In Prostate Cancer Cells the Interaction of C/EBPα with Ku70, Ku80, and Poly(ADP-ribose) Polymerase-1 Increases Sensitivity to DNA Damage*

Received for publication, October 13, 2005, and in revised form, January 26, 2006. Published, JBC Papers in Press, February 20, 2006, DOI 10.1074/jbc.MS11138200

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Prostate cancer cell lines were examined for proteins that partnered with the transcription factor C/EBPα by use of a pull-down assay with S-tagged C/EBPα combined with matrix-assisted laser desorption ionization time-of-flight mass spectroscopy analysis. Ku70, Ku80, and poly(ADP-ribose) polymerase-1 (PARP-1) were identified as proteins that associated with C/EBPα. The physical interaction of C/EBPα with these partner proteins was further demonstrated by glutathione S-transferase (GST) pull-downs using purified protein expressed in Escherichia coli. The strongest binding was between C/EBPα and PARP-1. Immunoprecipitation of C/EBPα expressed in prostate cancer cells co-precipitated Ku70, Ku80, and PARP-1. Deletion analysis of C/EBPα indicated that the C-terminus of C/EBPα was essential for the interaction of C/EBPα with Ku70, Ku80, and PARP-1. Functional analysis of the interaction between C/EBPα and the Ku proteins as well as PARP-1 showed that cells exhibiting these interactions had increased radiation sensitivity and decreased ability to repair double strand DNA breaks. Deficient DNA repair was dependent on the prostate cancer cell line tested, suggesting a complex process. We conclude that the association of C/EBPα with Ku proteins and PARP-1 raises the likelihood that C/EBPα-expressing prostate cancer cells may be more sensitive to DNA-damaging agents and may be important in the design of new prostate cancer therapies.

C/EBPα is a transcription factor belonging to a basic region-leucine zipper (BZIP) protein family (1–3). The intronless gene creates multiple protein isoforms with molecular weights of 42, 30, and 20 kDa by the differential use of multiple AUG initiation codons within the same open reading frame of a single mRNA (4). The 42-kDa isoform acts to inhibit cell growth and stimulate terminal differentiation. The truncated forms are generated through the mechanism of leaky ribosome scanning, and these isoforms may be important in aging and reaction to stress (5–7).

C/EBPα is expressed in many tissues including hepatocytes, adipose tissue, lung, small intestine, skin, mammary gland, adrenal gland, hematopoietic cells, and ovaries. C/EBPα plays essential roles in energy homeostasis and the differentiation of white adipose tissue and granulocytes (8–11). Mutations in the C/EBPα gene in some patients with acute myeloid leukemia give rise to proteins that are dominant negative and that impair myeloid differentiation (12). In leukemias with the (8:21) translocation, AML1-ETO blocks granulocytic differentiation by down-regulation of CEBPα (13, 14). Reduced expression of CEBPα has been observed in lung and skin cancer tissue, raising the possibility that in some tissues CEBPα may act as a tumor suppressor gene (15).

C/EBPα has been well demonstrated to act as an antiproliferation factor in a number of tissues. In animal models, the expression of C/EBPα is transiently decreased in regenerating liver after partial hepatectomy, and hepatocytes from C/EBPα knock-out mice manifest increased proliferation in culture. In several cancer cell lines, enforced expression of C/EBPα by transfection causes significant growth inhibition or arrest (16–18). The mechanisms of the antiproliferation effects of C/EBPα depend upon its interactions with cell cycle-related proteins (19). In other cell types the antiproliferative effects of C/EBPα result from C/EBPα binding to and inhibiting E2F; in hepatocytes, both inhibition of E2F and stabilization of p21 contribute to the growth arrest. Growth arrest by C/EBPα involves the stabilization of the cyclin-dependent kinase inhibitor p21 (20, 21), interaction with cdk2 and cdk4 (22, 23), disruption of E2F protein complexes (24, 25), and repression of c-Myc expression by interacting with the E2F binding site in the c-Myc promoter (26). Recent studies indicate that the inhibition of cell proliferation by C/EBPα is contingent upon its phosphorylation status, which when altered may allow C/EBPα to stimulate cell growth. In hepatocytes, activated phosphatidylinositol 3-kinase/AKT led to the nuclear accumulation of protein phosphatase 2A, which dephosphorylated C/EBPα at Ser-193. The dephosphorylated C/EBPα interacted with and sequestered the retinoblastoma protein (Rb), decreasing E2F-Rb complexes with a consequent acceleration of cell growth (27, 28). Another potential regulatory point for the anti-proliferation properties of C/EBPα is suggested by the interaction of C/EBPα with the chromosome remodeling factor, SWI/SWF, which is necessary for C/EBPα-mediated growth arrest (29).

