and the influence of these interactions on stress gene activation. We report here that both LEDGF/p75 and its short splice variant LEDGF/p52 interact with MeCP2, a methylation-associated transcriptional modulator, *in vitro* and in human cancer cells. These interactions were established by several complementary approaches: transcription factor protein arrays, pull down and AlphaScreen® assays, co-immunoprecipitation, and nuclear co-localization by confocal microscopy. MeCP2 was found to interact with the N-terminal region shared by LEDGF/p75 and p52, particularly with the PWWP-CR1 domain. Like LEDGF/p75, MeCP2 bound to and transactivated the Hsp27 promoter (Hsp27pr). Co-expression of LEDGF/p75 and MeCP2 significantly enhanced MeCP2-induced Hsp27pr transactivation in PC3 prostate cancer cells but not in U2OS bone cells. LEDGF/p52 repressed MeCP2-induced Hsp27pr activity. Interestingly, siRNA-induced silencing of LEDGF/p75 in both cell lines dramatically elevated MeCP2-mediated Hsp27pr transactivation, suggesting that LEDGF/p75 regulates MeCP2 transcriptional activity. Our results suggest that the LEDGF/p75-MeCP2 interaction differentially influences Hsp27pr activation depending on the intracellular levels of these proteins and

the cell context. These findings are of significance in understanding the contribution of this interaction to the activation of stress survival genes.

Introduction

LEDGF/p75 is a stress response protein with relevance to cancer, autoimmunity, inflammation, eye disease, and replication of the human immunodeficiency virus type 1 (HIV-1). LEDGF/p75 and its splice variant p52 are derived from the PSIP1 gene and were originally identified as transcription coactivators that interact with the RNA polymerase II transcription complex (1). LEDGF/p75 is also known as the dense fine speckled autoantigen of 70 kD (DFS70), which is targeted by autoantibodies in various human inflammatory conditions (2). Although initially proposed to be a growth factor for lens epithelial cells (3), subsequent studies revealed that LEDGF/p75 is a stress survival protein that protects against oxidative stress-induced cellular damage and death (4,5). As a key cellular co-factor for HIV-1 replication, LEDGF/p75 binds the HIV-1 integrase (IN) through its C-terminal integrase-binding domain (IBD) and tethers it to the chromatin, facilitating lentiviral integration to transcriptionally active regions of the host genome (6-8).

LEDGF/p75 is emerging as an oncoprotein in various human cancers. It is targeted by autoantibodies in patients with prostate cancer, and is overexpressed in prostate tumors and other human malignancies, including chemotherapy-resistant human acute myelogenic leukemia (9-11). In addition, it can be found as a fusion protein with NUP58 in leukemia patients (12). Its overexpression in tumor cells attenuates lysosomal cell death induced by stressors that trigger oxidative stress (e.g. certain chemotherapeutic

drugs, tumor necrosis factor, and serum starvation) (5,10,11,13). LEDGF/p75 also enhanced the tumorigenic potential of HeLa cells in xenograft models (10).

The mechanisms by which LEDGF/p75 promotes cell survival under stress are still unclear, although available evidence indicates that this protein transcriptionally activates genes encoding protective proteins such as heat shock protein 27 (Hsp27), αB-crystallin, peroxiredoxin 6 (PRDX6), and vascular endothelial growth factor c (VEGF-c) (14-16). Presumably, LEDGF/p75 transactivates these stress genes by binding to heat shock elements (HSE; nGAAn) and stress elements (STRE; A/TGGGGA/T) in their promoter regions (14-16). In leukemia cells, LEDGF/p75 interacts with oncogenic transcription complexes, such as the menin/MLL-HMT (mixed lineage leukemia histone methyltransferase) complex, to transactivate cancer-associated genes and facilitate leukemic transformation (17).

The transcriptional and pro-survival activities of LEDGF/p75 are attenuated by TGF-β1 (18), Bcl-2 (19), SUMOylation (20), and caspase-mediated cleavage (5). The pro-survival function of LEDGF/p75 is also regulated by alternative splicing since ectopic expression of its splice variant p52 antagonizes its transcriptional activity and induces apoptosis in cancer cells (21). LEDGF/p75 and p52 share N-terminal amino acids (aa 1-325); however, p52 has an intron-derived C-terminal tail (CTT, aa 326-333) implicated in its pro-apoptotic activity (1,21). The N-terminal region shared by both proteins contains a PWWP domain (aa 1-93), an entity implicated in chromatin binding, transcriptional repression, and methylation (22-24). The N-terminal region also has a positively charged domain (CR1) immediately after the PWWP domain that is followed by a nuclear localization signal (NLS) and two AT-hook (ATH) sequences that cooperate

with the PWWP domain for chromatin binding (25,26). A second charged region (CR2), also designated the supercoiled-DNA recognition domain (SRD) (aa 200-336), facilitates LEDGF/p75 binding to active transcription sites (27). The C-terminus of LEDGF/p75 (aa 347-429) encompasses both the IBD and the autoepitope recognized by human anti-LEDGF/p75 autoantibodies (8,28). This region is involved in protein-protein interactions and binding to HSE in promoter regions (17,29-32). Both the N- and C-terminal regions of LEDGF/p75 contribute to its transcription and stress survival functions (5,32).

Understanding the mechanisms by which LEDGF/p75 promotes tumor cell resistance to cell death and chemotherapy requires detailed knowledge of its cellular functions, particularly its interactions with other cancer-associated proteins, and its target genes. To date only a few cellular interacting partners of LEDGF/p75 have been identified. These include the PC4 transcription factor, menin/MLL, the Cdc7 activator of S-phase kinase (ASK), the pogZ (pogo transposable element derived protein with zinc finger) transposase, and the myc-interacting protein JPO2 (17,29-31). Using transcription factor protein arrays we identified several candidate interacting partners of LEDGF/p75. Among these, methyl-CpG binding protein 2 (MeCP2) was of particular interest because, like LEDGF/p75, it has been linked to prostate cancer progression (33,34). MeCP2 belongs to a family of methylated CpG binding proteins, and is mutated in Rett syndrome (35). MeCP2 contains an N-terminal methyl CpG binding protein (MBD) domain and a C-terminal transcriptional repression domain (TRD), and depending on its interacting partners either represses or activates gene transcription (35,36). In this study we characterized the interaction between LEDGF/p75 and MeCP2

in vitro and in human cancer cells. We provide evidence that the N-terminal region of LEDGF/p75 binds to MeCP2 and influences its transcriptional function.

Materials and Methods

Cell Lines, Antibodies and Plasmids

U2OS, PC3 and 293T cells were obtained from the American Type Culture Collection and cultured in McCoy's 5A medium or RPMI 1640 (Gibco), supplemented with 2 mM L-glutamine and penicillin/streptomycin, and 10% fetal bovine serum**.** PC3 cells stably expressing LEDGF/p75 (13), were grown in RPMI 1640, and supplemented with 10% (v/v) fetal bovine serum (FBS), 20 μg/μl of gentamicin, and 0.5 mg/ml of geneticin. Cells were grown with 5% CO_2 at 37°C.

The following antibodies were used: mouse monoclonals anti LEDGF/p75-p52 (BD Biosciences), anti-β-actin (Sigma); rabbit polyclonals anti-LEDGF/p75 (Bethyl Laboratories), anti-MeCP2 (ProteinTech Group), anti-HA (Santa Cruz Biotechnology), anti-eGFP (produced in Z. Debyser's laboratory); goat polyclonals anti-eGFP (produced in Z. Debyser's laboratory), anti-GFP (Santa Cruz Biotechnology), anti-GST (Pharmacia Biotech), anti-Flag-HRP (Sigma) and rat monoclonal horseradish peroxidase (HRP)-conjugated anti-HA (Roche Diagnostics). Human antibodies to LEDGF/p75 were a gift from Dr. Eng M. Tan (Scripps Research Institute, La Jolla, CA).

Plasmid pET28a-dfs70 encoding His-LEDGF/p75 was a kind gift from Dr. Edward Chan (University of Florida, Gainesville). Plasmids pDEST-GST-MeCP2 and pcDNA-Flag-MeCP2 were a kind gift from Dr. Adrian Bird (University of Edinburgh, UK). Plasmids pKB6H-p52, pMal™-p2x-BRD4-Ct, and p-eGFP-BRD4-Ct were

generated in Z. Debyser's laboratory. Plasmids pCruzHA-LEDGF/p75, pCruzHA-p52, and pGL3-Hsp27pr-Luc were generated as described (21). Plasmid eGFP-p52 was cloned by replacing the LEDGF/p75 cDNA in p-eGFP-LEDGF/p75 vector with the LEDGF/p52 cDNA at *XhoI* and *BamHI* restriction sites.

Purification of Recombinant LEDGF/p75, p52 and MeCP2

GST-tagged MeCP2 was produced from pDEST-MeCP2 in *E. coli* BL21 grown in the presence of sorbitol and betaine. Expression was induced in lysogeny broth (LB) medium at 37°C by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells harvested 3 h after induction were lysed by sonication in core buffer (50 mM Tris HCl , 0.5 M NaCl, pH7.5, 10 mM EDTA, 10 mM EGTA, 1% Triton X-100, and 20 μg/ml lysozyme). The fusion protein, captured on glutathione agarose (Sigma) or glutathione sepharose beads (GE Healthcare Life Sciences), was eluted with 20 mM glutathione in core buffer. His-tagged LEDGF/p75 and p52 were expressed from pET28a-dfs70 and pKB6H52 in *E. coli* BL21, respectively. Expression was induced with 1 mM and 3 mM IPTG, respectively, at 37°C for 3 h. Bacteria were lysed by sonication in B-PER® bacterial protein extraction reagent (Thermo Scientific). The recombinant proteins were captured on nickel columns (Novagen) or TALON® His-Tag Purification Resins (Clontech), and washed with 20 mM HEPES, 0.5 M NaCl, 2 mM KCl, 1% NP-40, and 5 mM imidazole. Proteins were eluted with 20 mM HEPES, 137 mM NaCl, 2 mM KCl, 300 mM imidazole, and dialyzed using D-tube™ dialyzer (Novagen).

Transcription Factor Arrays

Two transcription factor protein arrays that were commercially available at the time we initiated these studies, Active Protein Array™ (Active Motif), and TranSignal Protein Arrays I-III (Panomics), were used for identifying interacting partners of LEDGF/p75 following the manufacturer's instructions. These arrays contained a total of 170 transcription factors and co-activators, as well as RNA polymerase II, spotted on membranes in duplicates or triplicates. Briefly, membranes were blocked with 5% milk in tris-buffered saline tween-20 (TBS-T) buffer for 1 h. Recombinant His-LEDGF/p75 was incubated overnight with the membranes and after washes with TBS-T, the membranes were probed with human anti-LEDGF/p75 autoantibody for 2 h. Following washes with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies, and protein interaction signals were detected by chemiluminescence (Amersham).

Pull Down Assays

GST or GST-MeCP2 proteins bound to glutathione beads were blocked in HEPES buffer (20 mM HEPES, pH 7.4, 2 mM DTT, 137 mM NaCl, 2 mM KCl, 5% glycerol) with 0.1% bovine serum albumin at 4°C for 1 h. His-LEDGF/p75 or His-p52 were then incubated with the beads at 4°C for 1 h in HEPES buffer. The beads were then collected by centrifugation at 5,000 rpm for 30 seconds at 4°C, the supernatant was discharged, and the beads were washed two times with 1 ml of HEPES buffer + 0.1% NP-40 followed by HEPES buffer + 0.5% NP-40 + 0.5 M NaCl. Bound proteins were eluted in SDS-PAGE

sample buffer, separated by SDS-PAGE (10% Bis-Tris gel) and detected by immunoblotting.

Analysis of Protein-protein Interactions by AlphaScreen® Assay

The AlphaScreen® assay was performed according to the manufacturer's protocol (Perkin Elmer). Briefly, reactions were performed in 25 µl final volume in 384-well Optiwell™ microtiter plates. The reaction buffer contained 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Tween 20, 0.1% BSA. Varying concentrations of GST-MeCP2 in bacterial lysate and purified Flag-LEDGF/p75 were incubated in 15 µl reaction volume at 4°C for 1 h. The concentration of GST-MeCP2 in the lysates was estimated using BSA standards on SDS-PAGE gels stained with Coomassie Blue. Equal volume of bacterial lysate not expressing GST-MeCP2 was used as negative control. Subsequently, 5 µl of the diluted donor (glutathione) and acceptor (Flag) beads were added. After incubation for 1 h in the dark, light emission was measured in the EnVision® reader (Perkin Elmer) and analyzed using the EnVision® manager software.

Transient and Stable Transfection

293T cells were transfected in 10 cm^3 plates by polyethyleneimine (PEI) transfection. Ten µg of plasmid DNA were used to transiently transfect cells at >50% confluency. U2OS and PC3 cells were transfected using TransIt® 2020 (Mirus) transfection reagent. Transfected cells were grown for 24-48 h before analysis. Stable PC3 clones overexpressing LEDGF/p75 were generated by transfecting cells with

pcDNA-LEDGF/p75, or empty pcDNA vector for controls, and growing them in selection media containing geneticin (Calbiochem) as described (13).

Co-immunoprecipitation

U2OS cells were collected 48 h post transfection and lysed in RIPA buffer (Santa Cruz Biotechnology). Antibodies were incubated with cell lysates for 1 h before protein A/G^+ agarose beads (Santa Cruz Biotechnology) were added. The beads were collected by centrifugation and washed 3 times with core buffer (50 mM Tris pH 7.5, 300 mM NaCl, 1 mM $MgCl_2$, 5% glycerol, complete protease inhibitor EDTA free (Roche) and 0.1% NP-40. U2OS cells overexpressing Flag-MeCP2 and eGFP constructs were incubated with Flag-agarose beads. 293T cells were transiently transfected with p-eGFP-LEDGF/p75 and pcDNA-Flag-MeCP2. Whole cell lysates were collected 24 h post transfection and lysed in core buffer with 1% Triton X-100. Antibody against GFP was incubated with cell lysate overnight before protein G sepharose beads (GE Healthcare) were added. Immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting using appropriate antibodies.

Confocal Microscopy

Transfected cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100. Cells were washed with PBS after incubation with anti-MeCP2 antibodies and mounted with medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield). Confocal microscopy was performed using Zeiss LSM7 confocal

fluorescence microscope with 63x oil immersion objective and appropriate filters. Images were analyzed using ImageJ software.

Luciferase-based Transcription Reporter Assays

Hsp27pr luciferase transcription reporter assays were performed as described previously (21). Briefly, cells were co-transfected with plasmids encoding the proteins of interest or empty vector, and pGL3-Hsp27pr. At 48 h post-transfection, cells were lysed and luciferase assays were performed using the Luciferase Assay System (Promega). Relative light units were obtained in a MicroLumatPlus Lb 96V luminometer (Berthold Tech) and luciferase values were normalized to protein concentration of lysates from cells co-transfected with the empty vectors and pGL3-Hsp27pr. Student's *t* test analysis was performed using Microsoft Excel. Experiments were repeated at least three times.

LEDGF/p75 Knockdown by RNA Interference

Transient knockdown of LEDGF/p75 was carried out using synthetic siRNA oligos as described previously (37). The siLEDGF/p75 sequence corresponded to nucleotides 1340-1360 (5′- AGACAGCAUGAGGAAGCGAdTdT-3′) with respect to the first nucleotide of the start codon of the LEDGF/p75 open reading frame. Ambion Silencer® Negative Control siRNA #1 was used as scrambled control. siRNAs were transfected into U2OS cells using siQuest (Mirus). LEDGF/p75 knockdown was verified by immunoblotting.

Chromatin Immunoprecipitation Assays

U2OS cells were fixed in 1% formaldehyde for 10 minutes and subjected to chromatin immunoprecipitation (ChIP) assay using ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad). Anti-LEDGF/p75 antibodies (A300-848A, Bethyl), anti-MeCP2 antibodies (07-013, Millipore) and rabbit IgG (Santa Cruz Biotechnology) were used to immunoprecipitate protein-chromatin complexes. PCR was performed using primers to amplify Hsp27 promoter: set A forward 5'- CGC TTA AGC ACC AGG GCC GG -3 and reverse 5'- CCG GCC CTG GTG CTT AAG CG -3'; set B forward 5'-CTGGGCTCAAGCACCAGACTC -3' and reverse 5'-CAAATGAATTCGAGAGCGCGACGC-3'; set C: forward 5'-CAGGGTTTTGCTCTGTAG CC-3' and reverse 5'-CCACACGCGTGTGAGATAGAATGTG-3'; set D: forward 5'-CTCTGCCTTCTGGGGTTCAAG-3' and reverse 5'-TTGAACCCCGGTGAGTAGAG-3'.

Results

Identification of MeCP2 as a Candidate Interacting Partner of LEDGF/p75

We hypothesized that LEDGF/p75 interacts with other transcription factors to transactivate stress genes and promote tumor cell survival in the presence of stress. In order to identify novel cellular interacting partners of LEDGF/p75, we screened 170 different transcription factors for LEDGF/p75 binding using transcription factor protein arrays (Panomics and Active Motif). Purified recombinant His-tagged LEDGF/p75 (Fig.

1A) was incubated with recombinant transcription factors spotted on membranes, and protein-protein interactions were identified using a specific human autoimmune serum against LEDGF/p75 (Fig. 4B), followed by signal detection with chemiluminescence (Fig. 4C). We detected moderate to strong protein-protein interaction signals with 17 different transcription factors, with the strongest reactivity corresponding to the LEDGF/p75-MeCP2 interaction (Fig. 4C). One array (Active Motif) yielded strong signals with transcription factor PC4 and RNA polymerase II subunits (data not shown), consistent with the report that LEDGF/p75 co-purifies with these proteins (1).

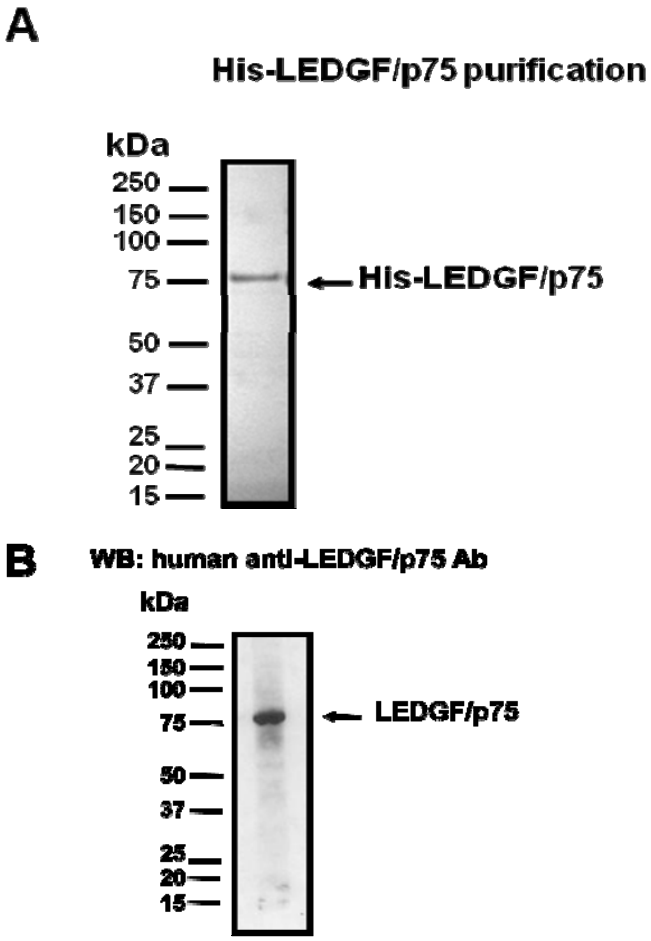

Figure 4. Identification of candidate interacting partners of LEDGF/p75 using transcription factor protein arrays. A) Coomassie blue stained SDS-PAGE gel showing purified His-LEDGF/p75. *E. coli* BL21 was transformed with pET28a-dfs70 encoding His-LEDGF/p75 and induced with IPTG. Lysate was passed through a nickel column to purify His-LEDGF/p75. B) Immunoblot showing the specificity of the human autoantibody against LEDGF/p75 used as detection reagent in the transcription factor protein arrays. The autoantibody reacts specifically with LEDGF/p75 in a PC3 prostate cancer cell lysate.

C

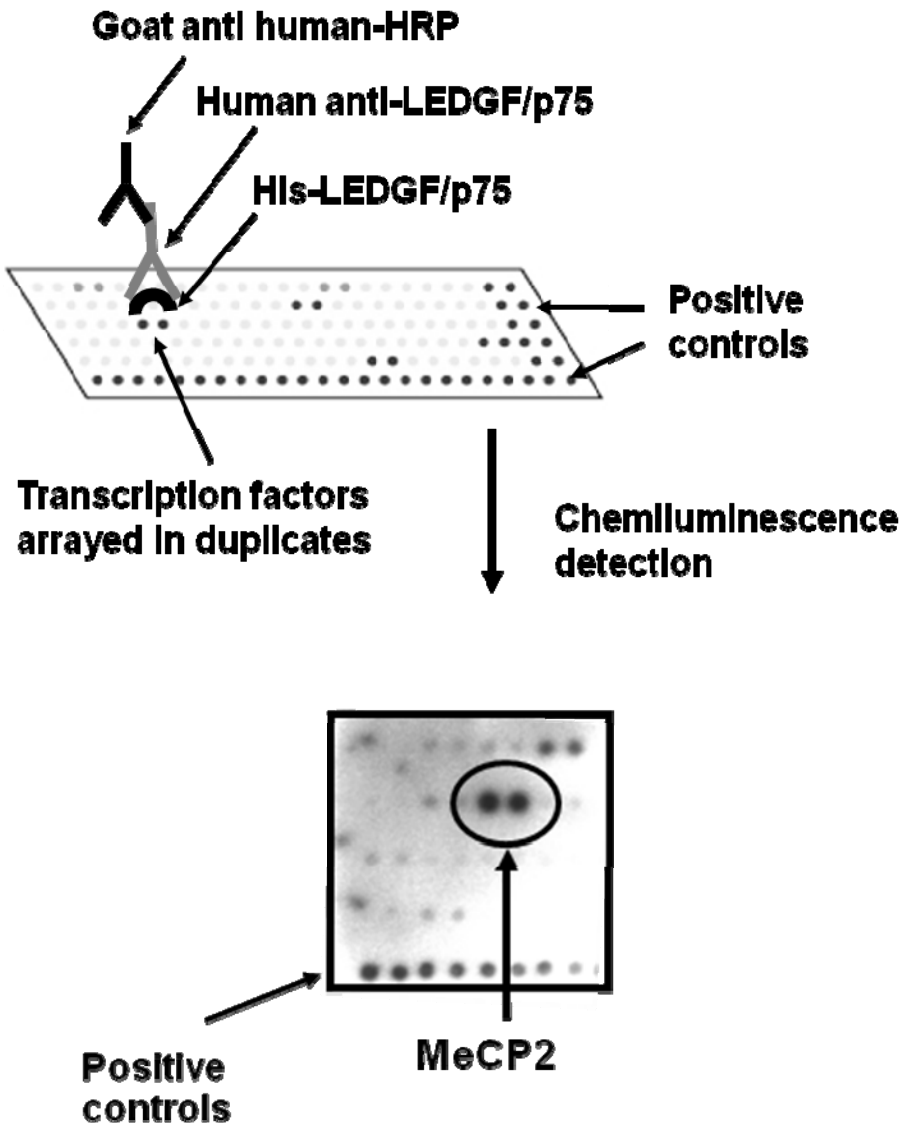

Figure 4. Identification of candidate interacting partners of LEDGF/p75 using transcription factor protein arrays. C) Transcription factor arrays were used to identify candidate interacting transcription factors of LEDGF/p75. Purified His-LEDGF/p75 was incubated with transcription factors spotted on membranes. Protein interactions were detected with human anti-LEDGF/p75 autoantibody and chemiluminescence. A section of the transcription factor array membrane containing MeCP2 is showed.

LEDGF/p75 Interacts with MeCP2 *in vitro*

To confirm the interaction between LEDGF/p75 and MeCP2 *in vitro*, pull-down experiments were performed using recombinant proteins. Beads with bound GST-MeCP2 or GST were incubated with His-LEDGF/p75, and interactions were detected by immunoblotting using anti-GST antibody. LEDGF/p75 was pulled down with GST-MeCP2 but not with GST (Fig. 5A). An irrelevant protein, His-FADD (Fas-associated protein with death domain), served as negative control.