In both normal and cancerous prostate epithelia the expression of C/EBPα has been verified at the RNA level (30, 31). We have observed that C/EBPα is differentially expressed between normal and cancerous prostate epithelia, that in prostate cancer cells C/EBPα down-regulated transcription of prostate-specific antigen, a marker of prostate differentiation (32), and that overexpression of C/EBPα stimulated proliferation of prostate cancer cell lines (33). These results suggest that C/EBPα might have tissue- and cell-specific functions in prostate epithelium. To determine whether C/EBPα exhibited unique functions in prostate cells, we screened prostate cancer cell lines for proteins that bound to C/EBPα and identified three DNA repair proteins, Ku80, Ku70, and PARP-1 as C/EBPα partner proteins. The interaction of C/EBPα with PARP-1, Ku80, and Ku70 interfered with the non-homologous ending joining (NHEJ) DNA repair and contributed to increased sensitivity of prostate cells to radiation and DNA damage from bleomycin and iron.
**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Antibodies against C/EBPα (sc-61 and sc-9314) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Ku80 (MS-285), Ku70 (MS-329), and PARP (RB-1680) were from Lab Vision Corp., Inc (Fremont, CA). RPMI 1640 medium was from Mediatech, Inc. (Herndon, VA). Bleomycin sulfate (Blenoxane) was from Nippon Kayaku Co. Ltd. (Tokyo, Japan).

**Cell Culture and Cell Transduction by Retrovirus-expressing C/EBPα—**The human prostate cancer cell lines PC3 and Du145 (ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells with stable expression of C/EBPα were established with a pantropic retroviral expression system (BD Biosciences Clontech, Palo Alto, CA). Briefly, the full-length rat C/EBPα cDNA was inserted into the retrovirus vector pLNCX and was co-transfected with Lipofectamine Plus (Invitrogen) into GP2–293 packaging cells with pVS-G, expressing an envelope glycoprotein of the vesicular stomatitis virus. After 48 h of transfection, medium was collected and filtered, and prostate cancer cell lines were transduced with a mixture of virus-containing medium and fresh medium at ratio of 1:2. Polybrene (Sigma-Aldrich) was added to the medium at 8 mg/ml for the first 24 h. Stable expressing clones were selected with Geneticin at 400 mg/ml (Mediatech Cellgro, Herndon, VA) for 2–3 weeks.

**Constructs and Protein Expression—**Full-length rat and human C/EBPα cDNA and C-terminal fragments (C1 and C2) were kind gifts from Daniel G. Tenen and Atsushi Iwama, respectively. The N-terminal fragment of C/EBPα was made by removing the NotI fragment from the C terminus of C/EBPα. The full-length cDNAs and N or C terminus-deleted fragments were subcloned into pET30 vectors (Novagen, EMD Biosciences, Madison, WI) and pGEX vectors (Amersham Biosciences) for expression of S tag, His tag, and GST fusion proteins. The full-length cDNAs of Ku80, Ku70, and PARP-1 were generated by reverse transcription-PCR from total RNA of LNCaP or Du145 cells and cloned into pCDNA3 and pET vectors. All constructs were sequenced, and a few errors in the sequence were corrected by the replacement of fragments. The clones with error-free cDNA of Ku80, Ku70, and PARP-1 were transfected into BL21 codonPlus™-(DE3)-RIL (Stratagene, La Jolla, CA), and expressed proteins were identified by Western analysis.