A protein-protein interaction assay using the AlphaScreen® technology (Perkin-Elmer) was used for additional confirmation of the LEDGF/p75-MeCP2 interaction *in vitro*. To prevent false positive signals due to rapid degradation of purified recombinant GST-MeCP2, we used GST-MeCP2 induced in the bacterial lysate. Enhanced bacterial expression of GST-MeCP2 was attained in the presence of sorbitol and betaine. Cross titration of increasing concentrations of recombinant Flag-LEDGF/p75 and GST-MeCP2 demonstrated binding between both proteins over a wide range of concentrations (Fig 5B). Optimum binding was observed between 11 nM LEDGF/p75 and estimated 1 nM GST-MeCP2 (Fig 5C).

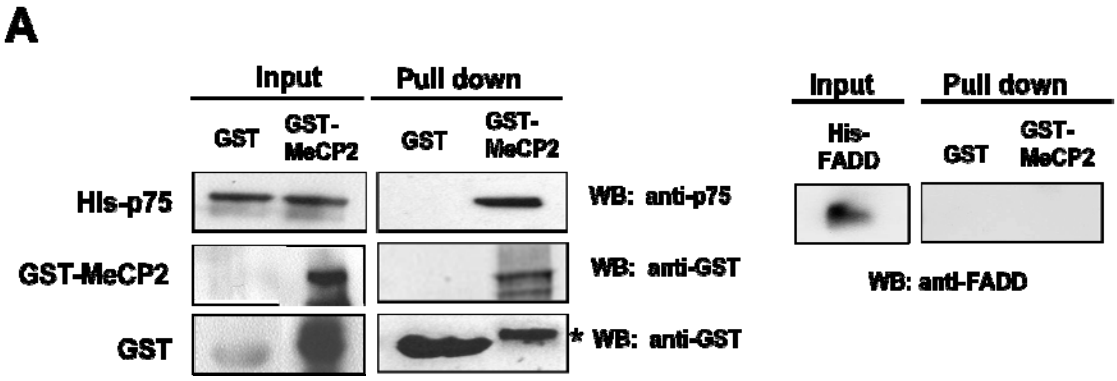

Figure 5. LEDGF/p75 interacts with MeCP2 *in vitro*. A) Pull down assays with His-LEDGF/p75 and GST-MeCP2. Recombinant His-LEDGF/p75 was incubated with GST or GST-MeCP2 bound to glutathione beads, and pulled down proteins were analyzed by immunoblotting using antibodies specific for GST or LEDGF/p75. His-FADD was used as irrelevant control. Protein input was determined by immunoblotting of whole cell extracts. *Denotes degraded GST-MeCP2.

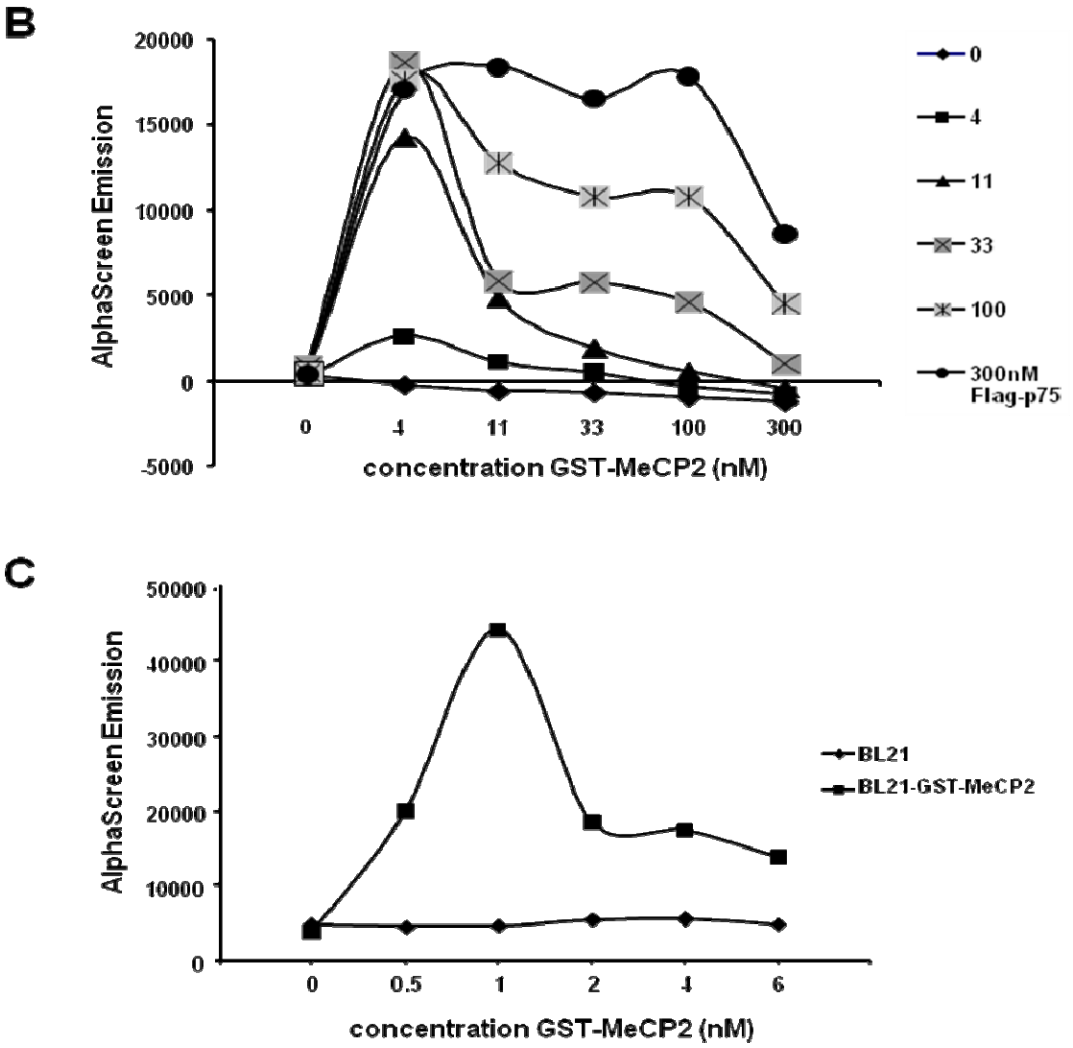

Figure 5. LEDGF/p75 interacts with MeCP2 *in vitro***.** B) Cross titration for Flag-LEDGF/p75 and GST-MeCP2 interaction as measured by AlphaScreen® assay. Interaction was measured at different concentrations of Flag-LEDGF/p75 as indicated on the vertical legend, and GST-MeCP2 as indicated on the X-axis. The experiment was performed in triplicate. C) An estimated 1 nM MeCP2 was sufficient to interact with 11 nM LEDGF/p75. *E.coli* BL21 lysate not expressing GST-MeCP2 was used as a negative control. Data is representative of three independent measurements.

LEDGF/p75 Interacts With MeCP2 in a Cellular System

The LEDGF/p75-MeCP2 interaction was confirmed in a cellular system by co-immunoprecipitation (co-IP) assays. Flag-MeCP2 and different eGFP-LEDGF/p75 constructs were co-transfected in 293T cells (for high transfection efficiency). Expressed proteins were then immunoprecipitated with anti-GFP antibody and pulled down by protein G agarose beads. Immunoprecipitated proteins were analyzed by immunoblotting with antibodies against eGFP and Flag tags. Immunoblotting analysis showed MeCP2 interaction with eGFP-LEDGF/p75, but not with eGFP-HIV-IN or the irrelevant protein eGFP-BRD4-Ct (carboxyl terminal fragment of the bromodomain containing protein 4) (Fig 6A). To verify that LEDGF/p75 and MeCP2 interact endogenously in cancer cells, co-IP experiments were performed using U2OS osteosarcoma cells, which express high endogenous levels of LEDGF/p75 (21). U2OS cell lysates were incubated with antibody against LEDGF/p75, and the immunoprecipitated proteins were detected by immunoblotting. Endogenous MeCP2 was detected in immunoprecipitates of endogenous LEDGF/p75 but not with IgG control (Fig 6B). The LEDGF/p75-MeCP2 interaction was also confirmed by reciprocal co-IP and immunoblotting in PC3 prostate cancer cells (data not shown).

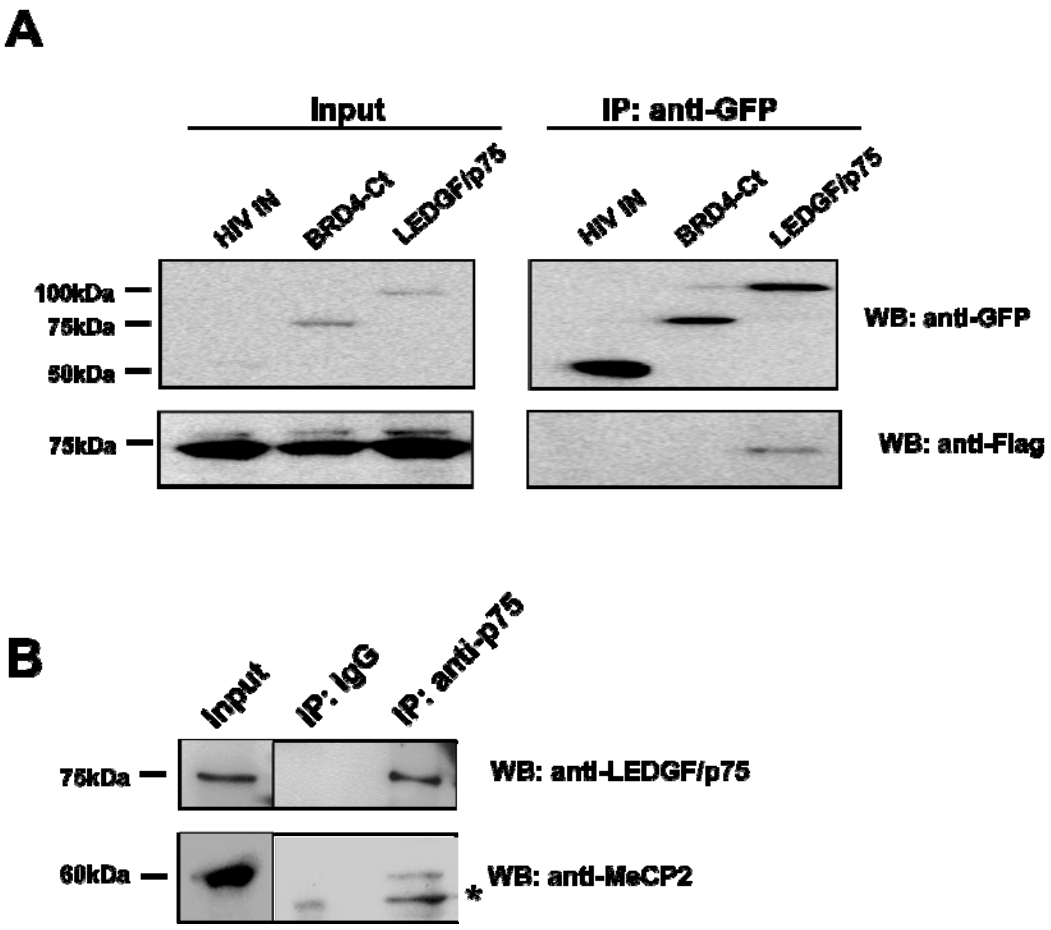

Figure 6. Co-immunoprecipitation of LEDGF/p75 and MeCP2. A) 293T cells co-transfected with Flag-MeCP2 and eGFP-LEDGF/p75, eGFP-HIV-IN, or eGFP-BRD4-Ct expression constructs were lysed 24 h post transfection. Proteins immunoprecipitated (IP) with antibody against GFP were resolved by SDS-PAGE and detected by immunoblotting using anti-GFP and anti-Flag antibodies. B) U2OS cell extracts were immunoprecipitated with mouse monoclonal anti-LEDGF/p75 antibody. Proteins were detected by immunoblotting with rabbit anti-LEDGF/p75 and anti-MeCP2 antibodies. *Denotes degraded MeCP2. Protein input was determined by immunoblotting of whole cell extracts.

Confocal microscopy analysis was performed to examine the intracellular co-localization of LEDGF/p75 and MeCP2. U2OS cells were transiently transfected with plasmids encoding HcRed-LEDGF/p75 and Flag-MeCP2. Both recombinant proteins displayed a distinctive nuclear speckled pattern and co-localized in the nucleus (Fig. 6C). Co-localization was also observed with co-expression of eGFP-LEDGF/p75 and Flag-MeCP2 (data not shown). Both proteins co-localized with DAPI-stained chromatin regions.

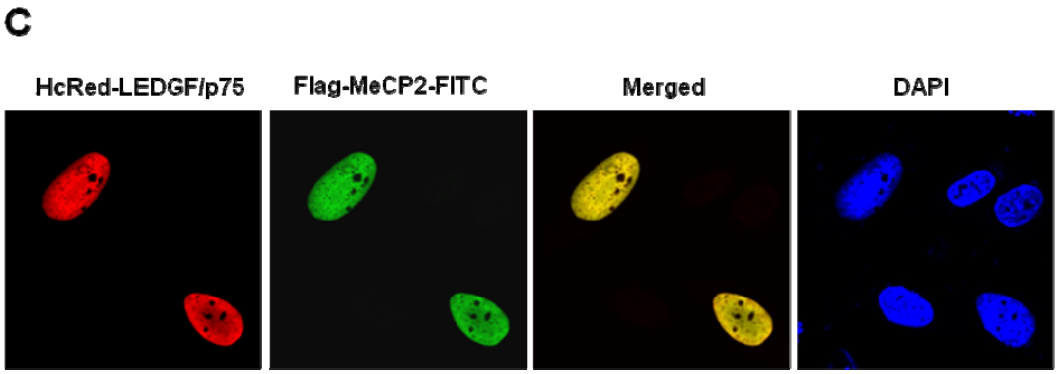

Figure 6. Nuclear co-localization of LEDGF/p75 and MeCP2. C) U2OS cells were transiently co-transfected with pcDNA-Flag-MeCP2 and pHcRed-LEDGF/p75. Ectopically expressed MeCP2 was detected 48 h post-transfection using anti-Flag antibodies and visualized with FITC-labeled secondary antibody. Nuclei were stained with DAPI, and fluorescent signals were analyzed by confocal microscopy.

The LEDGF/p75 Splice Variant p52 also Interacts with MeCP2

As mentioned above, LEDGF/p75 and its short splice variant p52 share their N terminal region, which contains the PWWP domain, CR1 and CR2, NLS, and AT-hooks that collectively facilitate DNA binding (Fig. 7A) [38, 39]. To determine if MeCP2 also binds to p52 we performed pull-down assays in which recombinant His-p52 was incubated with GST or GST-MeCP2 beads. Immunoblotting analysis showed that His-p52 was pulled-down by GST-MeCP2 but not by GST (Fig 7B). Confocal microscopy showed partial co-localization of HcRed-p52 with Flag-MeCP2 in nuclei of U2OS cells co-transfected with plasmids encoding these tagged proteins (Fig 7C). Taken together, these results indicated that p52 also interacts with MeCP2. Since p52 lacks the C-terminal portion of LEDGF/p75, the results also suggested that MeCP2 interacts with the N-terminal portion of LEDGF/p75.

The N-terminal Region of LEDGF/p75 Mediates the Interaction with MeCP2

Since LEDGF/p75 and p52 share the same N-terminal region (aa 1-325), we sought to map the minimal interacting region of these proteins with MeCP2. To accomplish this, deletion constructs comprising different regions of the LEDGF/p75 protein were used. Co-immunoprecipitation was performed in cells transiently co-transfected with plasmids encoding Flag-MeCP2 and one of the following eGFP-tagged constructs: LEDGF/p75 (aa 1-530), LEDGF/p52 (aa 1-333), PWWP domain (aa 1-93), ΔPWWP (aa 94-530), Δ1-325 (aa 325-530) or IBD (aa 347-429) (Fig 8A). Reciprocal Co-IP showed that Flag-MeCP2 co-precipitated with both full length LEDGF/p75 and p52 but not with any of the truncated LEDGF/p75 constructs (Fig 8B,C).

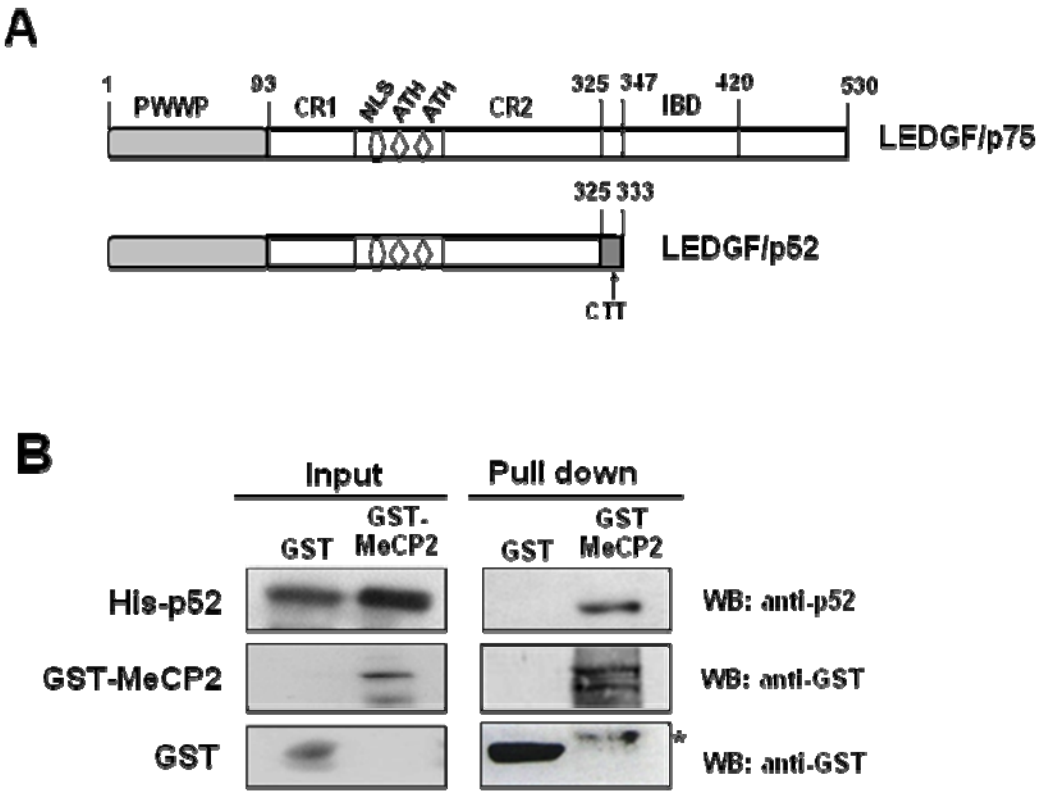

Figure 7. LEDGF/p52 interacts with MeCP2. A) Schematic domain structure of LEDGF/p75 and p52. B) Pull down assay was performed as described in the legend of Figure 5A using recombinant His-LEDGF/p52. Protein input was determined by immunoblotting of whole cell extracts.

C

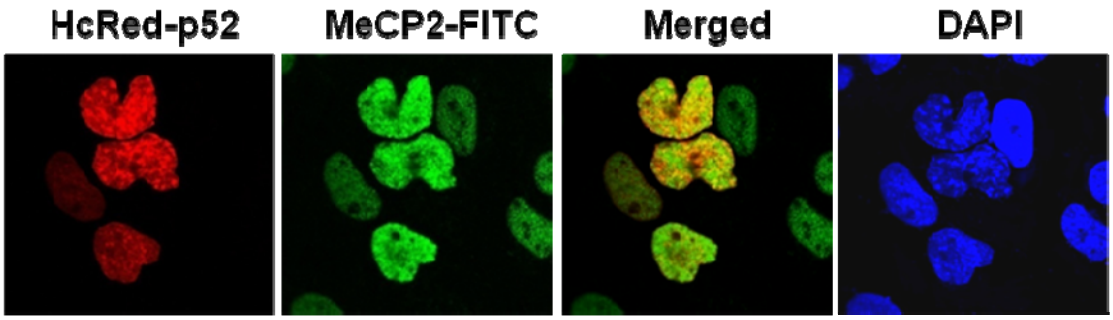

Figure 7. (C) LEDGF/p52 partially co-localizes with MeCP2 in the cell nucleus. Co-localization assay was performed as described in the legend of Figure 6C.

Since both LEDGF/p75 and p52 interacted with MeCP2, and the PWWP domain alone or ΔPWWP did not interact with MeCP2, we concluded that additional regions in the N-terminal portion of these proteins may be needed for MeCP2 binding. To identify these regions, deletion constructs consisting of the PWWP domain alone (aa 1-93), or in combination with its downstream CR1 region (aa 1-141), were used to examine MeCP2 binding. U2OS cell lysates containing endogenous MeCP2 were incubated with the following Flag-tagged recombinant proteins: LEDGF/p75, PWWP-CR1 (aa 1-141), or PWWP (aa 1-108) (Fig 8A). Pull-down was done using anti-Flag agarose beads. Immunoblotting analysis with anti-Flag antibody or anti-MeCP2 antibody showed that endogenous MeCP2 was pulled down with Flag-LEDGF/p75 and Flag-PWWP-CR1 but not with Flag-PWWP (Fig. 8D). This suggested that the extreme N-terminal region (aa 1-141) of LEDGF/p75 mediates its interaction with MeCP2.

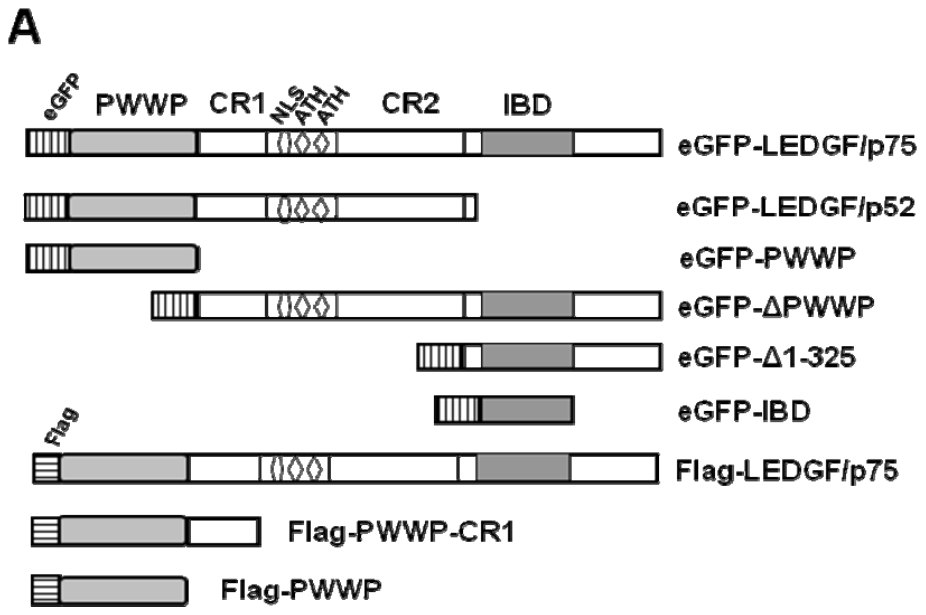

Figure 8. The N-terminus of LEDGF/p75 interacts with MeCP2. A) Diagram of LEDGF/p75 deletion constructs used to map interaction regions.

B

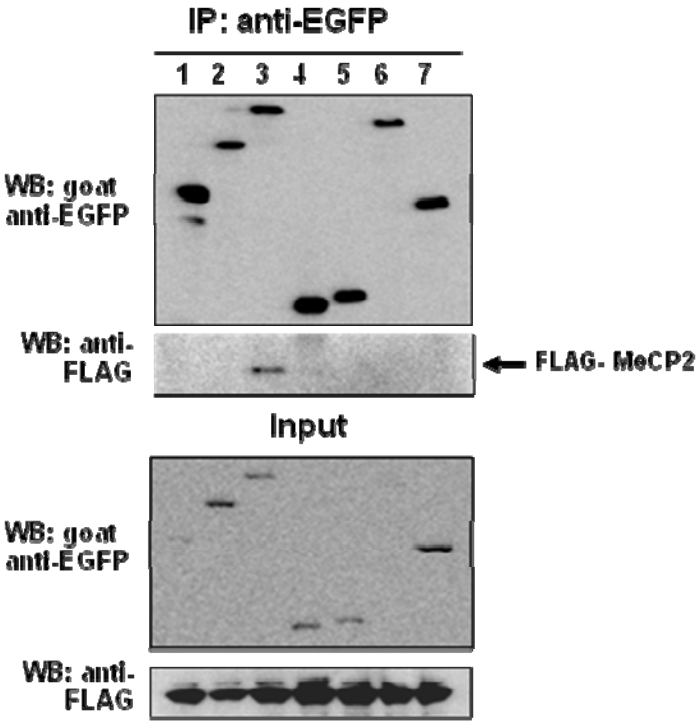

Figure 8. The N-terminus of LEDGF/p75 interacts with MeCP2. B) Flag-MeCP2 binds to eGFP-LEDGF/p75 but not to eGFP-tagged truncated LEDGF/p75 constructs. 293T cells ectopically overexpressing the tagged proteins labeled in the blot were immunoprecipitated with GFP antibody and visualized by immunoblotting with antibodies to GFP and Flag.

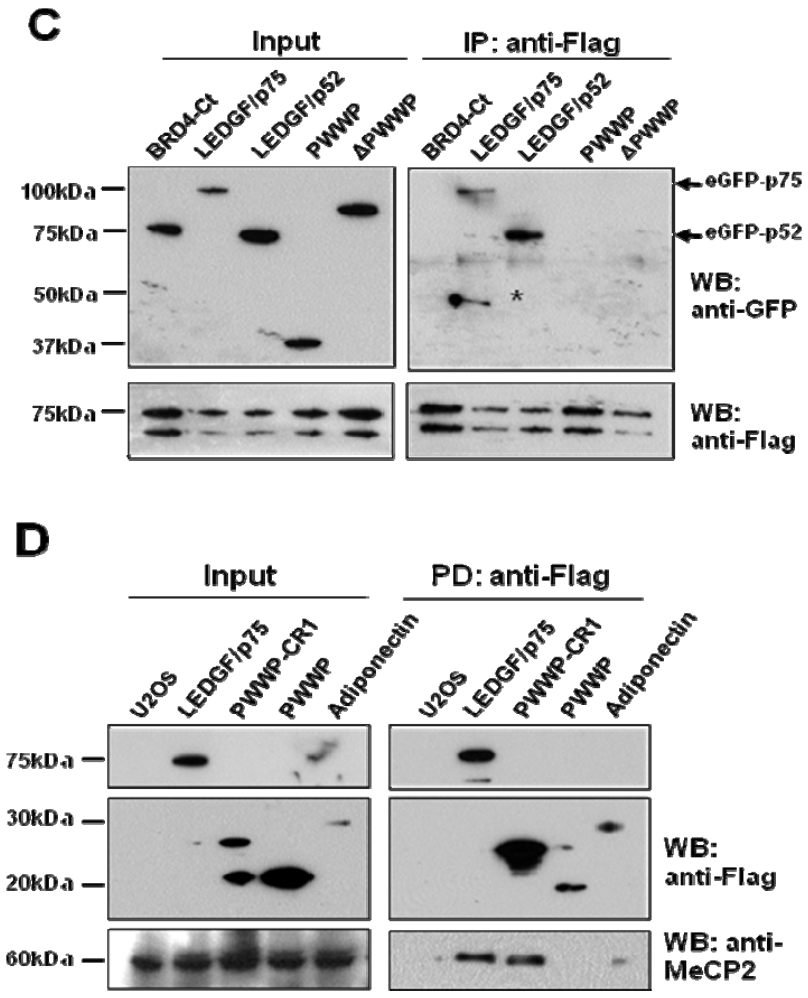

Figure 8. The N-terminus of LEDGF interacts with MeCP2. C) Flag-MeCP2 binds to eGFP-tagged LEDGF/p75 and LEDGF/p52 but not to truncated constructs. Proteins ectopically overexpressed in U2OS cells were immunoprecipitated with Flag antibody and visualized with both anti-GFP and anti-Flag antibodies. *Denotes degradation product of LEDGF/p75. D) Recombinant Flag-LEDGF/p75, Flag-PWWP-CR1 (aa 1-141) and Flag-PWWP (aa 1-101) were incubated with U2OS cell lysate. Proteins pulled down with anti-Flag affinity matrix were detected by immunoblotting. Endogenous MeCP2 in the cell lysate was pulled down by Flag-LEDGF/p75 and Flag-PWWP-CR1. Flag-Adiponectin and absence of recombinant proteins (U2OS only) served as controls.

MeCP2 Transactivates the Hsp27 Promoter

The interaction of LEDGF/p75 and p52 with MeCP2 suggested that these proteins are part of a transcription complex that activates and regulates stress genes such as *HSP27*. To examine this, we first determined if MeCP2 transactivates the Hsp27 promoter (Hsp27pr) in luciferase reporter assays. U2OS cells were transiently co-transfected with pGL3-Hsp27pr-Luc and either pcDNA-Flag-MeCP2, pCruz-HA-LEDGF/p75, or pCruz-HA-p52. Interestingly, transient overexpression of MeCP2 transactivated Hsp27pr at levels much higher (14 fold) (Fig 9A) than those induced by LEDGF/p75 and p52 (2-3 fold) (Fig 9B). All three proteins transactivated Hsp27pr in a dose dependent manner.

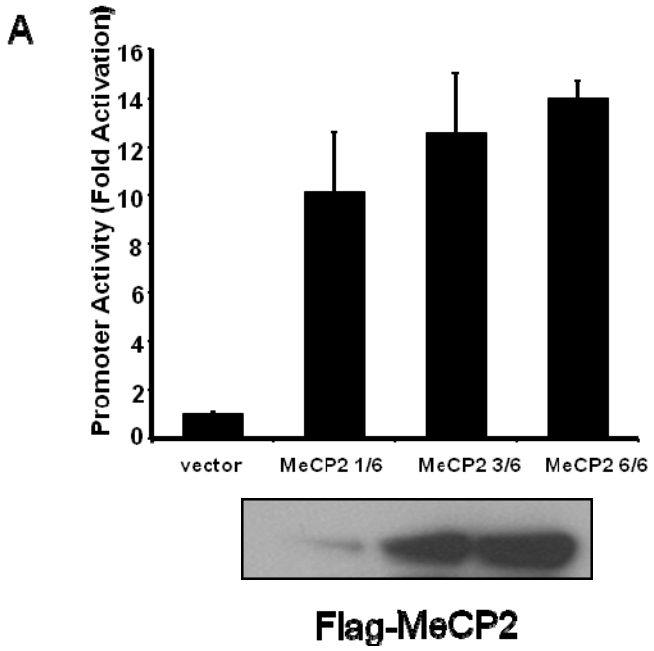

Figure 9. LEDGF/p75, LEDGF/p52, and MeCP2 transactivate Hsp27 promoter. A) U2OS cells were co-transfected with pGL3-Hsp27pr and increasing amount of pcDNA-flag-MeCP2. Promoter activity determined as luciferase light units/protein is expressed as fold activation compared to control activity (co-transfection of empty expression vectors). Data from each graph is representative of one experiment, each of which was repeated three or more times. Western blots show protein expression.

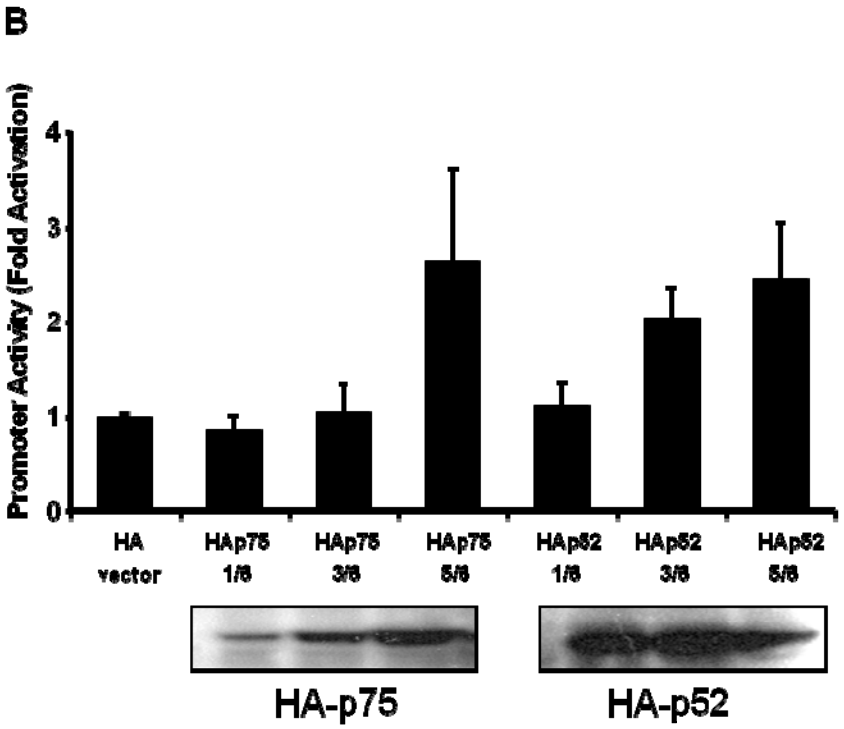

Figure 9. LEDGF/p75, LEDGF/p52, and MeCP2 transactivate Hsp27 promoter. B) U2OS cells were co-transfected with pGL3-Hsp27pr and increasing amount of pCruzHA LEDGF/p75 or pCruzHA p52. Promoter activity determined as luciferase light units/protein is expressed as fold activation compared to control activity (co-transfection of empty expression vectors). Data from each graph is representative of one experiment, each of which was repeated three or more times. Western blots show protein expression.

To establish if transactivation of Hsp27pr by LEDGF/p75 and MeCP2 was mediated by their binding to this promoter, chromatin immunoprecipitation (ChIP) assay was performed. Based on previous reports, LEDGF/p75 was predicted to bind HSE and STRE located in the proximal region (-185 to -111) of Hsp27pr (14), whereas MeCP2 was predicted to bind AT-rich repeats in the distal region (Fig. 9C) (38). ChIP assays using a specific MeCP2 antibody revealed binding of this protein to Hsp27pr regions C and D (bp -1071 to -382), located upstream of the HSE and STRE consensus sequences (region A, Fig. 9D). On the other hand, LEDGF/p75 bound to the entire Hsp27pr tested (bp -1071 to +18). ChIP with control rabbit anti-IgG did not produce any bands, while β-actin primers showed optimal enzymatic digestion of the chromatin. These results indicated that both LEDGF/p75 and MeCp2 bind to the Hsp27pr, with overlapping binding sites at the distal regions of the promoter.

C

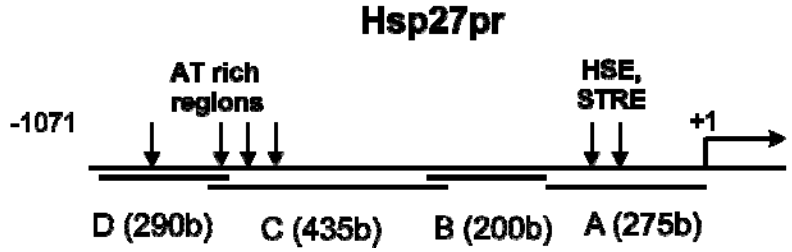

Figure 9. LEDGF/p75, LEDGF/p52, and MeCP2 transactivate Hsp27 promoter. C) Schematic diagram of Hsp27pr showing AT rich regions, and HSE and STRE sites. PCR primers targeted Hsp27pr regions A (bp -271 to +18), B (bp -480 to -220), C (bp -803 to -382), and D (bp -1071 to -781).

D

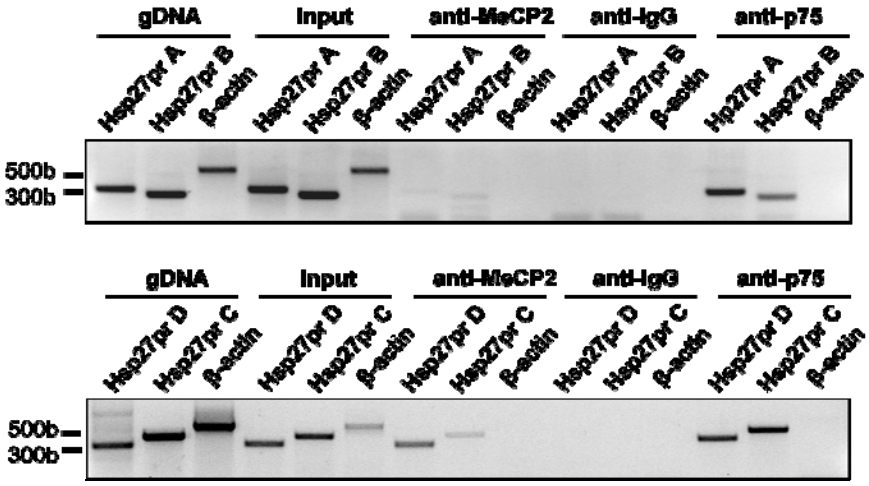

Figure 9. LEDGF/p75, LEDGF/p52, and MeCP2 transactivate Hsp27 promoter. D) Chromatin immunoprecipitation analysis of MeCP2 and LEDGF/p75 binding to Hsp27pr. Formaldehyde fixed chromatin from U2OS cells was precipitated with non-specific IgG, or antibodies specific for MeCP2 or LEDGF/p75. PCR amplifications of immunoprecipitated DNA derived from U2OS cells were carried out with primer sets specific for Hsp27pr regions A to D. Hsp27pr primers amplified DNA fragments precipitated by LEDGF/p75 antibody or MeCP2 antibody but not by IgG. Primers that target human β-actin controlled for optimal enzymatic digestion of chromatin. Results are representative of three experiments.

LEDGF/p75 and p52 Modulate the Transcriptional Activity of MeCP2

Given that LEDGF/p75 and p52 bind MeCP2, and all three proteins transactivate Hsp27pr in luciferase assays individually, with MeCP2 being the strongest activator (Fig. 9A,B), we sought to determine if LEDGF/p75 and p52 influence the ability of MeCP2 to transactivate Hsp27pr. Luciferase reporter assays were performed with co-expression of MeCP2 and LEDGF/p75, or MeCP2 and p52. Co-expression of induced LEDGF/p75 and MeCP2 in U2OS cells significantly enhanced, although only moderately and at low LEDGFp75/MeCP2 ratios, Hsp27pr transactivation levels above those by MeCP2 alone (Fig 10A). However, co-expression of MeCP2 with p52, resulted in significantly reduced Hsp27pr activity (Fig. 10B), suggesting that p52 represses MeCP2 transcriptional activity.

Since co-expression of LEDGF/p75 and MeCP2 did not result in robust increase on Hsp27pr activity in U2OS cells, we transiently silenced endogenous LEDGF/p75 using small inhibitory RNAs (siRNAs) oligonucleotides, while transiently overexpressing MeCP2, to further examine the functional relevance of the LEDGF/p75-MeCP2 interaction. Interestingly, U2OS cells with LEDGF/p75 knockdown and MeCP2 overexpression showed a dramatic and significant increase in Hsp27pr activation over cells transfected with control siRNAs, suggesting that LEDGF/p75 regulates MeCP2-driven Hsp27pr activity in these cells (Fig. 10C).

A

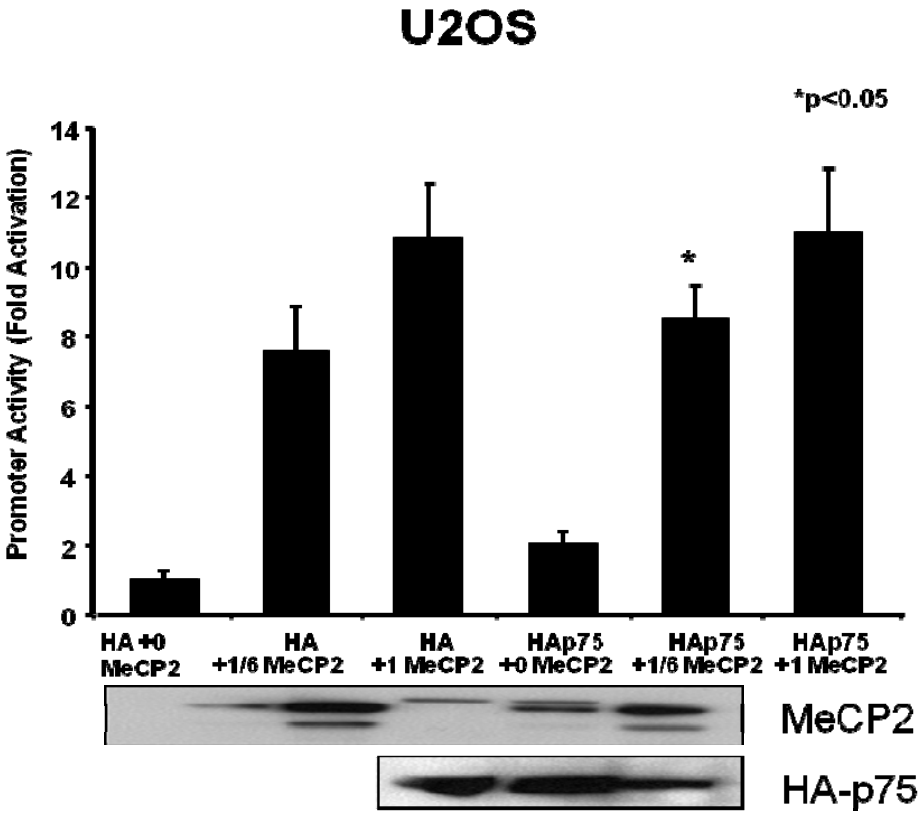

Figure 10. LEDGF/p75 and p52 influence MeCP2-induced transactivation of Hsp27pr. A**)** U2OS cells were co-transfected with 1.65g of pCruzHA or pCruzHA p75, and increasing amount of pcDNA-flag-MeCP2 and pGL3-Hsp27pr-luc. Western blots show protein expression. Data from graph is an average of five experiments. * indicates significantly different from control ($p<0.05$). Western blots show protein expression.

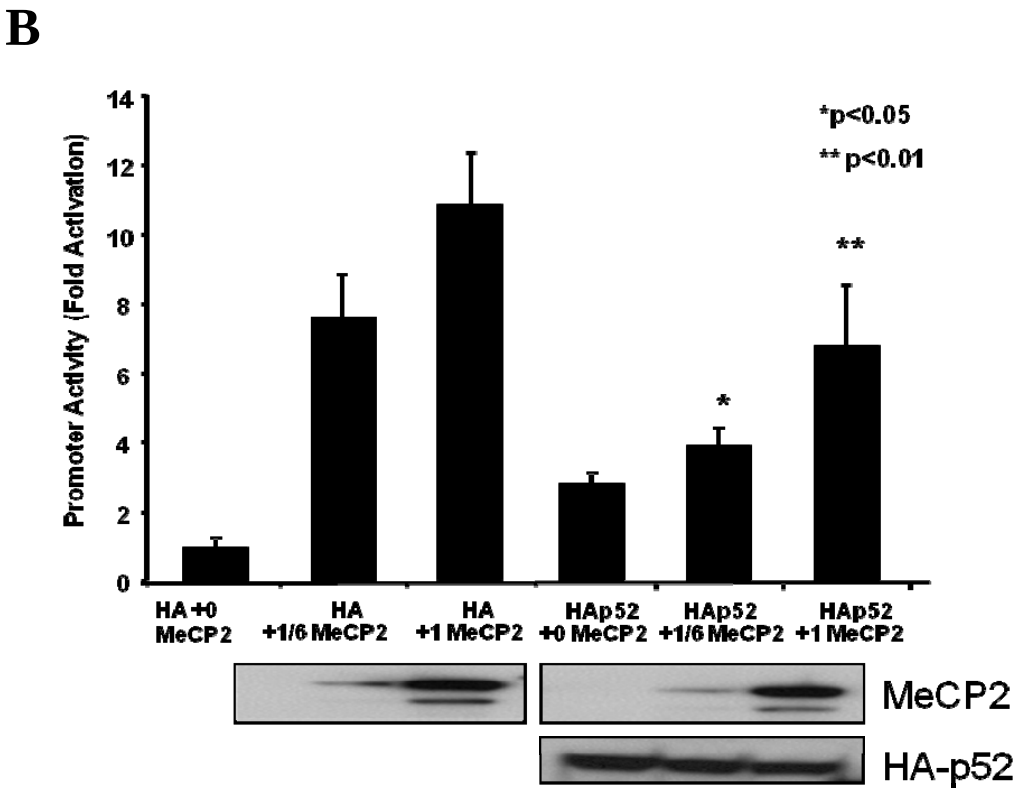

Figure 10. LEDGF/p75 and p52 influence MeCP2-induced transactivation of Hsp27pr. B) U2OS cells were co-transfected with 1.65g of pCruzHA or pCruzHA p52, and increasing amount of pcDNA-flag-MeCP2 and pGL3-Hsp27pr-luc. Western blots show protein expression. Data from graph is an average of five experiments. * indicates significantly different from control ($p<0.05$). Western blots show protein expression.

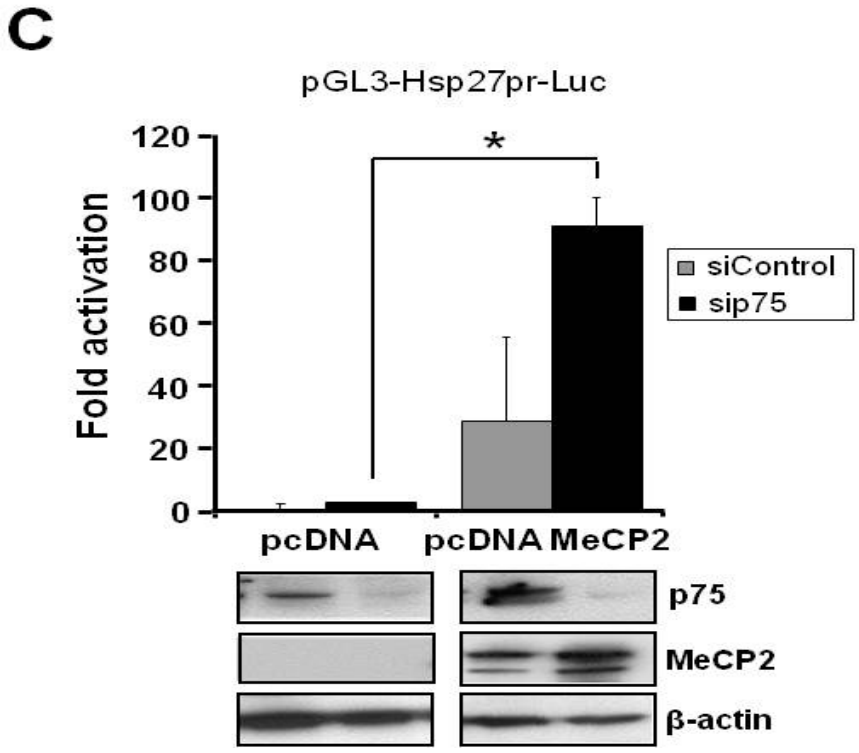

Figure 10. LEDGF/p75 and p52 influence MeCP2-induced transactivation of Hsp27pr. C) Transient knockdown of LEDGF/p75 results in increase of Hsp27pr activity in U2OS cells, which is amplified by overexpression of MeCP2. Luciferase reporter assay shows elevated Hsp27pr activity in the presence of MeCP2 when siRNA against LEDGF/p75 were employed. B) Western blots show protein expression.

Given that both LEDGF/p75 and MeCP2 have been implicated in prostate cancer cell growth and survival (33,34), we also examined the effects of their co-expression on Hsp27pr in PC3 cells. First, we evaluated Hsp27pr activation in PC3 cells stably overexpressing LEDGF/p75, and observed activation levels that were 3-5 fold above cells stably transfected with empty pcDNA vector (Fig. 10D,E). Transient MeCP2 overexpression in PC3 cells stably transfected with empty vector did not significantly increase Hsp27pr activation compared to cells without MeCP2 transfection (Fig. 10E), which could be due to the low transfection efficiency of these cells. However, PC3 cells overexpressing both LEDGF/p75 (stably) and MeCP2 (transiently) significantly enhanced Hsp27pr activation compared to cells without MeCP2 transfection (Fig. 10E). As observed with U2OS cells, PC3 cells with transient LEDGF/p75 knockdown and transient MeCP2 overexpression showed a significant increase in Hsp27pr activation over cells with normal LEDGF/p75 levels (Fig. 10F). Taken together, these results suggested that LEDGF/p75 and p52 influence differently MeCP2-driven Hsp27pr activity in human cancer cells.