**GST-C/EBPα in Vitro Protein Binding Assay—**GST-C/EBPα fusion proteins were expressed in Escherichia coli BL21-CodonPlus® (DE3)-RIL cells and purified with the GST Purification Module (Amersham Biosciences). The pull-down assay was conducted in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton-X100 by incubation of GST-C/EBPα with lysates of *E. coli* BL21-CodonPlus® (DE3)-RIL cells containing expressed Ku80, Ku70, PARP-1, and heat shock cognate protein 70 (HSC) proteins from pET vectors carrying full-length cDNAs coding for these proteins. The pull-down proteins were separated by SDS-PAGE and detected with the appropriate antibodies by Western blot analysis.

**Co-immunoprecipitation—**Cytoplasmic and nuclear proteins were extracted from cells stably expressing C/EBPα with NE-PER® nuclear and cytoplasmic extraction reagents (Pierce). Co-immunoprecipitation was conducted with an antibody against C/EBPα. Briefly, 4 μg of anti-C/EBPα antibody and protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) were added to 0.5 ml of cell lysate (about 600 μg protein) and incubated at 4 °C overnight with rotation. The precipitate was collected by centrifugation, and the pellet was washed 5 times with phosphate-buffered saline containing 0.5% Nonidet P-40 after which the pellet was resuspended in 2× SDS-PAGE loading buffer, boiled for 5 min, subjected to SDS-PAGE, and analyzed by Western blot analysis with antibodies against Ku80, Ku70, and PARP-1. For immunoprecipitation with human prostate cancer tissue, frozen samples were obtained under a LSIHSC Institutional Review Board-approved protocol from the Feist-Weiller Cancer Center tissue repository at LSUHSC. The tissue protein was extracted with NE-PER® nuclear and cytoplasmic extraction reagents (Pierce).

**RNA Isolation and Microarray Analysis—**RNA was isolated from retrovirus-transduced cells with TRI reagent®. RNA/DNA/protein isolation reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer’s protocol. The expression analysis with Affymetrix gene chip was conducted on the human U95A array (Affymetrix Inc., Santa Clara, CA) using 10 μg of total RNA. Synthesis of cRNA and subsequent hybridization was completed by the Research Core Facility at LSUHSC-S using the standard Affymetrix protocols. The human U95A array represents 12,256 oligonucleotides of known genes or expression tags. The raw data were collected and analyzed with the Affymetrix Microarray Suite with the scale set at 2500.

**Western Blot Analysis—**Whole cell extracts from prostate cancer cell lines were obtained with radioimmune precipitation assay buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS) containing 1× protease inhibitor mixture (Roche Applied Science). Protein concentration was determined by BCA protein assay kit (Pierce). Cell proteins were separated by electrophoresis on SDS-PAGE, transferred to Hybond × ECL nitrocellulose membrane (Amersham Biosciences), and blocked with 5% nonfat milk in 1× TBST (10 mM Tris-HCL, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The blots were then incubated at room temperature with rabbit anti C/EBPα antibody for 2 h, washed, and incubated with peroxidase-conjugated secondary antibody. The signal was detected with SuperSignal Dura Substrate (Pierce).