D

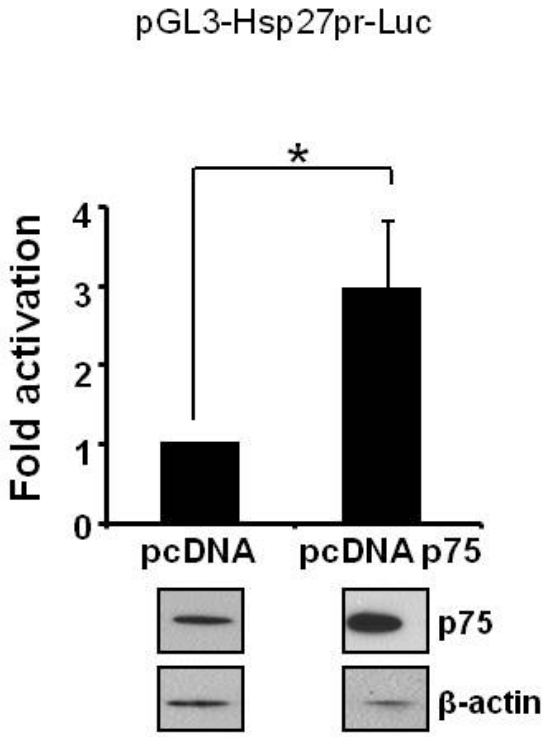

Figure 10. LEDGF/p75 and p52 influence MeCP2-induced transactivation of Hsp27pr. A) PC3 cells stably transfected with empty pcDNA vector or pcDNA-LEDGF/p75 were co-transfected with pGL3-Hsp27pr-luc and pMAX-GFP (transfection control). Promoter activity determined as luciferase light units/GFP is expressed as fold activation compared to control activity. Data represent the average of three or more experiments. Immunoblots show protein expression. *$p<0.05$

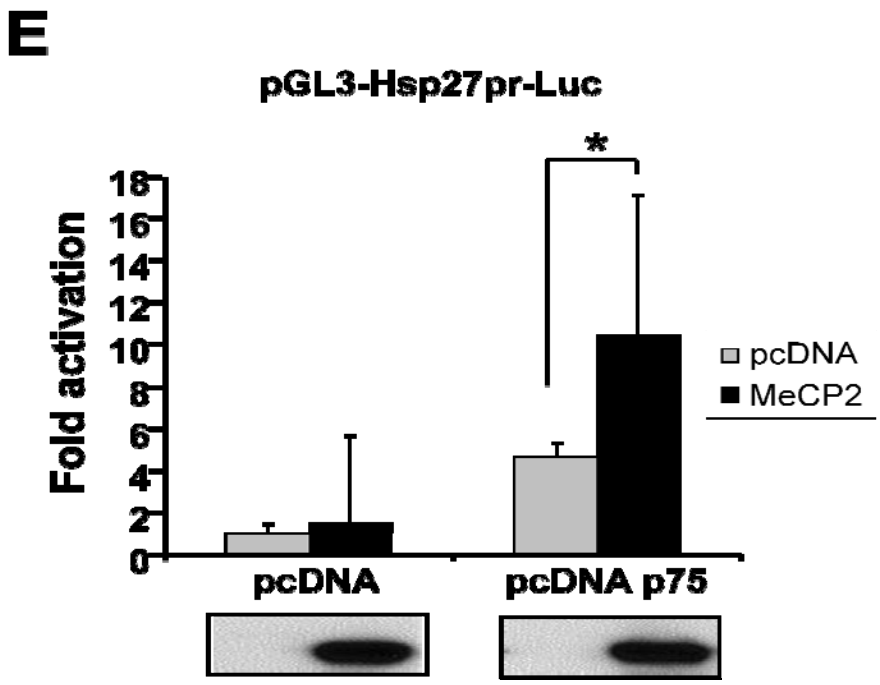

Figure 10. LEDGF/p75 and p52 influence MeCP2-induced transactivation of Hsp27pr.. B) PC3 cells stably transfected with empty pcDNA vector or pcDNA-LEDGF/p75 were co-transfected with pGL3-Hsp27pr-luc and pcDNA-Flag-MeCP2. Promoter activity determined as luciferase light units/GFP is expressed as fold activation compared to control activity. Data represent the average of three or more experiments. Immunoblots show protein expression. *$p<0.05$

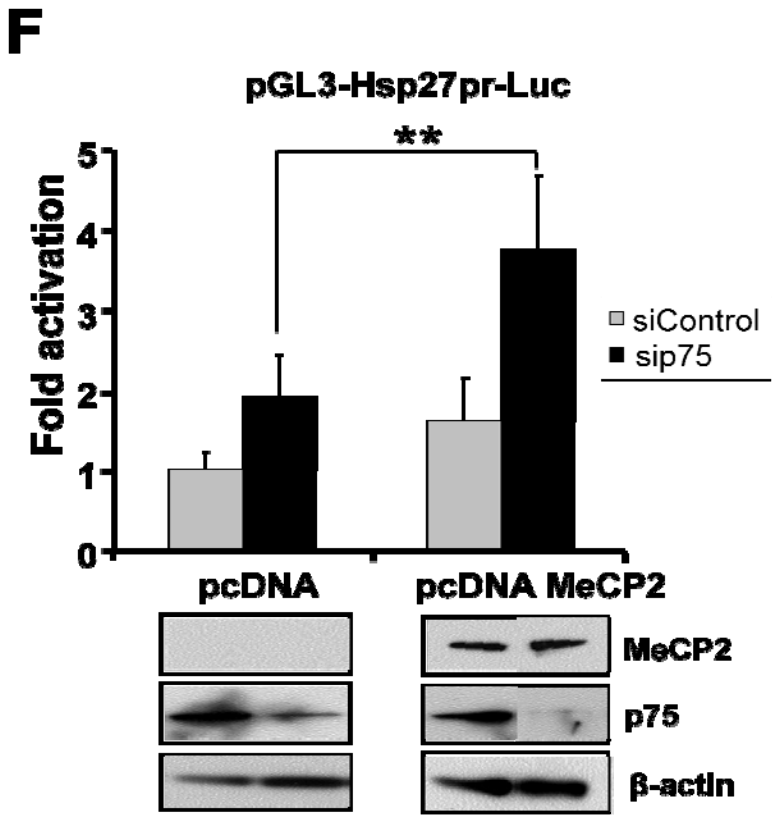

Figure 10. LEDGF/p75 and p52 influence MeCP2-induced transactivation of Hsp27pr. C) PC3 cells stably transfected with empty pcDNA vector or pcDNA-LEDGF/p75 were transfected with siRNA oligos to knockdown this protein. Cells were then co-transfected with pGL3-Hsp27pr-luc and pcDNA-Flag-MeCP2. Promoter activity determined as luciferase light units/GFP is expressed as fold activation compared to control activity. Data represent the average of three or more experiments. Immunoblots show protein expression. ** $p<0.01$.

Discussion

In this study we established MeCP2 as a cellular interacting partner of LEDGF/p75 using various complementary approaches. MeCP2 is a transcription factor that was initially reported to bind methylated CpG islands and repress transcription (35,39). More recently, it has been reported that MeCP2 binds DNA regardless of methylation status and does not necessarily function in transcriptional repression, since it can activate transcription in association with specific factors (36,40,41). We demonstrated that MeCP2 binds to the extreme N-terminal region of LEDGF/p75, which implies that it does not compete with HIV1-IN for LEDGF/p75 binding. Our results suggested that the PWWP domain of LEDGF/p75 alone is not sufficient to bind MeCP2 and that the downstream CR1 domain is also required. The CR2/SRD domain, also present in LEDGF/p52, may stabilize the interaction with MeCP2 by providing additional binding points.

To the best of our knowledge, these results represent the first direct demonstration that the PWWP-CR1 domain of LEDGF/p75 is involved in protein-protein interactions. This region was initially thought to bind directly to DNA, but was later shown to be insufficient for DNA binding (32). Instead, the NLS and AT-hooks are the main motifs involved in DNA binding, leaving the PWWP domain free to interact with chromatin proteins (25,26). The PWWP domain is conserved in some DNA binding proteins, and appears to be targeted to specific regions of chromatin for interaction, not necessarily with DNA, but with different chromatin proteins (42,43).

We examined whether the LEDGF/p75-MeCP2 interaction influences the transactivation of Hsp27pr in luciferase reporter assays. Hsp27 is known to be a target

gene of LEDGF/p75 and has been implicated in prostate cancer resistance to cell death and chemotherapy (14,21,44,45). We have previously shown that overexpression of LEDGF/p75 or p52 upregulates Hsp27 transcript, correlating with its promoter transactivation (21). Our results revealed that MeCP2 overexpression robustly transactivated Hsp27pr in U2OS cells, surpassing the levels of transactivation induced by LEDGF/p75 or p52. However, we did not observe a robust synergistic effect between MeCP2 and LEDGF/p75 on Hsp27pr activation in U2OS cells. This could be due to high endogenous levels of LEDGF/p75 in this cell line, which may have reached saturation levels. We cannot exclude the possibility that the lack of further activation by the interaction between these two proteins is due to limitations of the assay. However, co-expression of both proteins in PC3 prostate cancer cells resulted in significantly enhanced promoter transactivation when compared to individual protein expression. These results suggest that the LEDGF/p75-MeCP2 interaction enhances Hsp27pr activation depending on the intracellular levels of these proteins or the cellular context.

Co-expression of LEDGF/p52 with MeCP2 in U2OS cells resulted in reduced transactivation of Hsp27pr, suggesting that p52 represses the transcriptional activity of MeCP2. Bueno et al. (20) recently reported that mutations impairing SUMOylation of LEDGF/p75 increase its transcriptional activity, but not that of p52, suggesting that these splice variants may activate Hsp27pr via different molecular mechanisms. Considering the antagonistic functions of LEDGF/p75 (pro-survival) and p52 (pro-apoptotic) (21), it is possible that the latter may compete with LEDGF/p75 in binding to MeCP2 and Hsp27pr, thus preventing their interaction with the general transcription complex. In addition, p52, which lacks the C-terminal domain of LEDGF/p75 implicated in pro-

survival functions (1,5,21,32), may be unable to promote cellular survival because of its inability to interact with survival proteins. It is possible that interaction of the C-terminal region of LEDGF/p75 with other proteins, such as the Myc-interacting protein JPO2 (30), is important to fine tune transcription regulation of stress and survival genes. The binding of p52 to MeCP2 could also form a growth suppressor unit, down-regulating the activation of Hsp27pr, similar to that of the growth suppressor unit formed by interaction of JunD with menin (46). Alternatively, reduced Hsp27pr activity may be due to p52-induced apoptosis, which leads to inactivation of transcription signals.

Our observations that transient LEDGF/p75 silencing in both U2OS and PC3 cells induced a dramatic increase in MeCP2-driven Hsp27pr activity, suggested that MeCP2 is a main modulator of this promoter, with LEDGF/p75 playing a regulatory role. It is possible that LEDGF/p75 could tether MeCP2 to the chromatin, as it does with HIV-IN and the menin/MLL complex (6,17), and regulate its transcriptional activity through interaction with other transcription factors or co-activators. Since siRNA-mediated silencing is not 100% efficient, low endogenous levels of LEDGF/p75 could still be sufficient for MeCP2 binding to the promoter region. Alternatively, LEDGF/p75 may be part of a feedback loop by competing with MeCP2 or blocking other transcription co-factors from promoter regulatory sites in the presence of high MeCP2 levels, as in the feedback loop observed between HSF1 and Hsp27 (47).

Consistent with our promoter reporter assays, we demonstrated the binding of LEDGF/p75 and MeCP2 to Hsp27pr by ChIP assays. Interestingly, binding of LEDGF/p75 was not limited to the region where HSE and STRE are located (bp -271 to +18), but also to the entire Hsp27pr region tested (bp -1071 to -220). This observation

sheds some light into the question of whether LEDGF/p75 binding to promoter regions is mainly restricted to STRE and HSE as reported by Singh's group (14,32), but not observed by Engelman's group (26). Recently, members of this research team used the DamID technology, focusing on the highly annotated ENCODE (encyclopedia of DNA Elements) region, to demonstrate that LEDGF/p75 binding to DNA occurs primarily downstream of active transcription unit start sites (48). LEDGF/p75 binding was not restricted to STREs, but correlated with active chromatin markers and RNA polymerase II binding (18). However, STREs appear to be important for LEDGF/p75 mediated VEGF-c promoter transactivation (16). It is plausible that LEDGF/p75 binding to particular promoter regions might be influenced by interactions with specific proteins, and the cellular type and microenvironment.

Binding of MeCP2 to Hsp27pr was observed upstream of HSE and STRE sites in AT-rich regions (bp-1071 to -382) as predicted (38), sharing this region with LEDGF/p75. Prior to this study, there have been no reports documenting MeCP2 binding to and activation of Hsp27pr. In agreement with our results, MeCP2 has been shown to directly bind promoter sites of proteins and enhance their function, as demonstrated by its association with Creb1 on promoters of transcribed genes (36). It is possible that MeCP2 interacts with other transcription factors on Hsp27pr in a manner similar to its association with Creb1 at promoter sites, contributing to up-regulation of Hsp27pr activity in human cancer cells.

Conclusion

In conclusion, we validated MeCP2 as a specific cellular interacting partner of both LEDGF/p75 and p52, and a novel transactivator of Hsp27pr. Since MeCP2 represses or enhances gene activation in association with other transcription factors, it is possible that its binding to LEDGF/p75 and p52 may result in targeting specific promoters to either protect cells from stress-induced cell death or trigger cell death (49). Although additional work is required to fully establish the role of LEDGF/p75, p52, and MeCP2 in stress gene regulation in tumor cells, our results lay the groundwork for future studies focused on modulating the transcriptional functions of these proteins to circumvent tumor resistance to cell death and chemotherapy.

Acknowledgments

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CHAPTER THREE

RESULTS IN PROGRESS NOT INCLUDED IN PUBLICATIONS

Introduction

LEDGF/p75 is a known survival protein that has been linked to attenuation of cell death induced by DTX and oxidative stress [1, 2]. This protection might be related to the protein's ability to upregulate stress related proteins. Its interaction with MeCP2 shows transactivation of Hsp27pr activity (Chapter 2). Other stress response proteins such as oxidoreductase ERp57 has been implicated in attenuation of cell death induced by oxidative stress in various systems [3]. Insulin-like growth factors binding protein 5 (IGFBP-5) is the major IGFBP that binds IGF, the most abundant growth factors in the bone matrix. PSA derived from PCa cells was reported to enhance IGF bioavailability in the bone microenvironment of PCa metastasis, thereby permitting PCa survival and malignant progression in the bone microenvironment [4]. To examine if LEDGF-MeCP2 complex modulates other stress response proteins in PCa, we examined their function on ERp57pr and IGFBP5pr. The transactivation functions of cleaved constructs of LEDGF/p75 were also examined. In addition, we examined if LEDGF/p75 selectively protects against stress-induced non-apoptotic cell death. The results presented in this chapter were obtained from preliminary experiments relevant to Chapter two and other publications currently in preparation. Some of these results are likely to lead to new ideas for research projects.

Materials and Methods

Induction of Cell Death

Cell death was induced by treatment with TBHP (150 μM), TRAIL/actinomycin D (100 ng/ml each), or 4 μM STS for up to 24 h. Cells morphology was visualized on an Olympus IX70 microscope equipped with Hoffmann Modulation Contrast (Olympus American). Images were acquired using a digital Spot Imaging System (Diagnostic Instruments).

Cell Viability Assays

Cells seeded in 96-well plates (3×10^4 cells per well) were treated with cytotoxic drugs, washed with phosphate buffered saline (PBS), and fixed in 4% paraformaldehyde for 1 h at 4°C. Cells were then washed three times with distilled water, and Accustain Crystal Violet solution (Sigma-Aldrich) (1:4) was added to each well followed by incubation for 20 minutes at room temperature. Plates were washed with distilled water to remove excess dye and then dried at room temperature. Acetic acid (10% v/v) was added to each well for 10 minutes and absorbance was measured at 570 nanometers (nm) using a μQuant microplate reader (Bio-tek Instruments).

Measurement of ROS by Flow Cytometric Analysis

Generation of reactive oxygen species (ROS) were assessed based on the intracellular oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen) to form the fluorescent compound 2',7'-dicholorofluorescein (DCF). Cells were seeded in a 6-well plate at a density of 3×10^5 cells per well and cultured for 24 hours. Treatment of cells with the drugs was done for up to 12 h. Then, DCFH-DA (0.5

μM) was added to the cells, followed by incubation for 20 minutes at 37°C. The cells were washed with PBS (2X), and then resuspended in 0.5 mL of PBS. Fluorescence intensity was determined by flow cytometry using the FACScalibur (BD Biosciences).

Results

Additional Studies on the LEDGF-MeCP2 Interaction

Binding of LEDGF PWWP CR1 to MeCP2

U2OS cell lysates containing endogenous MeCP2 were incubated with recombinant Flag-LEDGF/p75, Flag-PWWP CR1 (res 1-141) or Flag-PWWP (res 1-108). This mixture was pulled down with anti-Flag agarose. Immunoblotting using antibody against MeCP2 showed MeCP2 being pulled down with Flag-p75 and Flag-PWWP CR1 but not with Flag-PWWP. This suggests that the extreme N-terminal (108 res) part of LEDGF mediates interaction with MeCP2 (Chapter 2 Fig 8). To further characterize these interactions, we conducted AlphaScreen® assays. Due to high degradation of MeCP2 and difficulties in using purified GST MeCP2 in AlphaScreen® assays, we opted to use GST MeCP2 present in bacterial lysate. Induction of GST MeCP2 was increased by the addition of sorbitol and betaine, which creates osmotic stress for intake of stabilizing biomolecules. The solutes also assist in protein folding in a chaperone-like manner. AlphaScreen® counts showed binding of purified flag-PWWP CR1 to GST MeCP2 in bacterial lysate, although in relatively lower binding capacity compared to full length LEDGF/p75 binding to MeCP2 (Fig 11A,B). Non-transformed bacterial lysate served as negative control (Fig 11C).

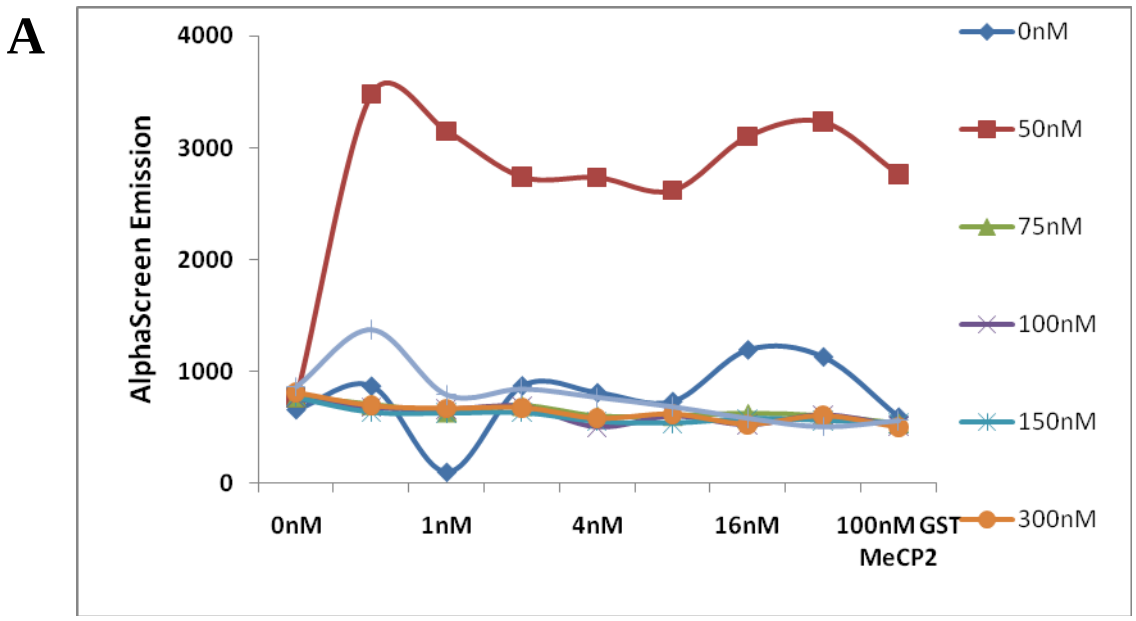


Figure 11. The N-terminus of LEDGF interacts with MeCP2. PWWP CR1 binds MeCP2 as confirmed by AlphaScreen® assay. Purified flag PWWP CR1 was incubated with GST-MeCP2 in bacterial lysate for 1 h. Glutathione donor beads and flag acceptor beads were then added, and plates were read on an EnVision multilabel plate reader after 2 h incubation period in the absence of light. **A)** Cross titration for Flag-LEDGF-PWWP-CR1 and GST-MeCP2 shows interaction as measured by AlphaScreen® assays. Interaction was measured at different concentrations of Flag-LEDGF-PWWP-CR1 as indicated in the legend and GST-MeCP2 as indicated on the X-axis.

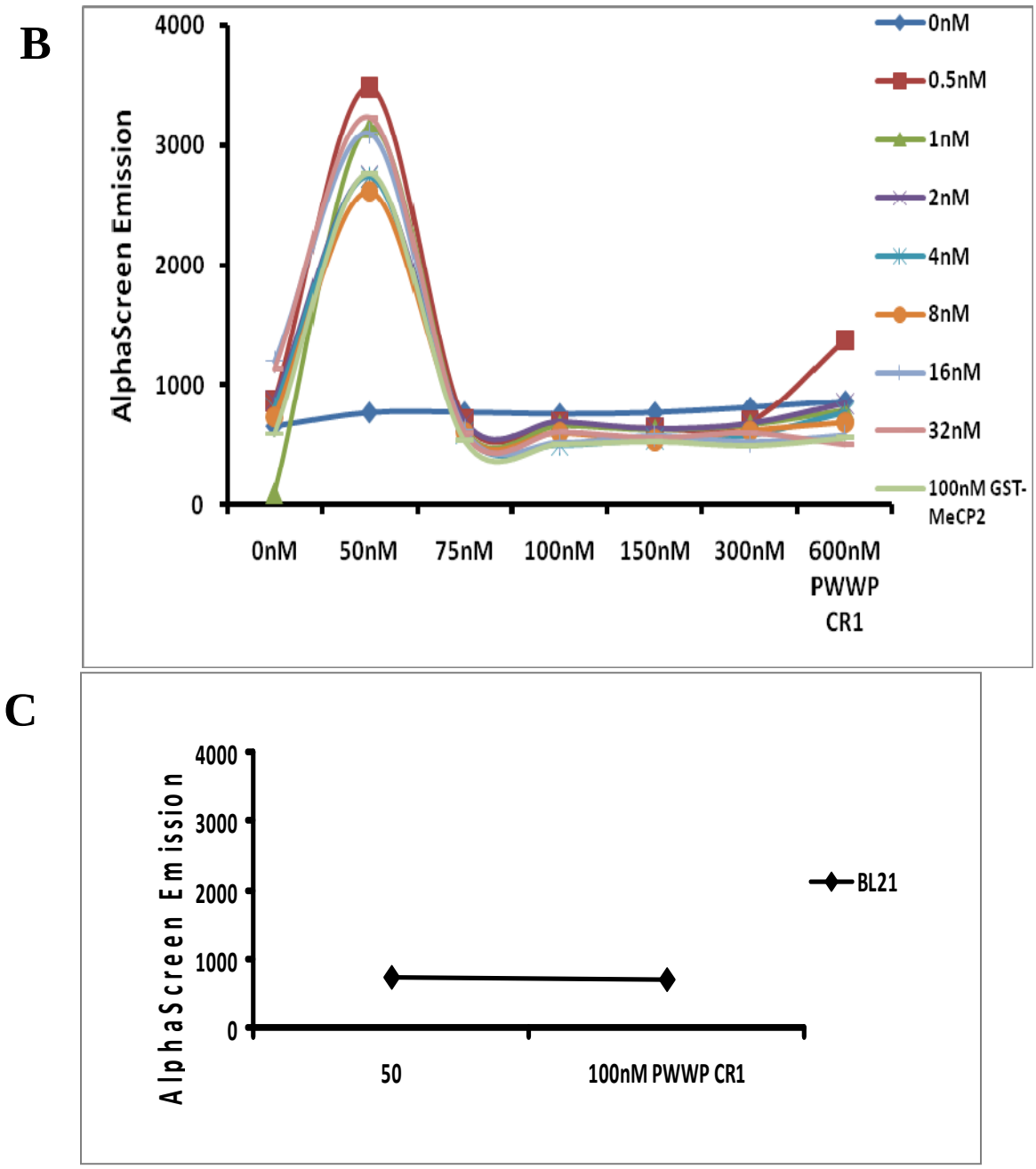
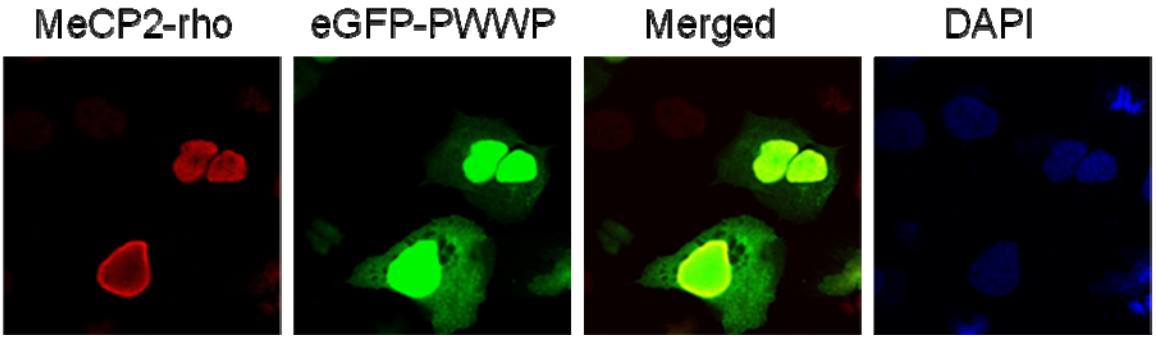


Figure 11. The N-terminus of LEDGF interacts with MeCP2. **B)** 50nM of PWWP CR1 is required to bind MeCP2. **C)** Non-transformed bacterial lysate control does not bind to MeCP2.

Co-localization assays were also performed to confirm protein interactions. U2OS cells were transfected with pEGFP-PWWP or pEGFP-ΔPWWP with pcDNA-flag-MeCP2. Rhodamine dye was used to detect MeCP2 localization. Confocal fluorescence microscopy was used to visualize proteins, showing partial co-localization of both LEDGF constructs with MeCP2 (Fig11D,E).

D



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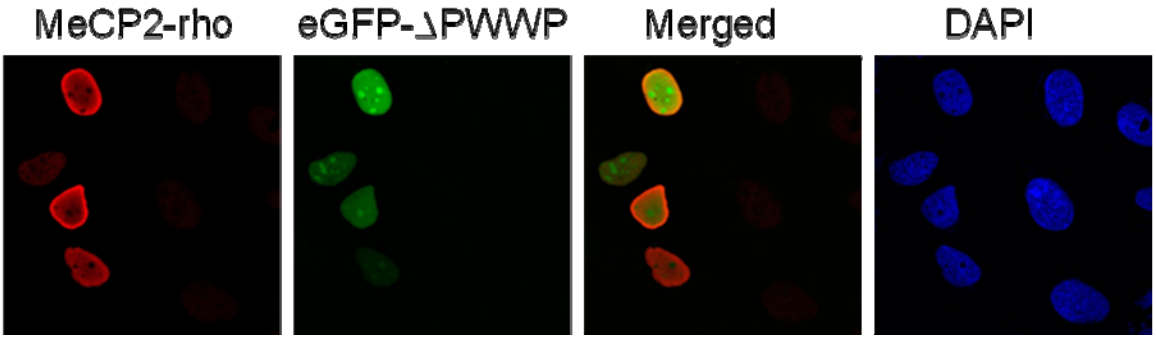


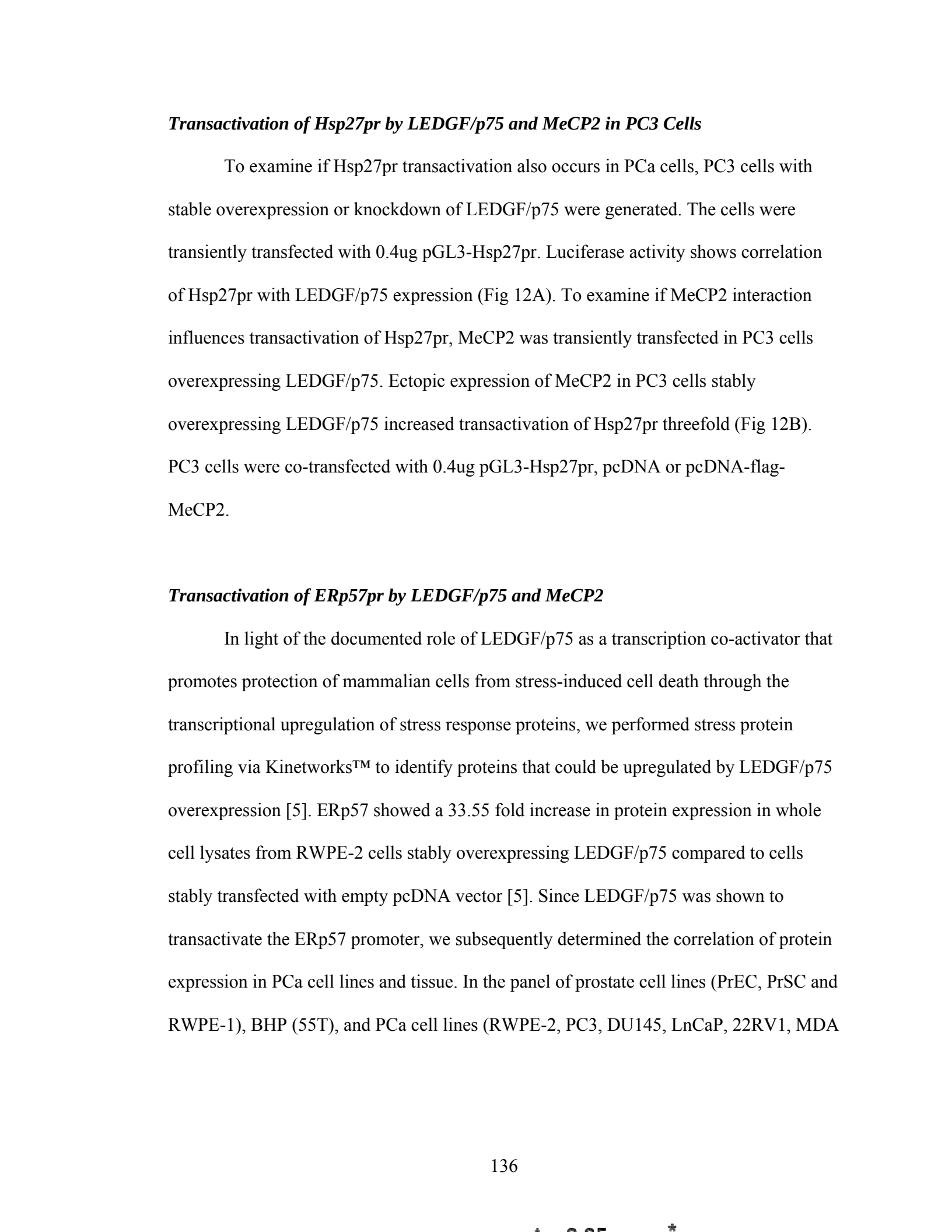
Figure 11. The N-terminus of LEDGF interacts with MeCP2. Localization of PWWP and MeCP2 in the nucleus. U2OS cells plated on chamber slides were transfected with pcDNA-Flag-MeCP2 and **D)** peGFP-PWWP or **E)** peGFP-ΔPWWP using Trans-It® 2020 (Mirus). Ectopically expressed LEDGF or MeCP2 were detected using specific antibodies, followed by specific anti-rhodamine antibody. The nuclei were subsequently stained with DAPI and fluorescent signals were analyzed by confocal fluorescence microscopy 48h post-transfection.

Transactivation of Hsp27pr by LEDGF/p75 and MeCP2 in PC3 Cells

To examine if Hsp27pr transactivation also occurs in PCa cells, PC3 cells with stable overexpression or knockdown of LEDGF/p75 were generated. The cells were transiently transfected with 0.4ug pGL3-Hsp27pr. Luciferase activity shows correlation of Hsp27pr with LEDGF/p75 expression (Fig 12A). To examine if MeCP2 interaction influences transactivation of Hsp27pr, MeCP2 was transiently transfected in PC3 cells overexpressing LEDGF/p75. Ectopic expression of MeCP2 in PC3 cells stably overexpressing LEDGF/p75 increased transactivation of Hsp27pr threefold (Fig 12B). PC3 cells were co-transfected with 0.4ug pGL3-Hsp27pr, pcDNA or pcDNA-flag-MeCP2.

Transactivation of ERp57pr by LEDGF/p75 and MeCP2

In light of the documented role of LEDGF/p75 as a transcription co-activator that promotes protection of mammalian cells from stress-induced cell death through the transcriptional upregulation of stress response proteins, we performed stress protein profiling via Kinetworks™ to identify proteins that could be upregulated by LEDGF/p75 overexpression [5]. ERp57 showed a 33.55 fold increase in protein expression in whole cell lysates from RWPE-2 cells stably overexpressing LEDGF/p75 compared to cells stably transfected with empty pcDNA vector [5]. Since LEDGF/p75 was shown to transactivate the ERp57 promoter, we subsequently determined the correlation of protein expression in PCa cell lines and tissue. In the panel of prostate cell lines (PrEC, PrSC and RWPE-1), BHP (55T), and PCa cell lines (RWPE-2, PC3, DU145, LnCaP, 22RV1, MDA



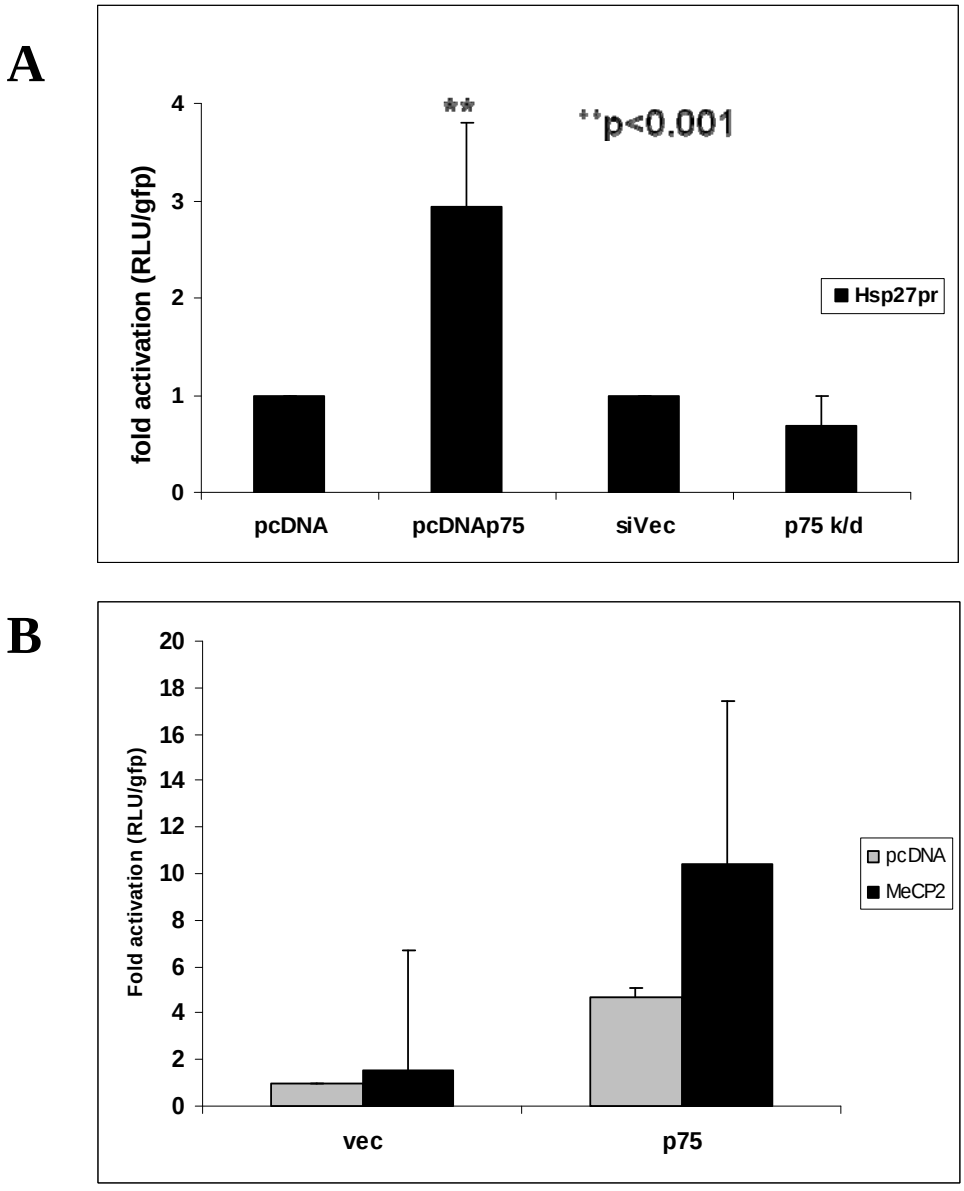


Figure 12. MeCP2 up-regulates Hsp27pr activity in PCa cells. **A)** Hsp27pr activity of PC3 cells with stable overexpression and knockdown of LEDGF/p75 was analyzed using luciferase reporter assay. **B)** pcDNA MeCP2 was overexpressed in PC3 cells stably overexpressing LEDGF/p75. Increased Hsp27pr activity was observed in the presence of LEDGF/p75 and MeCP2.

and 41T) examined, no evident correlation between the expression of both LEDGF/p75 and ERp57 was found [6]. Contrary to this, immunohistochemical analysis of prostate tissue microarrays (TMA) showed correlation of LEDGF/p75 and ERp57 expression at the tissue level (p value < 0.0001, Kendall's tau b analysis) (Basu,A et al., unpublished results). However, the expression of ERp57 in two PCa cell lines (PC3 and RWPE-2) with LEDGF/p75 transient siRNA-mediated knockdown showed no downregulation of ERp57 when LEDGF/p75 was knocked down (manuscript in preparation). Nevertheless, we cannot rule out the fact that minimal levels of LEDGF/p75 are sufficient to stimulate ERp57 expression, since total repression of HIV1 replication was only achieved when LEDGF/p75 knockdown was over 97%.

To examine if LEDGF/p75-MeCP2 similarly transactivates ERp57pr in other cell types, luciferase reporter assays were performed. U2OS cells were transiently co-transfected with pcDNA-Flag-MeCP2, pCruzHA-LEDGF/p75 or pCruzHA-p52 or pCruzHA-p38 and a promoter construct containing -262 to -22 bp of the human ERp527 gene in pGL3 luciferase reporter. MeCP2 overexpression up-regulated ERp57pr activity seven fold (Fig 13B), compared to two fold of LEDGF/p75 (Fig 13A). p52 and p38 transactivated ERp57pr to a lesser degree. Co-expression of MeCP2 with all LEDGF constructs showed further up-regulation of ERp57pr that corresponds to MeCP2 transactivation (Fig 13C).

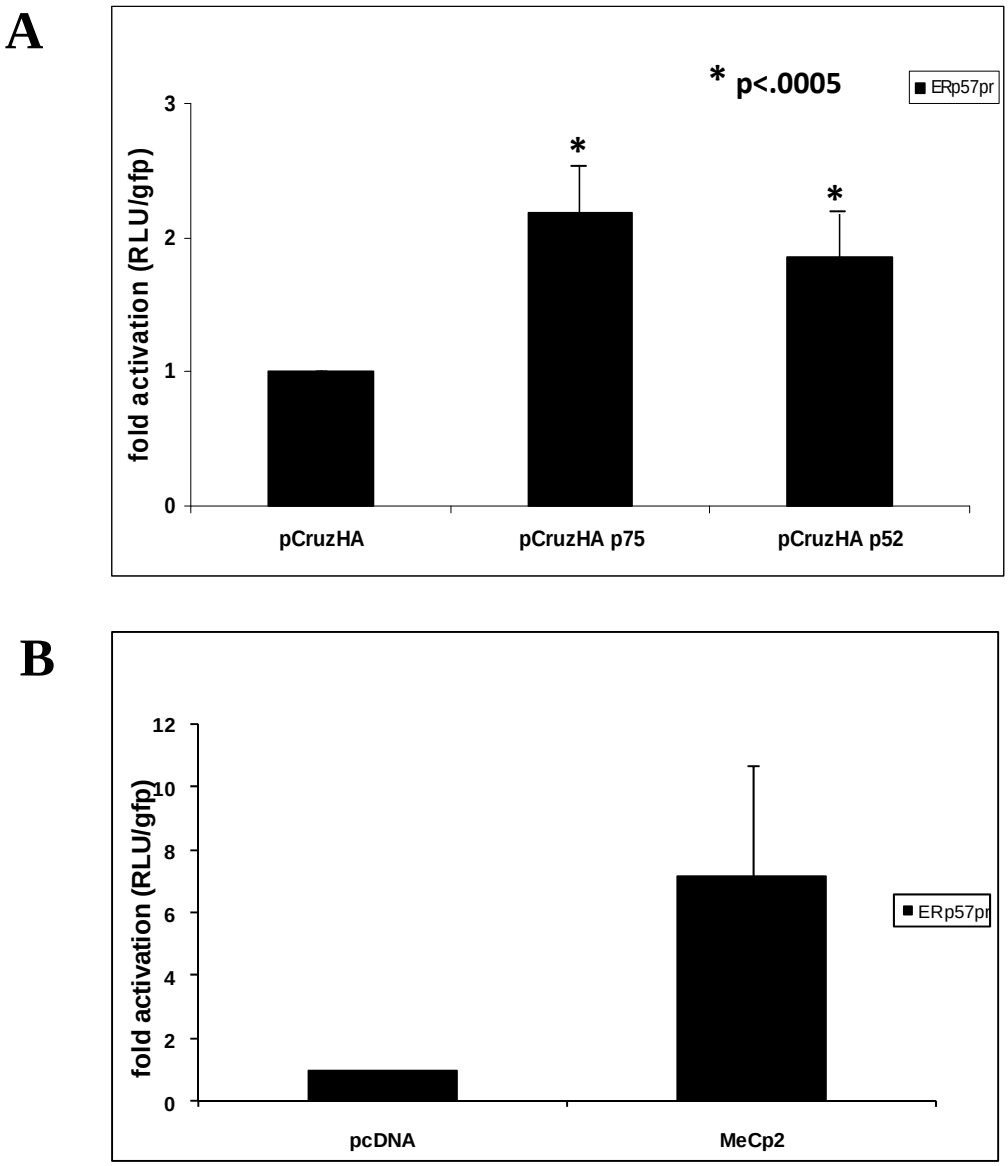


Figure 13. LEDGF/p75 and MeCP2 upregulate ERp57pr activity. **A)** Transient transfection of pCruzHA-p75 or pCruzHA-p52 **B)** pcDNA-MeCP2, with pGL3-Hsp27pr-Luc in U2OS resulted in significant increase of ERp57pr activity.

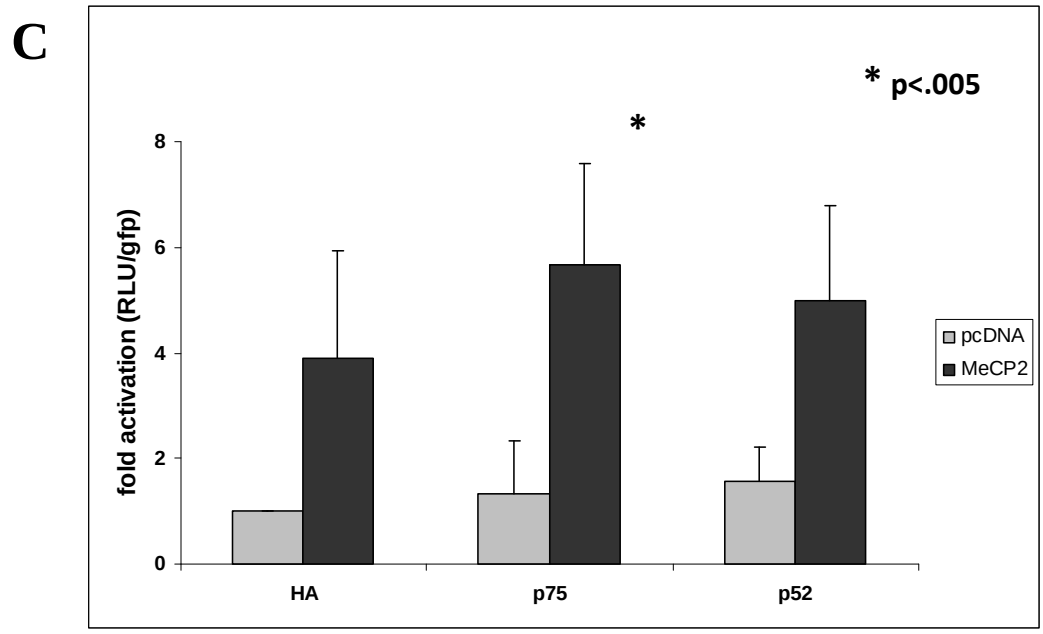


Figure 13. LEDGF/p75 and MeCP2 upregulate ERp57pr activity. **C)** Co-expression of the plasmids in U2OS. GFP was transfected as control for transfection efficiency. Promoter activity determined as luciferase light units/gfp is expressed as fold activation compared to control activity (co-transfection of empty expression vectors). Results are average of three or more experiments.

In PC3 cells, stable overexpression of LEDGF/p75 upregulated ERp57pr up to three fold (Fig 13D). However, PC3 cells with stable knockdown of LEDGF/p75 showed similar upregulation of ERp57pr. Overexpression of MeCP2 in PC3 cells stably overexpressing LEDGF/p75 increased transactivation of ERp57pr another four fold (Fig 13E). Fold activation is relative luciferase units normalized against gfp for transfection efficiency.

Transactivation of IGFBP5pr by LEDGF/p75 and MeCP2

To explore if LEDGF-MeCP2 interaction modulates transactivation of Hsp27pr but not ERp57pr, we examined another protein implicated in PCa, IGFBP5pr. U2OS cells were transiently co-transfected with pcDNA-flag-MeCP2, pCruzHA-LEDGF/p75 or pCruzHA-p52 or pCruzHA-p38 and a promoter construct containing -252 to +24 bp of the human IGFBP5pr gene in pGL3 luciferase reporter as described in Perez-Casellas et al. MeCP2 overexpression up-regulated IGFBP5pr activity twelve fold (Fig 14B), compared to six fold of LEDGF/p75 (Fig 14A). p52 and p38 transactivated IGFBP5pr to a lesser degree. In PC3 cells, stable overexpression of LEDGF/p75 upregulated IGFBP5pr three fold, while stable knockdown of LEDGF/p75 downregulated IGFBP5pr to basal levels (Fig 14C).