**NHEJ Assay—**The NHEJ assay was conducted according to previous work with slight modifications (34–36). Briefly, the nuclear extract was prepared as above with NE-PER® nuclear extraction reagents and dialyzed against 20 mM Tris-HCL, pH 8.0, 100 mM potassium acetate, 10% glycerol, 0.5 mM EDTA, and 1 mM dithiothreitol. Then 20–25 μg of nuclear protein was mixed with 400 ng of BSA/1- and Xhol-digested pBlueKs plasmid DNA in 50 mM Tris-HCL, pH 8.0, 40 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EDTA, 1 mM ATP, 1 mM dithiothreitol, 200 mM dNTPs, and 100 mg/ml bovine serum albumin. The 50-μl reactions were incubated at 37 °C for 2 h followed by protease K treatment and extraction with phenol/chloroform. One-half of each reaction was separated on a 0.8% agarose gel and stained with Gelstar (Cambrex Corp., East Rutherford, NJ). The stained gels were scanned, and the percentage of end rejoining was calculated by dividing the sum of dimer and multimer by sum of monomer, dimer, and multimer.

**S-tagged C/EBPα Protein Capture and MALDI-TOF-MS—**S-tagged C/EBPα fusion proteins were expressed in pET-30A-C/EBPα in *E. coli* BL21-CodonPlus® (DE3)-RIL cells after induction with 0.8 mM isopropyl 1-thio-β-D-galactopyranoside and purified with S-tagged agarose beads. The fusion proteins bound to agarose beads were incubated overnight at 4 °C with 400 μl (about 1 mg of protein) of cell lysates from Du145 and PC3 cells, obtained by extraction with NE-PER® nuclear and cytoplasmic extraction reagents. The beads were pelleted and washed, and the “pull-down” proteins were separated on SDS-PAGE and stained with GelCode® Blue. The proteins bound to S-tagged C/EBPα fusion proteins were analyzed by MALDI-TOF-MS in the Research Core Facility of LSUHSC-Shreveport with the Voyager-DE™ PRO Biospectrometry™ Work station (Applied Biosystems, Foster City, CA). Briefly, the proteins captured by the S-tag C/EBPα fusion protein were separated by SDS-PAGE and stained with GelCode® Blue. The protein bands of interest were sliced from the gel and subjected to peptide extraction.
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and elution with C18 zip-tips. The peptides were analyzed with three different instrument settings: Proteomics_autosample.bic, Insulin_linear.bic, and peptide_negative_reflector.bic. The analyses of spectra data was performed with Data Explore Software (version 3.2.1) for algorithms to correct the base line and remove the noise at 2 S.D. The match of peptide data was conducted against protein databases, NCBI and Genpept, using Auto MS-Fit software.

Radiation, Cell Proliferation, and Clonogenic Survival Assays—80 × 10^5 cells grown on 35-mm plastic dishes were suspended in 2 ml of RPMI1640 medium in a 15-ml plastic tube and exposed at room temperature to radiation at doses of 4 and 8 Gy from a Varian 6-MV linear accelerator. For proliferation assays after exposure to radiation, cells were grown in 96-well plates at initial densities of about 1000 cells/well for 0 and 4 Gy and 3000 cells/well for 8 Gy exposure in RPMI 1640 containing 10% fetal bovine serum and 400 μg/ml Geneticin. The cell proliferation assay was conducted 6 days after radiation exposure with the CellTiter 96® AQuncial non-radioactive cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. For colony formation assays cells were exposed to radiation and plated at density of 500 cells per well in 6-well plates in RPMI 1640 containing 10% fetal bovine serum and 400 μg/ml Geneticin, and after 10 days the cells were stained with 0.05% crystal violet, and colonies enumerated under a dissecting microscope.