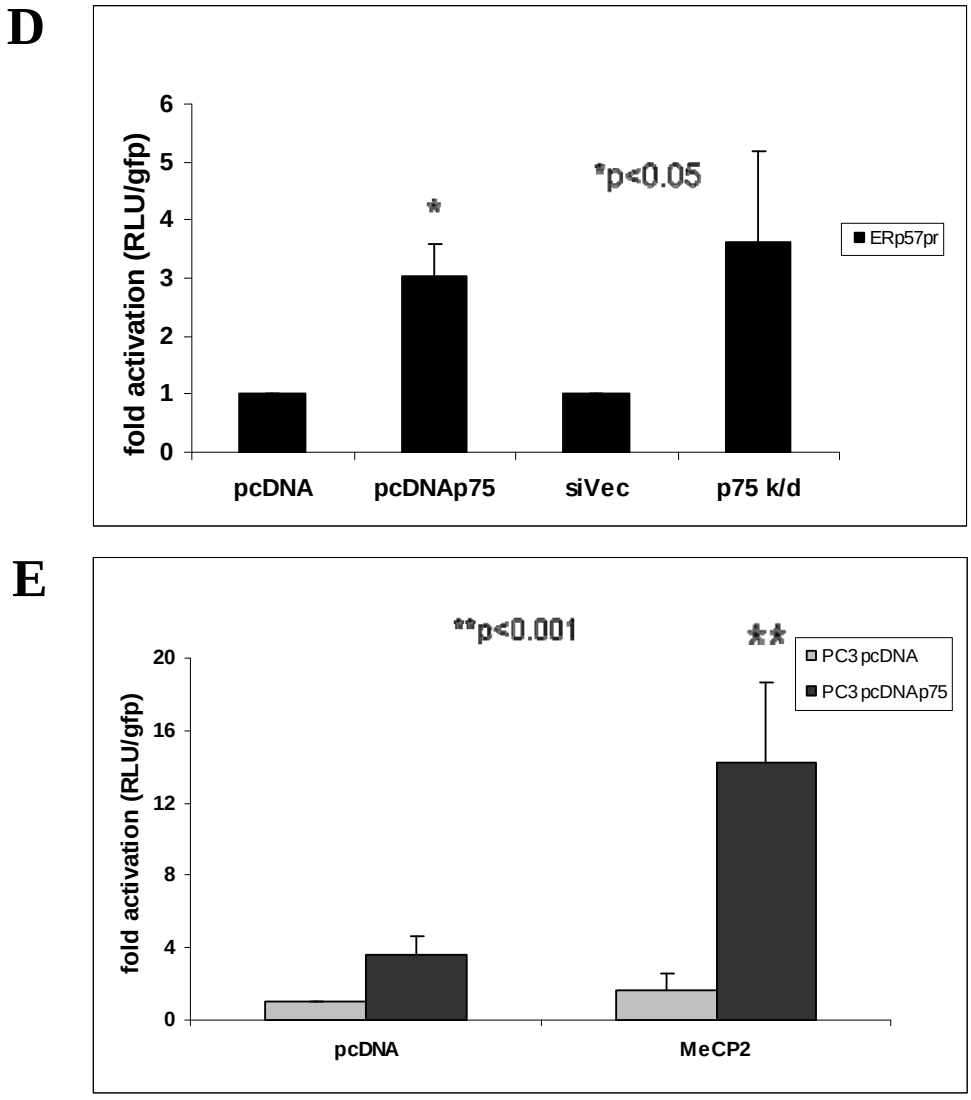


Figure 13. LEDGF/p75 and MeCP2 up-regulate ERp57pr activity. **D)** PC3 cells with stable overexpression or knockdown of LEDGF/p75 were transfected with pGL3-ERp57pr-luc. ERP57pr acitivity does not correlate with LEDGF/p75 expression. **E)** MeCP2 was transiently overexpressed in PC3 cells stably overexpressing LEDGF/p75. Co-expression of both proteins upregulated Hsp27pr threefold. GFP was transfected as control for transfection efficiency. Promoter activity determined as luciferase light units/gfp is expressed as fold activation compared to control activity (co-transfection of empty expression vectors). Results are average of four or more experiments.

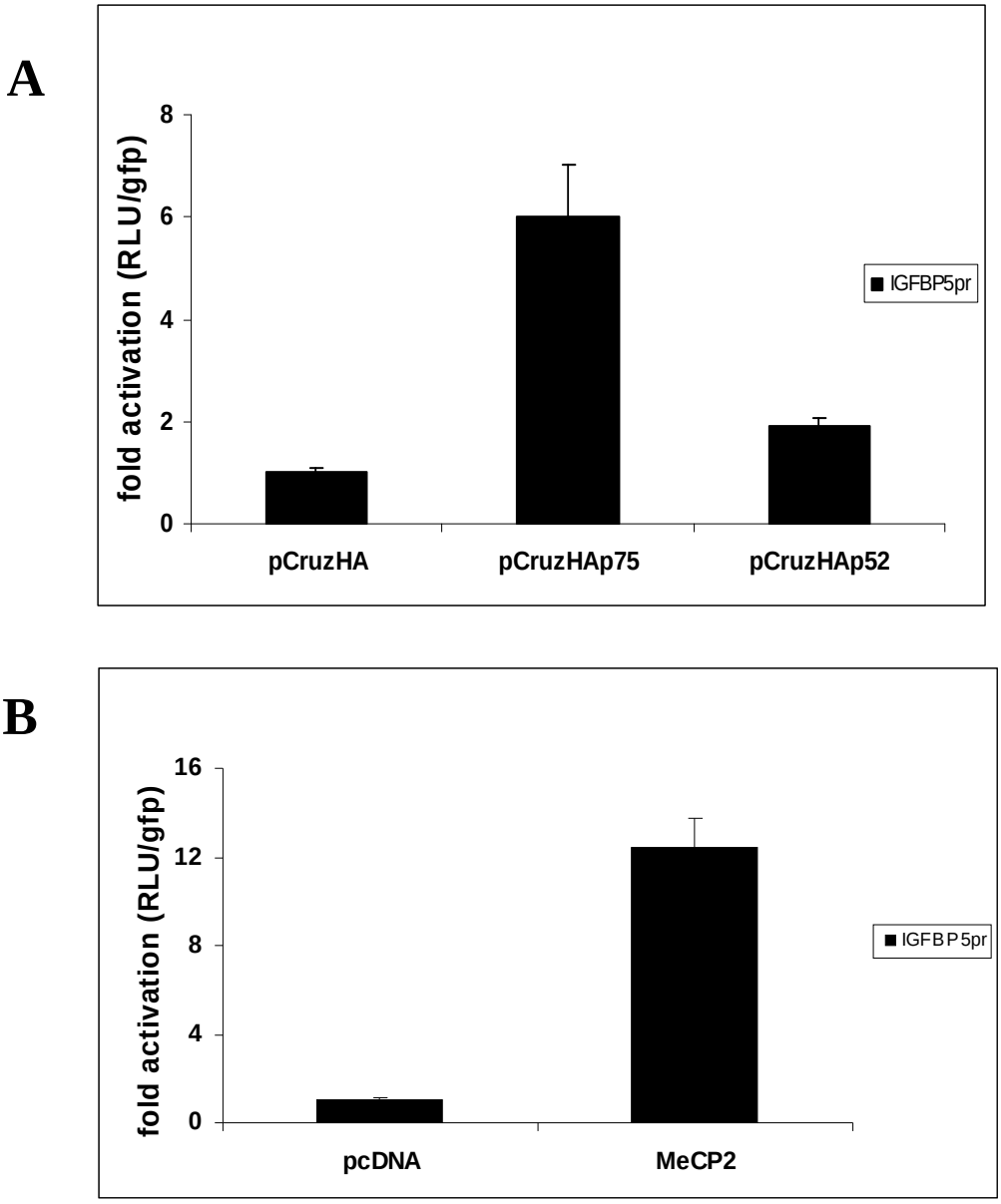


Figure 14. LEDGF/p75 and MeCP2 up-regulate IGFBP5pr activity. **A)** Transient transfection of pCruzHA-p75, pCruzHA-p52, or **B)** pcDNA-MeCP2with pGL3-Hsp27pr-Luc in U2OS resulted in significant increase of Hsp27pr activity. Results are representative of three experiments.

C

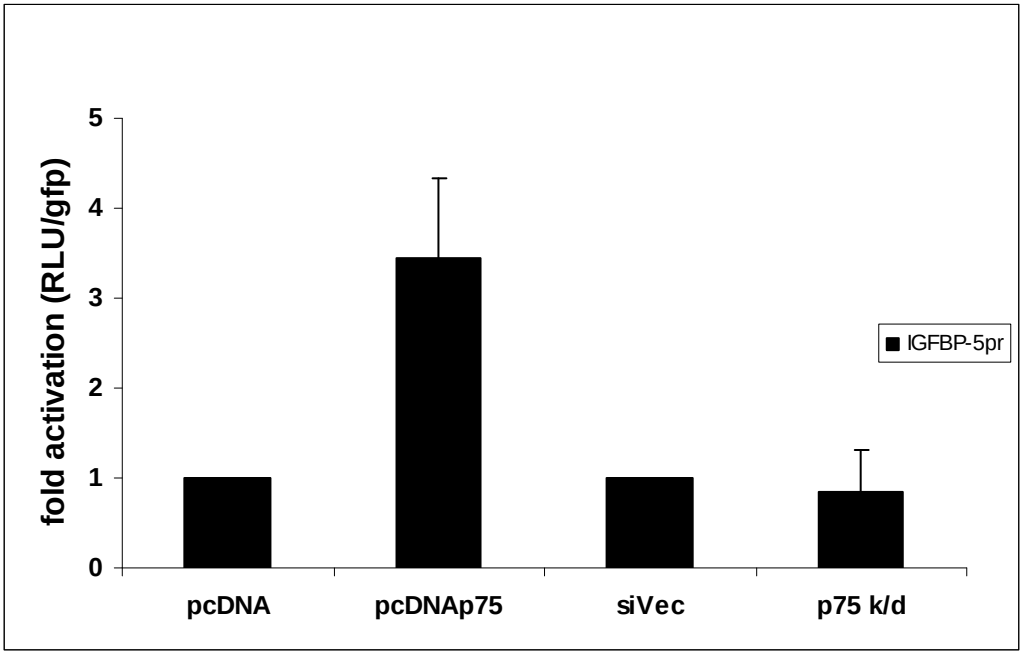


Figure 14. LEDGF/p75 and MeCP2 up-regulate IGFBP5pr activity. **C)** PC3 cells with stable overexpression or knockdown of LEDGF/p75 were transfected with pGL3-IGFBP5pr-luc. GFP was transfected as control for transfection efficiency. Promoter activity determined as luciferase light units/gfp is expressed as fold activation compared to control activity (co-transfection of empty expression vectors). Results are average of three experiments.

Transactivation of Hsp27pr by LEDGF/p75 Cleaved Constructs

During apoptosis, LEDGF/p75 is cleaved by caspases into different fragments (Fig 15A), which abolishes its pro-survival functions [7]. Cleavage of N-terminal domain of LEDGF/p75 was shown to upregulate Hsp27pr transactivation [8], suggesting a repressive function. To examine if MeCP2 modulates this transactivation, we transiently overexpressed LEDGF/p75 cleaved constructs in the presence and absence of MeCP2 with Hsp27pr. The removal of N-terminal 30aa results in an increase in Hsp27pr transactivation (Fig 15B). However, further removal of C-terminal 44aa showed a slight decrease in this activation, suggesting the involvement of this region in transactivation (Fig 15B). Co-expression of MeCP2 further enhanced Hsp27pr activation up to three fold (Fig 15C).

Regulation of LEDGF/p75 by MeCP2

While confirming protein expression of LEDGF/p75 and MeCP2 in luciferase reporter assay, we observed that co-expression of MeCP2 and LEDGF/p75 shows decreased levels of MeCP2. To further examine this, we co-expressed pCruzHA, pCruzHA-p75, pCruzHA-p52 in the absence and presence of pcDNA-flag-MeCP2 in U2OS cells. Co-expression of LEDGF/p75 and MeCP2 shows down-regulation of MeCP2 protein levels (Fig 16).

A

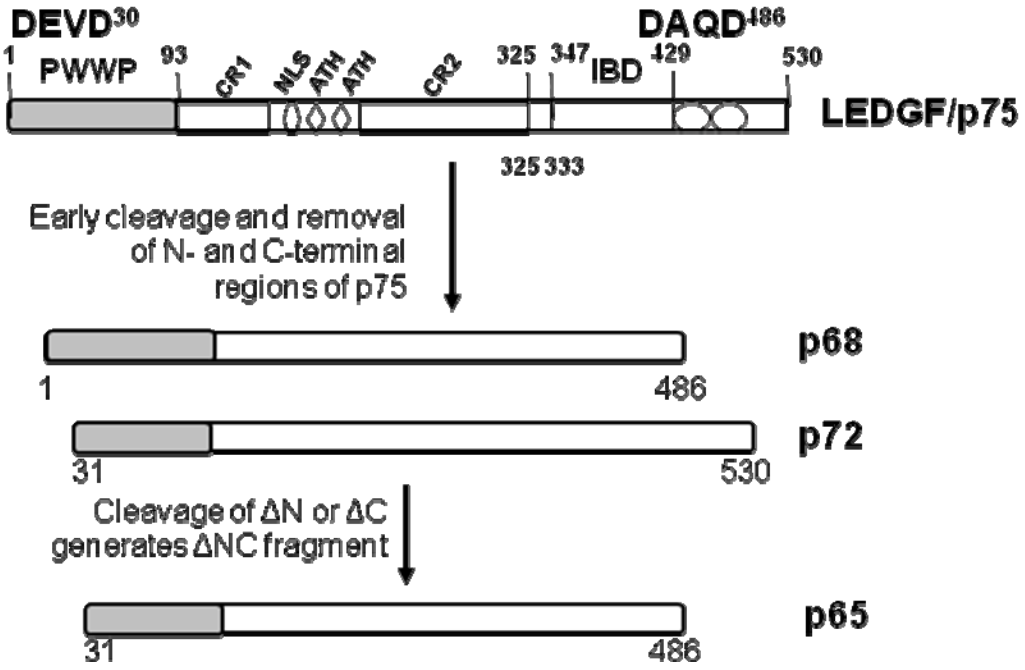


Figure 15. Transactivation of Hsp27pr by LEDGF/p75 constructs. During apoptosis, LEDGF/p75 is cleaved by caspases-3 at its N-terminal and C-terminal region.

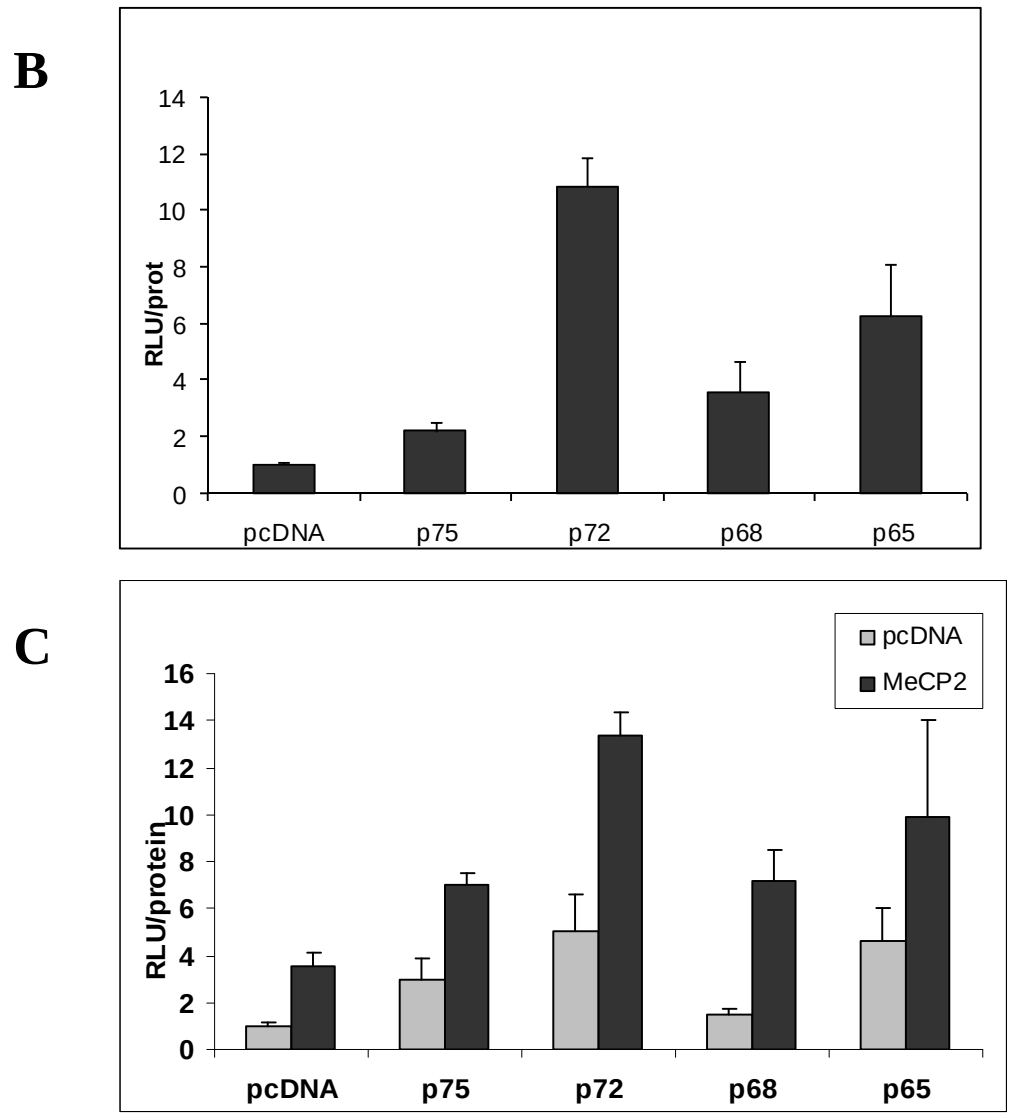


Figure 15. Transactivation of Hsp27pr by LEDGF/p75 constructs. **B)** Transient transfection of pCruzHA-p75, pCruzHA-p72, pCruzHA-68, or pCruzHA-p65 with pGL3-Hsp27pr-Luc in U2OS in U2OS cells. Significant increase of Hsp27pr activity was observed with p72 and p65 overexpression. Promoter activity determined as luciferase light units/protein is expressed as fold activation compared to control activity (co-transfection of empty expression vectors). **C)** Co-expression of pcDNA-MeCP2 with LEDGF/p75 constructs mentioned in A with pGL3-Hsp27pr-Luc in U2OS. Results are representative of three experiments.

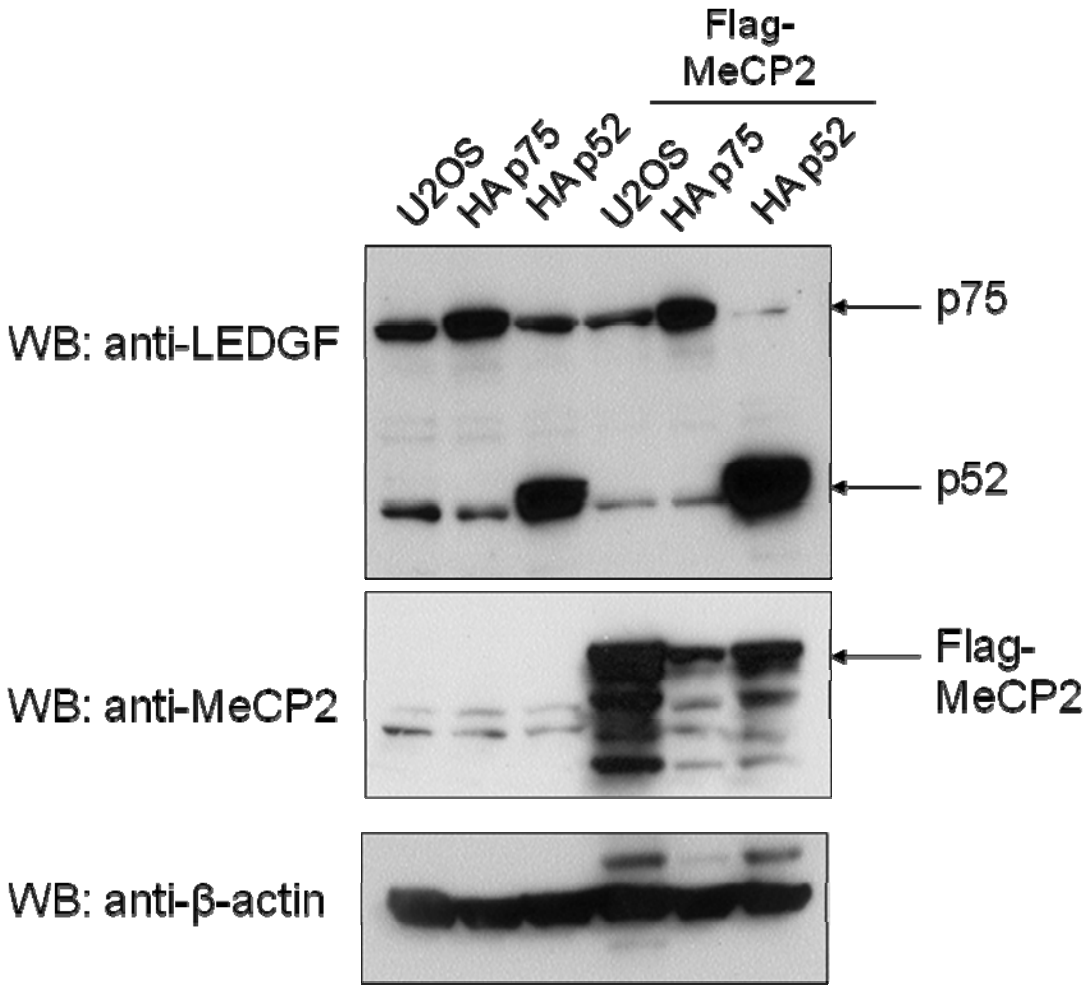


Figure 16. Regulation of LEDGF/p75 by MeCP2. Western blot shows protein expression levels of LEDGF/p75, p52 and MeCP2. Co-expression of LEDGF/p75 with Flag-MeCP2 (lane 5) shows decreased levels of MeCP2 compared to Flag-MeCP2 overexpression alone (lane 4).

Interaction of Menin with MeCP2

Menin/MLL was reported to interact with LEDGF/p75, which facilitates its integration to chromatin and allows transcription and leukemic transformation [9]. To examine if menin interacts with LEDGF/p75, pull down assays were performed using GST-LEDGF/p75 with U2OS or PC3 cell lysate. Western blots show LEDGF/p75 binding to menin (Fig 17A, B). To examine if menin also interacts with MeCP2, U2OS lysate was incubated with recombinant GST MeCP2. Pull down assays showed binding of MeCP2 to menin (Fig 17C). This suggests that LEDGF/p75 may interact in a complex with MeCP2 and menin.

Role of Overexpressed LEDGF/p75 in Protection Against Oxidative Stress-induced Cell Death

LEDGF/p75 Overexpression Protects Cells from TBHP Treatment

We have shown that LEDGF/p75 protects cells from serum deprivation [7] and DTX-induced cell death [1]. LEDGF/p75 also protects RWPE-2 (transformed prostate) cells from TBHP-induced non-apoptotic cell death (unpublished data). To examine if this protection occurs in PC3 cells, we generated PC3 cells stably overexpressing LEDGF/p75. The cells were then treated with increasing doses of TBHP for 6, 12, and 24h. Cell survival was analyzed using crystal violet assay (Fig 18A). Pictures of cellular and nuclear morphology show protection of LEDGF/p75 against TBHP treatment (Fig 18B). To understand how LEDGF/p75 protects against TBHP-induced cell death, we examined ROS levels measured using DCFH-DA staining via flow cytometry. LEDGF/p75 overexpression reduced ROS generated by TBHP, STS and TRAIL (Fig 18C).

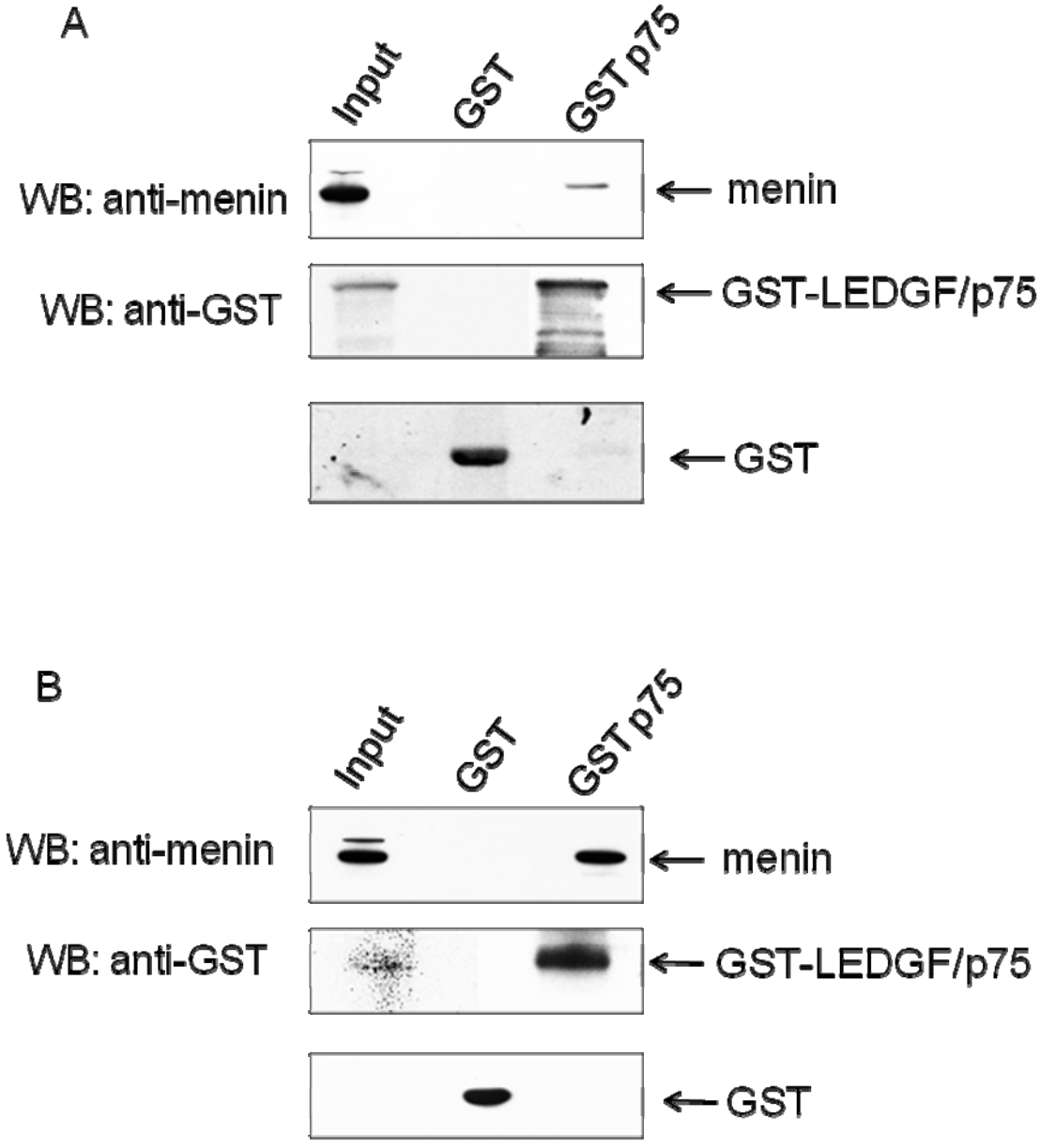


Figure 17. Interaction of Menin with LEDGF/p75. Pull down assays of GST-LEDGF/p75 and menin. GST-MeCP2 or GST beads were incubated with A) PC3 or B) U2OS lysates. Samples brought down by glutathione beads were analyzed by western blot. Left panel shows immunoblot of input proteins. Pull down Assays were performed by Leslimar Rios-Colon, graduate student in our laboratory.

C

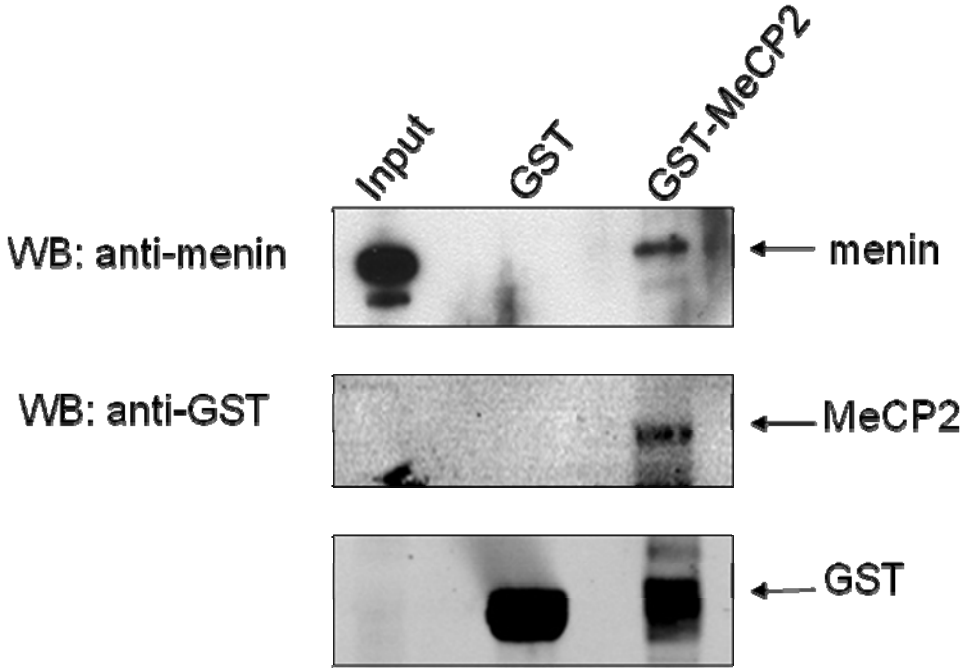


Figure 17. Interaction of Menin with MeCP2 in U2OS cells. C) Pull down assays of GST-MeCP2 and menin. GST-MeCP2 or GST beads were incubated with U2OS lysates. Samples brought down by glutathione beads were analyzed by western blot. Left panel shows immunoblot of input proteins.

A

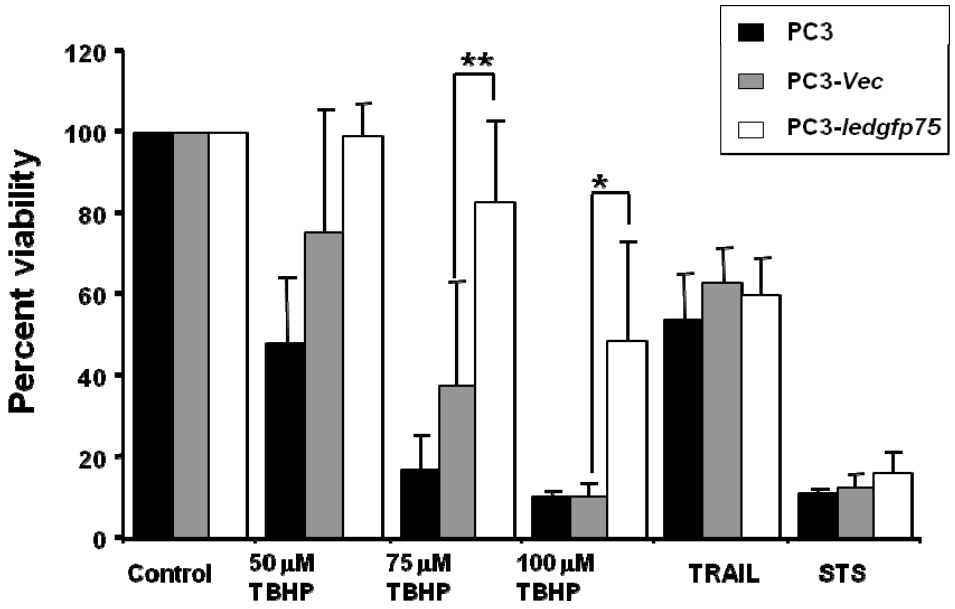


Figure 18. LEDGF/p75 overexpression protects against TBHP treatment. **A)** Percentage of surviving PC3 cells treated with 50, 75 or 150 µM TBHP, 100 ng/mL TRAIL/Act.D or 4 µM STS. Cell viability was determined by crystal violet staining. Absorbance was measured at 570 nm and the values were normalized against those of untreated cells, which were assumed to be 100% viable. Errors bars represent the standard deviation of at least three independent experiments done in triplicate. * $p<0.05$; ** $p< 0.01$

B

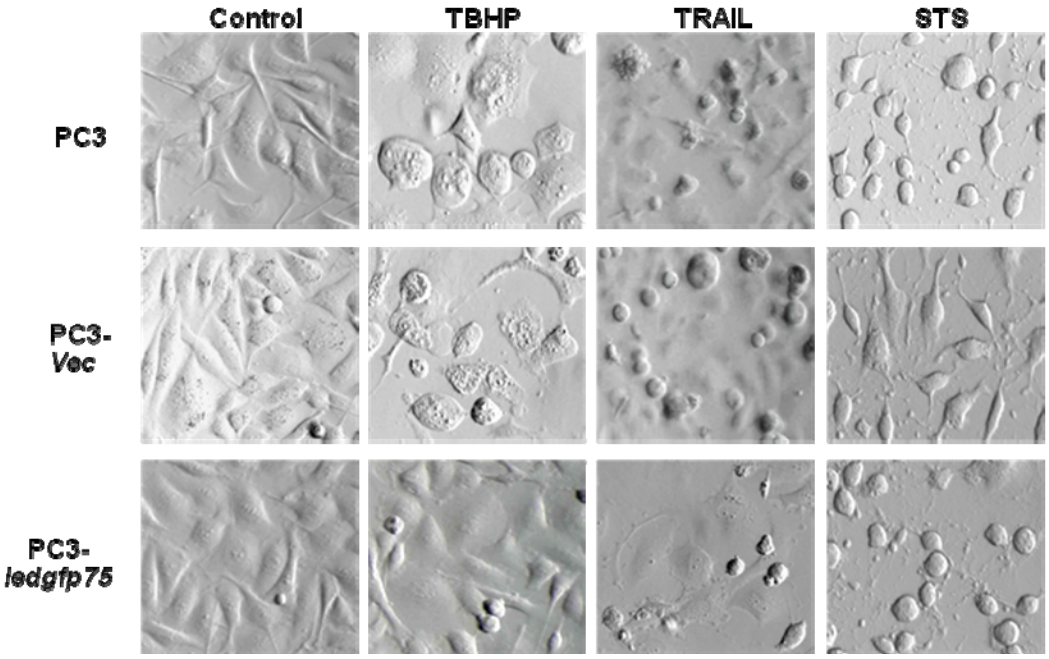


Figure 18. LEDGF/p75 overexpression protects against TBHP treatment. **B)** Cellular morphology of PC3 cells treated with TBHP, TRAIL/Act.D and STS as in **A)** for 12 h. Cell morphology visualized by Hoffman modulation microscopy.

C

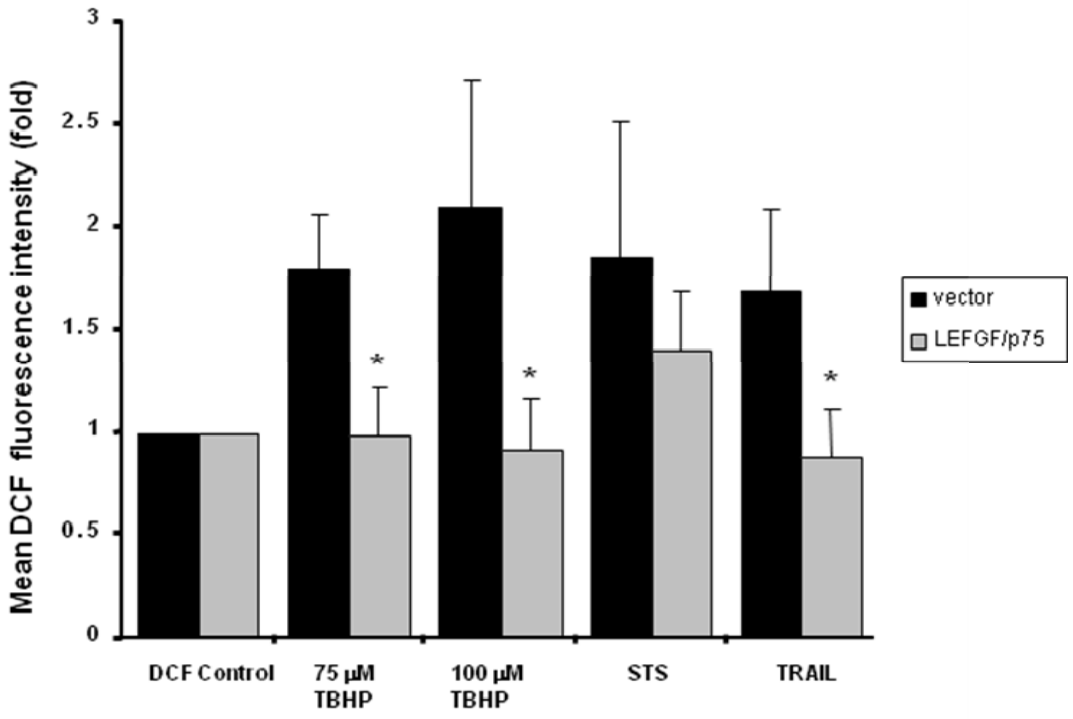


Figure 18. LEDGF/p75 overexpression protects against TBHP treatment. **C)** LEDGF/p75 overexpression reduces ROS levels. PC3 cells stably overexpressing LEDGF/p75 were treated with 75, 100 µM TBHP, 100 ng/mL TRAIL/Act.D or 4 µM STS for 6 h. Cells were incubated with DCFH-DA dye and analyzed via flow cytometry. Results show reduction of ROS in the cells. Results are representative of three experiments. * $p<0.5$ compared to vector control.

Discussion

The binding of MeCP2 to the N-terminal region of LEDGF/p75, PWWP CR1, is likely a weak interaction which is probably strengthened by the additional region (CR2) present in p52. Both PWWP and ΔPWWP constructs partially co-localize with MeCP2 in the cell nucleus.

LEDGF/p75 and MeCP2 upregulate Hsp27pr in PC3 cells in a manner similar to that observed in U2OS cells, suggesting that this interaction occur in PCa. Interestingly, co-expression of LEDGF/p75 and MeCP2 led to synergistic transcriptional activity of Hsp27pr in PC3 cells not observed in U2OS cells (Chapter two), suggesting a cell type specific regulation. As an example of a precedent for this phenomenon, HoxA10 expression was reported to increase activity of IGFBP1pr in endometrial stromal and glandular cells but not in decidual cells [10]. In addition, SOX proteins Likewise, ectopic expression of MeCP2 also resulted in a higher increase of ERp57pr activation compared to overexpression of LEDGF/p75. However, ERp57pr activity remained upregulated when LEDGF/p75 levels were repressed, suggesting that MeCP2 could be the main transactivator of ERp57pr. In this situation, it could bind a different response site that is not blocked by LEDGF/p75. Further experiments should examine MeCP2's role in ERp57pr transactivation by repressing its expression. Similar to Hsp27pr and ERp57pr, IGFBP5pr activity was highly upregulated by MeCP2. The knockdown of LEDGF/p75 was shown to downregulate IGFBP5pr activity. Future experiments should examine modulation of IGFBP5pr in the presence of both LEDGF/p75 and MeCP2. Our results suggest that LEDGF-MeCP2 interacts with various stress proteins in a different manner, and that their transactivation function may involve other cofactors.

Caspase-mediated cleavage of intracellular proteins occurs commonly in apoptosis. Many transcription factors, regulators of cell growth, proliferation and apoptosis are cleaved by caspase-3, converting them into functionally inactive fragments or fragments exhibiting dominant-interfering functions that amplify the cell death process [11]. For example, cleavage of Forkhead transcription factor FOXO3a results in fragments with reduced transcriptional activity [12]. However, removal of LEDGF/p75 N-terminal 30aa increased transactivation of Hsp27pr, suggesting a repressive function of this region. On the other hand, further removal of C-terminus region resulted in a slight decrease in elevated transactivation, suggesting its involvement in transactivation. The addition of MeCP2 greatly enhanced this transactivation, suggesting that it plays a major role in upregulation of Hsp27pr. However, correlation between pro-survival function and Hsp27pr transactivation remains unknown.

LEDGF/p75 overexpression protects PC3 cells from TBHP treatment but not from the classical apoptosis inducers TRAIL and STS. However, this overexpression reduced ROS levels generated by all three treatments. Additional experiments are needed to examine if this effect is general or chemospecific. Further experiments should include examining pathways upstream of ROS generation. Since LEDGF/p75 protects cells from oxidative stress-induced cell death, further experiments should examine if overexpression of MeCP2 contributes to this protection against TBHP and DTX, or if the protection is conferred through LEDGF's interaction with other proteins. The decreased expression of MeCP2 in the presence of LEDGF/p75 suggests negative regulation or presence of a feedback loop. We cannot rule out the possibility that protein expression was regulated by the cells to avoid toxicity as a result of protein saturation. The interaction of menin

with MeCP2 suggests its possible involvement in transactivation of stress proteins. Examining the menin-LEDGF-MeCP2 complex will lead to deeper understanding of the interactome that controls transcription and allow for multiple target points in therapy.

Regulation of transcription involves multiple players and variations in different contexts. These studies show that the interaction between LEDGF and MeCP2 and their modulation of stress protein transactivation requires further investigation.

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CHAPTER FOUR

OVERALL DISCUSSION

Interaction of LEDGF/p75 and MeCP2

LEDGF/p75 shares the following similarities with high mobility group (HMG) family members: abundant charged and proline residues; presence of AT-hook motifs; and little secondary structure [1]. Based on these similarities, it is speculated that they might share similar functions, namely in multiple interactions with proteins and DNA/chromatin. In Sry-related HMG box (SOX) proteins, the HMG domain doubles as both the DNA-binding moiety and the major interface for protein–protein interaction, in agreement with previous reports that HMG domain interacts with other transcriptional co-regulators and in nuclear import [2]. Further examinations revealed that the NLS and AT-hooks are the main players in LEDGF/p75 chromatin binding, with PWWP playing a supporting role [3] and available for protein-protein interactions.

LEDGF/p75 gene expression is upregulated in PCa cell lines and tissue [4] . Its protein expression is upregulated in response to heat and oxidative stress, and promotes resistance of mammalian cells to stress induced cell death [5-9]. In addition, overexpression of LEDGF/p75 conferred a protective effect against DTX- and TBHP-induced cell death [10] (Chapter Three). It is presumed that the stress survival functions of LEDGF/p75 are associated with its ability to transcriptionally activate stress genes via interaction with heat shock elements (HSE) and stress response elements (STRE) in promoter regions of stress proteins such as antioxidant protein 2 (AOP2/Prdx6),

involucrin, alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), Hsp27, and αB-crystallin [8, 11-14]. Since the mechanisms by which it confers resistance to oxidative stress and chemotherapeutic agents are not well understood, we examined its interaction with other transcription factors.

MeCP2 was initially discovered to bind methylated CpG islands and repress transcription [15]. It also protects mice from N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)-induced excitotoxicity and hypoxic-ischemic insult, possibly through repressing caspase-3 activation [16]. Binding of LEDGF/p75 to MeCP2 might be part of a transcription complex that serves to protect cells from stress-induced cell death by regulating the expression of stress protective genes. LEDGF/p75 was shown to bind MeCP2 through its N-terminal regions (Chapter two). This interaction was mapped to PWWP CR1, and retained the protein's chromatin association. The interaction between LEDGF/p52 and MeCP2 was stronger compared to that with PWWP CR1, possibly due to the additional CR2 region present in LEDGF/p52, which may stabilize interaction with MeCP2 by having additional interaction points. Given the lack of secondary structure of both LEDGF/p75 and MeCP2, the binding of LEDGF/p75 to MeCP2 may cause conformational changes that allow it to bind transcription sites, thus modulating their functions and interactions with other proteins.

Functional Implications of LEDGF/p75 and MeCP2 Interaction

As mentioned previously, LEDGF/p75 is a survival protein and transcriptional co-activator upregulated in PCa cell lines and tissues [4]. Early studies on LEDGF/p75

uncovered its pro-survival properties in lens epithelial cells (LECs) [14], where overexpression of LEDGF/p75 was shown to protect against thermal and oxidative stress [14]. LEDGF/p75 overexpressing cells showed upregulation of Hsp27 and αB-crystallin [14], enhanced growth rate [17], and prolonged cell survival in the absence of serum [17]. Since LEDGF interacts with MeCP2, we examined the functional impact of this interaction on transcriptional activity.

Transactivation Functions of LEDGF-MeCP2

Binding of MeCP2 to LEDGF/p75 could lead to recruitment of transcription activator proteins such as CREB1, which subsequently activates genes that confer resistance to oxidative stress-induced cell death in PCa cells. In light of our data, which shows MeCP2 as the main transactivator of Hsp27pr (Chapter Two), we examined if this effect is also observed with thiol-disulfide oxidoreductase ERp57pr and tumor suppressor IGFBP5pr. Our results (Chapter Three) suggested that MeCP2 is a main transactivator of these proteins, compared to LEDGF/p75. Overexpression of MeCP2 greatly transactivates Hsp27pr, surpassing the level of transactivation induced by LEDGF/p75 or p52 up to three fold. Knockdown of endogenous LEDGF/p75 expression further increased Hsp27 promoter activity (up to four fold) in the presence of ectopic MeCP2 in U2OS cells, compared to siRNA control knockdown cells, suggesting that MeCP2 is a strong modulator of Hsp27pr activity. LEDGF/p75 seems to repress transactivation of Hsp27pr activity by MeCP2 in U2OS, suggesting that it plays a regulatory role. It is not clear if this phenomenon is promoter and cell type specific.

Generally, transcription coactivators interact with DNA–binding transcription factors to confer synergistic activation of gene expression [18]. This seems to occur with Hsp27pr and ERp57pr in PC3 cells overexpressing both LEDGF/p75 and MeCP2 (Chapter Three). In this context, MeCP2 may associate with CREB1 to facilitate transcription. On the other hand, LEDGF/p75 may interfere with MeCP2 transactivation in U2OS cells by blocking it from binding to Hsp27pr, or by blocking its activation site. Alternatively, up-regulation of Hsp27pr might be dependent on a different combination or order of assembly of co-factors to its promoter site, as in the case of MTF-1 activator [19]. Furthermore, it is possible that LEDGF/p75-mediated repression of MeCP2 activity could be modulated by another transcription factor. We cannot exclude the possibility that LEDGF/p75-MeCP2 may exist in a feedback loop, with high levels of MeCP2 stimulating repression by LEDGF/p75, as seen in the feedback loop observed between heat shock transcriptional factor 1 (HSF1) and heat shock proteins [20, 21]. LEDGF may regulate persistent, increased activity of MeCP2 to avoid induction of cell death as in the case of Stress-activated protein kinase (SAPK) [22]. Further experiments should examine MeCP2 transactivation function in the presence and absence of LEDGF/p75 and other co-factors.

Novel Binding of LEDGF/p75 and MeCP2 to Hsp27pr

LEDGF/p75 has been shown to bind stress response elements and heat shock elements in Hsp27 promoter region in electrophoretic mobility shift assay (EMSA) assays using both purified protein and HeLa nuclear extracts [23]. However, this binding could not be confirmed by other investigators who suggested it was non-specific

[3](unpublished data). A recent report showed LEDGF/p75 binding mainly correlating with active chromatin markers and RNA polymerase II binding and not restricted to STRE [24]. LEDGF/p75 was reported to bind VEGF-c promoter in H1299 cells (in ChIP assays) [25] and upregulate its activity, similar to transactivation of the Hsp27 promoter. VEGF-c promoter activation was not affected when one STRE site on the promoter was mutated, but decreased when both STRE sites were mutated. This agrees with our data that LEDGF/p75 binding to promoter sites may not strictly depend on one HSE or STRE region, but may depend on interaction with other regions and factors (Chapter Two).

The role of LEDGF/p75 in tethering proteins to chromatin was reported to resemble a dynamic scan-and-lock mechanism [26]. Non-specific chromatin scanning/hopping is a common phenomenon of transcription factors. This likely facilitates targeting of LEDGF binding proteins to random active transcription regions. Probable existence of low-concentration higher-affinity chromatin-bound states were also observed, which might result from association with stress-responsive genes promoters, or from association downstream of transcription start sites of active transcription units [24].

A recent report shows that LEDGF/p75 guides its binding partners to active transcription sites through recognition of negative supercoils generated around it [27]. This chromatin binding is facilitated by the novel supercoiled DNA-recognition domain (SRD), designating a new role for the N-terminal CR2 region (res 200-336). LEDGF/p75 charged regions CR1 and CR2 were previously suggested to be involved in electrostatic interaction with DNA and chromatin [28]. Expression of LEDGF/p75 fused to *E. coli* Dam methylase in HeLa cells showed a typical distribution pattern in the nucleus, overlapping mostly with condensed chromatin regions associated with low transcriptional

level [24]. However, determination of DNA sites bound by the protein, as mapped in the ENCODE region by the DamID technique, revealed that its most frequent sites are transcription units of active genes [24]. The LEDGF/p75-bound chromatin islands also correlated well with the HIV-1 integration sites. Likewise, MeCP2 has been recently reported to associate with active promoter sites [29]. It is possible that only a small fraction of LEDGF is targeted to transcription active chromatin sites and the protein's ability to recognize superhelical structure would be a driving force for chromatin targeting.