RESULTS

Screening for Proteins That Partner with C/EBPα in Prostate Cancer Cells—Putative protein partners of C/EBPα were identified by using a pull-down assay in which an S-tagged C/EBPα fusion protein was incubated with lysates containing both cytoplasm and nuclear proteins from the prostate cancer cell lines PC3 and Du145 (Fig. 1). Analysis of the proteins that co-precipitated with C/EBPα by separation by SDS-PAGE identified four protein bands that appeared to be specifically pulled down by C/EBPα. These protein bands were neither seen with incubation of the S-tag protein alone with cell lysates from PC3 and Du145 cells (Fig. 1, lanes 1 and 3) nor with the S-tag C/EBPα fusion protein without cell lysate (Fig. 1, lane 5). MALDI-TOF-MS analysis of each protein band demonstrated that the proteins of approximate molecular masses 113, 82, and 70 kDa were PARP-1, Ku80, and HSC, respectively. Based on analysis with Auto MS-Fit, the MOWSE (Molecular Weight SEarch) scores for all identified proteins were greater than 10^5. In addition, a band staining with a molecular mass of about 20 kDa was not successfully identified.

The Further Identification of the Interaction of C/EBPα with Putative Partner Proteins—To confirm the physical interaction between C/EBPα and the proteins identified in the pull-down assay, we conducted an in vitro GST pull-down assay by incubating a GST-C/EBPα fusion protein with Ku80, HSC, and PARP-1 expressed in the E. coli BL21 system. Considering that Ku80 functions as a heterodimer with Ku70, we also examined the interaction between Ku70 and C/EBPα. Among the four proteins, Ku80, Ku70, and PARP-1 were pulled down by GST-C/EBPα fusion protein (Fig. 2A), whereas HSC did not show any interaction with C/EBPα in the GST pull-down assay (data not shown). Furthermore, by taking advantage of the ability to express PARP-1, Ku70, and Ku80 in E. coli, we could determine whether the addition of one of these proteins competed for the binding of the others to C/EBPα (Fig. 2B). The presence or absence of Ku70/80 had no effect on the binding of PARP-1 to C/EBPα (Fig. 2B, left panel). Likewise, in the absence of PARP-1 or in the presence of PARP-1 at two different concentrations, the binding of Ku70 or Ku80 to C/EBPα was not affected (Fig. 2B, right panel). Therefore, it is most likely that C/EBPα forms a complex with PARP-1, Ku70, and Ku80 proteins rather than forming dimers with each individual protein. In all experiments a greater percentage of PARP-1 appeared bound to C/EBPα than seen with Ku70 and Ku80. For example, by densitometric scanning of the Western blots of the pull-down proteins and the corresponding inputs in Fig. 2A, the ratio of bound protein over input was 2.4 for PARP-1, 0.63-fold for Ku70, and 0.28-fold for Ku 80. These data imply that PARP-1 binds more strongly to C/EBPα than the two Ku proteins, but it is not known if the differences in binding have any functional consequence. To determine whether the interactions between C/EBPα and Ku80, Ku70, and PARP-1 also occurred in vivo in intact cells, we first examined the expression levels of Ku80, Ku70, and PARP-1 in various prostate and non-prostate cell lines. As shown in Fig. 2C, Ku80 and Ku70 were expressed in all the cell lines examined. PARP-1 was expressed in all cell lines except U937 cells. These cell lines also expressed low levels of endogenous C/EBPα protein, although only the p42 isoform was detected (Fig. 2D, left panel). To determine whether the endogenous Ku80, Ku70, and PARP-1 proteins would interact with C/EBPα, several prostate cell lines were constructed that overexpressed C/EBPα. Clones of the prostate cancer cell lines LNCaP, Du145, and PC3 were generated through retrovirus transformation using the full-length rat C/EBPα cDNA inserted into the retrovirus vector PLNMX. The increased expression of C/EBPα was seen in all transduced cell lines. Although the p42 isoform was the predominant isoform, the p30 isoform was detected in transduced LNCaP cells (Fig. 2D, right panel). As shown in Fig. 2E, in all three lines Ku70, Ku80, and PARP-1 co-precipitated with C/EBPα. Among the three transduced cell lines PC3 showed the weakest interaction of C/EBPα with Ku proteins and PARP-1 based on the ratio of co-precipitated proteins to the input in the cell lysates. However, the interaction could be strengthened by the induction of double-strand DNA breaks by exposure of PC3 cells to bleomycin and iron. After incubation of the PC3 cells with bleomycin and iron for 24 h, expression of C/EBPα increased by 3.4-fold, and the amount of Ku70, Ku80, and PARP-1 that co-precipitated with C/EBPα increased as well by 6.6, 2.7, and 1.7-fold, respectively. Ku70, Ku80, and PARP-1 also co-precipitated with endogenous C/EBPα as was demonstrated in LNCaP cells (Fig. 2F, top panel) and in human prostate cancer tissue (Fig. 2F, middle and lower panels). The expression of C/EBPα and the two Ku proteins was...
detected in all three samples of prostate cancer. However, PARP-1 was only detected in two of the samples (Fig. 2F, middle panel), and in a third sample, PARP-1 was detected as only small fragments, presumably resulting from proteolysis, of less than 50 kDa apparent molecular mass (data not shown). In the cancer tissue the interaction of C/EBPα with Ku70, Ku80, and PARP-1 was seen by co-immunoprecipitation (Fig. 2F, bottom panel). These in vitro and in vivo binding assays demonstrate that Ku70, Ku80, and PARP-1 proteins may universally interact with C/EBPα. In the lung cancer cell line H358, Ku70 and Ku80 also coprecipitated with C/EBPα when C/EBPα was overexpressed (data not shown). These results also suggest that formation of a C/EBPα-Ku70–80-PARP-1 complex could depend on the specific cell type and perhaps the status of the cell.