In our studies, we showed for the first time the binding of MeCP2 to Hsp27pr region (Chapter Two) upstream of HSE and STRE sites. Yasui et al. reported that the majority of MeCP2 binding sites are intergenic or intronic, outside of transcription units and CpG islands, [30]. MeCP2 was recently reported to bind DNA regardless of methylation status and does not function primarily in silencing methylated promoters [30], but also activates transcription through association with other proteins such as CREB1 [29]. In addition, DNA binding of MeCP2 is enhanced by hydration near a run of four or more A/T [31]. Even though MeCP2 binding sites have also been shown to be distant from genes, commonly over 10kb away; our data shows binding of LEDGF and MeCP2 on the promoter of Hsp27 in close proximity, within 1kb. In order to resolve this, we examined Hsp27pr region and identified potential MeCP2 binding sites (Appendix I). In agreement with our data, sequences containing four or more A/T correlated with MeCP2 location as seen via ChIP assay. We cannot exclude the possibility that transient interactions might not be captured using formaldehyde crosslinking in ChIP assays, and only stable MeCP2-DNA interactions are visualized [32].

Possible Mode of Interactions between LEDGF and MeCP2

Given that LEDGF binds MeCP2 on its N-terminal region, as opposed to C-terminal binding of HIV-IN and menin/MLL complex, its role here might differ from its typical cargo-chromatin tethering function. LEDGF/p75 may bind chromatin through interaction with MeCP2, which preferentially recognize hydrated AT runs [31]. Alternatively, as LEDGF/p75 was observed to facilitate HIV integration in AT rich regions [33], both proteins might compete for DNA binding at AT rich regions and regulate each other's function. It is also possible that LEDGF/p75 binding to chromatin blocks activation site of MeCP2 and disrupts its association with transcription co-activators, consequently repressing transcription (Fig 19).

Alternatively, the many response element binding sites on Hsp27pr suggest the participation of multiple co-factors in transcription regulation. This interaction could vary according to promoter context, explaining the different consequences of LEDGF-MeCP2 interaction on the three promoters we examined [34]. For example, SOX proteins form stable transcription factor complexes with a variety of co-regulators to activate or repress gene transcription [35]. They could possibly simultaneously recruit more than one transcription factor to regulate gene expression from a single promoter or enhancer [36]. Similar to our observations with U2OS and PC3, transcription regulation of SOX proteins are also cell-type dependent [35]. Moreover, dynamic pattern of tissue-specific gene expression coupled with partner protein availability and selectivity, are mechanisms that underlie specific changes in gene expression associated with cell death decisions [37]. Selective DNA binding of promoters activated by sox proteins and their co-factors is enhancer context-dependent.

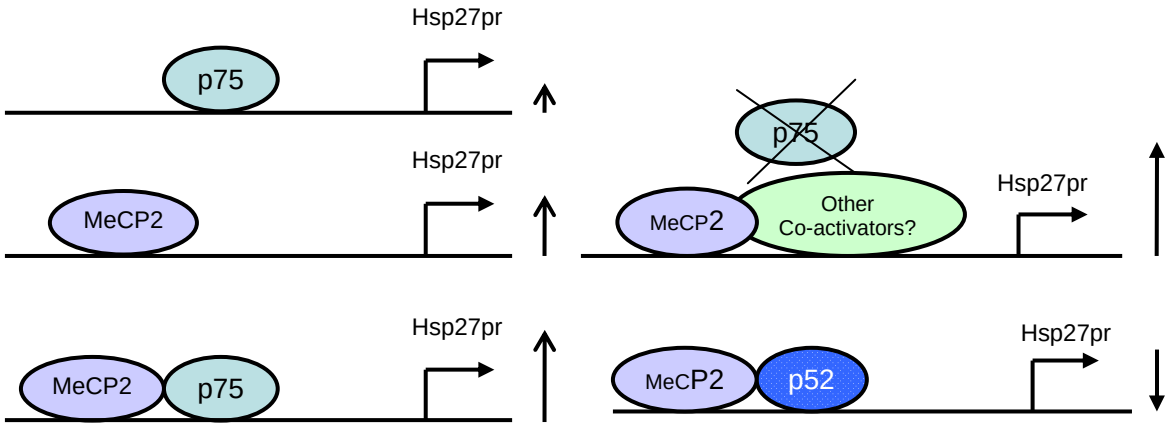

Figure 19. Proposed model of Hsp27 promoter transactivation. We propose that LEDGF/p75-MeCP2-mediated transactivation of Hsp27pr is dependent on intracellular protein levels and cellular context. In the presence of LEDGF/p75, MeCP2 is prevented from interacting with co-activators required for transactivation of Hsp27pr. In the absence of LEDGF, MeCP2 binds to co-activators such as menin and up-regulates Hsp27pr activity. Binding of p52 to MeCP2 seems to repress Hsp27pr activation, possibly due to interaction with a different set of proteins that potentially induces apoptosis.

Another example is Cyclic AMP-dependent transcription factor 3 (ATF3), whose role in transcriptional regulation is decided by the presence or absence of other ATF/CREB family members [38]. ATF3 is also involved in oncogenesis by increasing proliferation in DU145 PCa cells [39] and promoting motility and invasiveness of PC3 metastatic derivative MM cells [40]. Conversely, its overexpression results in increased apoptosis of PC3 cells [41]. Furthermore, the transactivation properties of LEDGF/p75 may depend on its ability to form homodimers or heterodimers with other transcription co-factors, similar to its interaction with tetramers of HIV IN [42]. LEDGF/p52 , which antagonizes LEDGF/p75 and has pro-apoptotic functions [43] may compete with LEDGF/p75 in binding to MeCP2 and Hsp27pr. Following this assumption, the C-terminal domain lacking p52 may not confer survival because of its inability to interact with survival proteins. We cannot exclude the possibility that these proteins might bind to each other and stress promoter regions only during stress conditions.

Role of the PWWP Domain in Chromatin and Protein Binding

The PWWP domain is a member of the Tudor "Royal Family" implicated in chromatin remodeling during DNA repair, replication, transcription and recombination [44]. This domain is highly conserved in proteins with diverse functions, including the hepatoma-derived growth factor (HDGF) family, DNA repair and methylation proteins, transcription factors, and chromatin-associated proteins. DNA methyltransferase proteins (Dnmt) associates with chromatin via PWWP domains to establish genomic DNA methylation patterns during development [45]. The Nuclear magnetic resonance (NMR) solution structure of the PWWP domain of HDGF family shows a five-stranded

antiparallel β-barrel with a solvent-exposed hydrophobic cavity suggested to bind chromatin. This chromatin binding property is supported by its electrostatic charge distribution, which surrounds the binding cavity [46]. Recently, the PWWP domain has been shown to be crucial for locking LEDGF/p75 and its binding protein on chromatin [26]. On the other hand, the β-sheets of the bHLH-PAS family (Per-Arnt/AhR-Sim basic helix-loop-helix), which has a structure similar to that of the PWWP domain, have been implicated in inter- or intra-protein interactions [47]. In HDGF protein, nuclear localization signals downstream of its PWWP domain were required for its stimulation of DNA replication [48, 49]. HDGF was also showed to repress SMYD1 (SET and MYND domain containing 1) gene expression through its binding to C-terminal binding protein [50]. Likewise, the similar structure of LEDGF PWWP suggests its involvement in chromatin and protein binding.

PWWP Domain Represses Transcription

As mentioned before, caspase-mediated cleavage of proteins convert them into functionally inactive fragments or fragments exhibiting dominant-interfering functions that amplify the cell death process [51]. Cleavage may impair post translational modifications necessary for transcriptional activity. For example, caspases-mediated cleavage of NF-κB p65 (RelA) subunit produces a dominant-negative fragment that is capable of binding DNA but has no transactivating potential [52]. The cleavage of N-terminal region of LEDGF/p75 resulted in substantial up-regulation of Hsp27pr (Chapter Three); however, the cleavage of both N- and C-terminal of LEDGF/p75 appears to result in a fragment with reduced transactivation function. This suggests that cleavage of

LEDGF/p75 at residue 30 abolished the repressive function of N-terminal region. At the same time, this suggests that the C-terminal region is implicated in transactivation function. This concurs with previous report, which suggests that N-terminal domain (res 5-62) exhibits auto-transcriptional repression activity and is involved in stabilizing the LEDGF-DNA binding complex [23].

The PWWP domain has been shown to be involved in recognition of methylated histones as in the case of Pdp1 (*Schizosaccharomyces pombe* protein PWWP domain protein 1), human BRPF1 (bromo and plant homeodomain finger-containing protein 1) and murine Dnmt3a DNA methyltransferase [53-55]. This suggests PWWP domain targets either transcriptionally active (H3K36me3) or inactive (H4K20me) signatures, probably depending on the structural difference among each protein. It is unknown whether the PWWP domain recognizes methylated histones or other unknown modifications enriched in heterochromatic regions. Other chromatin factors such as MeCP2 might be needed for its chromatin interaction.

Post-translational Modifications and Transcriptional Regulation

Both LEDGF/p75 and MeCP2 are predicted to migrate around 60 kDa. However, immunoblots show LEDGF/p75 migrating around 75 kDa, correlating with migration of ectopic expression of Flag-tagged MeCP2. Since both proteins are relatively unstructured [1, 56] , this suggests the involvement of post-translational modifications. MeCP2 has been associated with binding methylated CpG islands, and represses transcription through interaction with histone modifying enzymes such as histone deacetylases (HDACs) and its co-repressor mSin3A, or through chromatin condensation [57]. However, the role of

LEDGF in methylation remains unknown. Phosphorylation of MeCP2 was suggested to regulate its intracellular localization during neuronal cell differentiation, and play a crucial role in its transcription function [58, 59]. This suggest that phosphorylation of MeCP2 and LEDGF/p75 could potentially modify their transcription of Hsp27. Predicted post-translational modification sites of LEDGF/p75 were analyzed using the Accelrys Omiga 2.0 program (Appendix II) [1]. However, functional consequences of LEDGF/p75 phosphorylation remain to be determined. Further analysis should include phosphorylation and its effect on the interaction of these proteins with Hsp27pr.

SUMOylation of LEDGF and MeCP2

Bueno et al. recently reported that mutations impairing SUMOylation (Small Ubiquitin-like Modifier) of LEDGF/p75 increases its transcriptional activity, but not that of p52, suggesting that different molecular mechanisms might be used to activate Hsp27 promoter [60]. SUMOylation of HDGF was reported to abolish its binding to chromatin [61]. SUMOylation of Dnmt3a disrupts its ability to interact with histone deacetylases (HDAC1/2), but not with another interaction partner, Dnmt3b [62]. We cannot exclude the possibility that SUMOylation of a different position could either inhibit or promote protein-protein interactions. In addition, SUMO-1 modification modulating the biological effects of HDAC1 by potentiating its histone deacetylase activity [63], while SUMOylation of HDAC4 is needed for its transcription repression and histone deacetylase activity [64]. Sumoylation of coREST (corepressor of RE1 silencing transcription factor) contributed to its transcriptional repression [65] and might be involved in regulating histone methylation [66] and Brain-derived neurotrophic factor

(BDNF) levels in MeCP2 deficient brain [67]. Further experiments should explore the transactivation functions of LEDGF/p75 SUMOylated mutants.

Immunoblotting analysis showed SUMOylation of MeCP2 in neurons, which likely permits its interaction with DNA-methylation-based gene regulators such as Dnmt3 and HDACs [59]. However, the location of this modification and its function was not identified. We analyzed potential SUMOylation sites of MeCP2 using a SUMO prediction software, SUMOsp 2.0. Consensus and non-consensus motifs with high probability of SUMO modification were located at its N and C terminus (Appendix III). Analysis of MeCP2 SUMOylation could shed light to its transactivation function and its interaction with LEDGF/p75 and p52.

Other Implications of LEDGF-MeCP2 Interaction

LEDGF and MeCP2 share similarities through their binding to other proteins with similar functions, suggesting that their interaction in a complex may extend to other contexts. We will discuss some that are closely linked below.

mRNA Splicing

Besides conferring a survival advantage to tumor cells, it is possible that the LEDGF-MeCP2 interaction could modulate RNA splicing activity. MeCP2 is involved in RNA splicing regulation through its association with RNA-binding protein Y box-binding protein 1 [68]. LEDGF/p52 have been reported to interact with the essential splicing factor ASF/SF2 to modulate ASF/SF2-mediated pre-mRNA splicing [69]. Both proteins show localization in the nucleus, exhibiting dense fine speckles pattern, similar

to co-localization pattern of cellular pre-mRNA splicing factors with the RNA polymerase II transcription, supporting the close link between transcription and pre-mRNA splicing [70-74].

Regulation of Olfactory Receptors

Studies of nasal epithelium from patients with Rett syndrome show that the maturation of olfactory receptor neurons is impeded prior to the time of synapse formation [75]. Likewise, we have observed down-regulation of several olfactory receptor genes in Affymetrix global microarray analysis using mRNA from cells transiently depleted of LEDGF/p75 (Basu, unpublished data). Homozygous *LEDGF* mutant mice in C57BL/6 background resulted in perinatal lethality [76], most possibly because of impeded development of olfactory receptor, which resulted in failure to nurse. These mice also exhibited craniofacial and skeletal abnormalities similar to those seen in mice with mutated Hoxa genes, suggesting the link between LEDGF and Hox gene. This was confirmed by the association of LEDGF to Hoxa9 expression through menin/MLL complex [77], suggesting the existence of a complementing interacting complex consisting of LEDGF/p75, MeCP2 and menin/MLL complex. It will be of interest to explore whether this complex functions in the regulation of olfactory receptors, some of which have been recently implicated in signal transduction in PCa cells [78-80].

Interaction with Menin

LEDGF/p75 was reported to transport Menin/MLL complex to the nucleus and bind chromatin [77]. To examine if menin exists in a complex with LEDGF and MeCP2,

we performed pull down assays and showed binding of menin to MeCP2 (Chapter three). Like LEDGF/p75, menin is also required for HIV-1 Tat transactivation [81] through its association with the Ski-interacting protein SKIP. MeCP2 has been reported to bind co-repressor c-Ski, which is required for its transcriptional repression [82]. It will be interesting to examine if MeCP2 modulates HIV-1 transactivation. In addition, menin was shown to bind estrogen receptor, and enhances its activity in breast cancer cells, subsequently conferring resistance to tamoxifen [83]. It will be of interest to examine the role of menin in Hsp27pr transactivation and resistance to stress-induced cell death.

Conclusion and Final Perspectives

The studies presented here show that LEDGF binds to MeCP2 through its N-terminal domain. This interaction also resulted in modulation of LEDGF-MeCP2 transactivation function. In U2OS cells, MeCP2 appears to be a more potent activator of Hsp27pr than LEDGF/p75, which appears to regulate MeCP2-mediated transactivation of Hsp27pr. On the other hand, preliminary data shows that LEDGF/p75, in conjunction with MeCP2, activate ERp57pr and IGFBP5pr in PC3 cells. Therefore, the effects of LEDGF/p75 on MeCP2-mediated transactivation might be dependent on other co-activators and repressors, and varies with different promoters and cell types. Further studies on the effect of LEDGF/p75 and MeCP2 on their transactivation function are necessary for a better understanding of their mechanism.

Even though ChIP binding assays shows binding of protein complexes to DNA, it does not reveal the exact binding location for each protein. LEDGF/p75 might bind directly onto DNA response elements on Hsp27pr, or it might bind to other proteins

associated with the DNA. The same can apply to MeCP2. Alternatively, the presence of LEDGF/p75 or MeCP2 could strengthen the interaction between the transcription factors and co-factors, allowing or disrupting their association with chromatin restructuring proteins. Other experiments such as EMSA should be performed to identify direct binding of these proteins to Hsp27pr. We cannot exclude the possibility that many transcription co-factors are involved in Hsp27pr activation, and that a specific combination of factors are required for transcription initiation, depending on cell type or environmental stressors.

The mechanism by which LEDGF/p75 interacts with MeCP2 to modulate transactivation requires further investigation. LEDGF/p75 may serve to tether MeCP2 to the chromatin, as it does with HIV-1 IN and the Menin/MLL complex. Alternatively, these proteins might compete for DNA binding and regulate each other's transcription function. Furthermore, these interactions could have different consequences on different promoters, cell type and stress stimulators. Since HSF1 upregulates Hsp70 and Hsp27 expression, it is important to identify the interplay between this proteins and the LEDGF/p75-MeCP2 complex. Future experiments should include examining how LEDGF/p75 and MeCP2 fit into HSF1-mediated stimulation of Hsp27 and Hsp70 and their negative feedback control. Requirement of additional factors might be needed to determine promoter activation or repression. Alternatively, binding of splice variants or cleaved fragments with antagonistic functions might result in the different outcomes. The presence of menin in relation to LEDGF/p75-MeCP2 complex and its role in transactivation function modulation in tumor cells also deserves further investigation.

Overall, these studies provide us with clues to understanding the interaction of transcription complexes involving LEDGF/p75 and their influence on the activation of stress genes that protect cancer cells from stress-induced cell death. It also emphasizes the complexity of transcription modulation and the need for extensive studies in developing novel therapeutic strategies aimed at targeting the transcription proteins that regulate stress survival pathways and chemoresistance in PCa.

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APPENDIX A

POSSIBLE MECP2 BINDING REGIONS ON HSP27 PROMOTER

Modified from Oesterreich, 1996

CAT-Box
cggctcactgcaacctctgccttctgggttcaag**caatt**ctcctgcctcagcctccccagcagctgcg
SP1
aatacaggcg**cccgcc**accacacccagc**taatttttg**t**attttta**gtagagatggggtttcaccatgttg
ERE ERE
gccaggct**ggtct**caaactcc**tgacc**tctggtgatcctcccacctcggtctcccaaagtgctgggattaca

ggcgtgagccaccacgcccagcccagactgcc**ttatttttg**t**atttg**t**atttatt**c**atttac**t**tatttt**gagacag

ggttttgctctgtagcccaggctgaagtgcagtggtgcaatccagctcaccacagcctctactcaccggg

gttcaaaggatcctcctgcttcagcctctggagtagctggggccacaggcatgcaccaccatgcccagct

aatttttaaatattttttggtagaagtagggtctcactatgttgcccagactggtctcaaactcctagcctcaag

ggacccttctgccttggcctcccaaagtgctgagattacaggcatgagccatgcacccagcccc**ttttaa**

aattttttttgagagacaagactttgatctgttgcctaggctggagtgcagtggtgagatcatagctcactgca

gcctcaactcctgggctcaagcaccagactccttttatcacattctatctcacacgcgtgtggttccaatcct

gcctctgccacttctcagttgtatgccccaacccaacctgtctggctctgtcctccttaacagaaggacggc

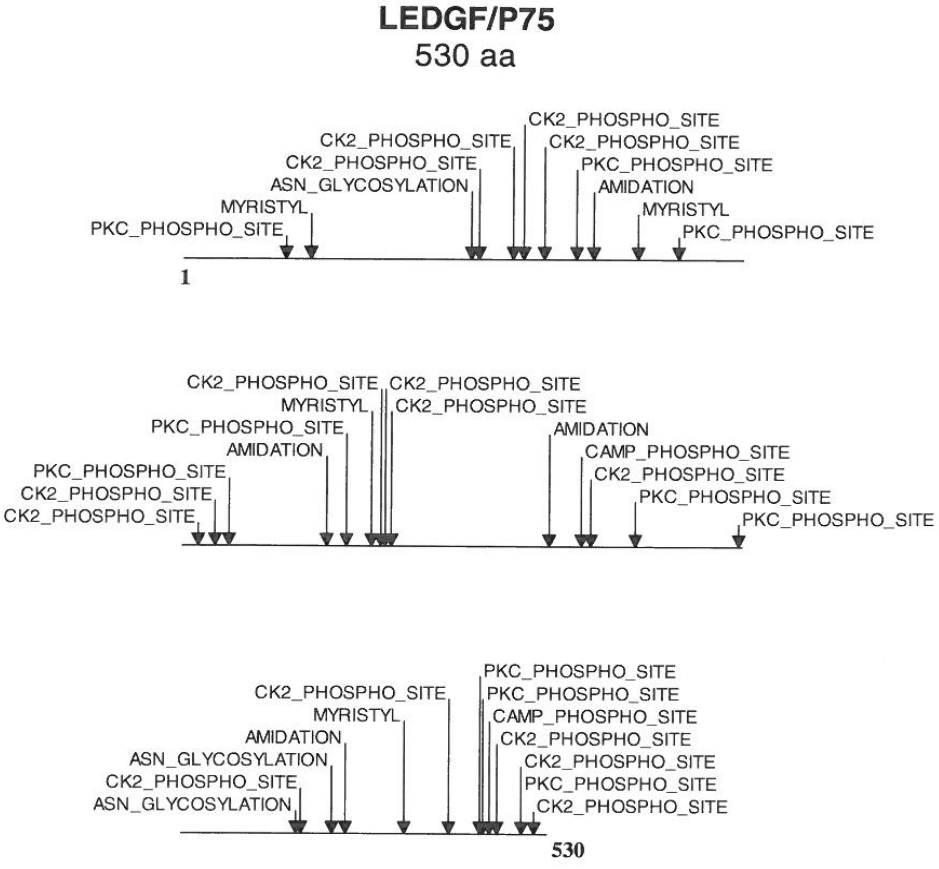
cctggccacgggccacagccagcaacgcttaagcaccagggccggcgagtgccctgccgtggcacg
HSE
gctccagcgtcgcgctctcGAATTCATTTGCTTTCCTTAACGAGA**GAAGGTTCC**AG
SP1/AP2
ATGAGGGCTGAACCCTCTTCGC**CCCGCCCACGGCCC**CTGAACGCTGGG
STRE SP1 ERE TATA
GGAGGAGTGC**ATGGGGAGGGGCGGC**CCTCAAACG**GGTCA**TTGCC**ATTA**
TATA
ATAGAGACCTCAAACACCGCCTGCTAAAAATACCCGACTGGAGGAGC**AT**

AAAAGCGCAGCCGAGCCCAGCGCCCCGCACTTTTCTGAGCAGACGTCCA
Met
GAGCAGAGTCAGCCAGCATG

APPENDIX B

PREDICTED POST-TRANSLATIONAL MODIFICATION OF LEDGF/P75

Predicted post-translational modification of LEDGF/p75 using Accelrys Omiga 2.0**.** Only phosphorylation, amination, glycosylation, and myristylation sites are shown here.



APPENDIX C

PREDICTED SUMOYLATION SITE OF MECP2 BY SUMOSP 2.0

NP_001104262.1

MAAAAAAAPSGGGGGGEEERLEEKSEDQDLQGLKDKPLKFKKVKKDKKEEKE
GKHEPVQPSAHHSAEPAEAGKAETSEGSGSAPAVPEASASPKQRRSIIRDRGPMY
DDPTLPEGWTRKLKQRKSGRSAGKYDVYLINPQGKAFRSKVELIAYFEKVGDTS
LDPNDFDFTVTGRGSPSRREQKPPKKPKSPKAPGTGRGRGRPKGSGTTRPKAATS
EGVQVKRVLEKSPGKLLVKMPFQTSPGGKAEGGGATTSTQVMVIKRPGRKRKA
EADPQAIPKKRGRKPGSVVAAAAAEAKKKAVKESSIRSVQETVLPIKKRKTRETV
SIEVKEVVKPLLVSTLGEKSGKGLKTCKSPGRKSKESSPKGRSSSASSPPKKEHHH
HHHHSESPKAPVPLLPPLPPPPPEPESSEDPTSPPEPQDLSSSVCKEEKMPRGGSLE
SDGCPKEPAKTQPAVATAATAAEKYKHRGEGERKDIVSSSMPRPNREEPVDSRT
PVTERVS

Position	PeptideScore		Cutoff	Type
44	KKVKKDK	3.824	2.64	TypeII: Non-consensus
47	KKDKKEE	3.176	2.64	TypeII: Non-consensus
48	KDKKEEK	3.456	2.64	TypeII: Non-consensus
375	SPPKKEH	0.578	0.13	TypeI: ψ-K-X-E
426	SVCKEEK	2.691	2.64	TypeII: Non-consensus

Ψ: hydrophobic amino acid
K: Lysine
X: any amino acid
E: Glutamic acid