The C Terminus of C/EBPα Is Essential for the Binding of Ku80, Ku70, and PARP-1

A

B

C

D

E

F

Native LNCaP cells

C/EBPα

Ku80

Ku70

PARP-1

C/EBPα

Co-IP

IgG

CE

WB

IgG

CE

WB

Ku80

Ku70

PARP-1

Input

GST C/EBPα

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C/EBPα Interacts with Ku70, Ku80, and PARP-1

A

![Diagram of C/EBPα fragments](image)

B

![Diagram comparing Ku70, Ku80, and PARP-1](image)

FIGURE 3. Identification of Ku70, Ku80, and PARP-1 binding sites on C/EBPα. A, schematic illustration of C/EBPα polypeptides generated to determine the region(s) of C/EBPα that allows for interaction of C/EBPα with Ku70, Ku80, and PARP-1. B, upper panel, the pull-down assay with S-tagged C/EBPα fragments was conducted with LNCaP whole cell extracts, the proteins were absorbed to S-agarose beads separated by SDS-PAGE, and the presence of Ku70, Ku80, and PARP-1 was examined by Western blot analysis with specific antibodies. S, S peptide alone; F, full-length C/EBPα; N, N-terminal fragment of C/EBPα; C1, truncated fragment of C/EBPα; C2, C-terminal fragment of C/EBPα containing basic leucine zipper DNA binding domain alone; B, lower panel, purified S-tagged C/EBPα fragments were examined on the same blot as in the upper panel with antibodies to N and C termini of C/EBPα to verify the presence of the fragments in the pull-down assay.

terminus. Both the N and C termini have been shown to have different roles in regulation of gene transcription, cell differentiation, and proliferation. To determine which domain is necessary for the binding of Ku80, Ku70, and PARP-1, we expressed several different polypeptides of C/EBPα (Fig. 3). These peptides included an N-terminal fragment with two of the transactivation domains intact (Fig. 3A, N), a truncated C/EBPα p30, which can be physiologically translated from an internal AUG codon in some cells (Fig. 3A, C1), and a C-terminal fragment containing the basic leucine zipper domain alone (Fig. 3A, C2). All three fragments were expressed as S-tagged proteins and purified with S-protein-agarose. The C/EBPα fragments (Fig. 3B, lower panel) were incubated with whole cell extracts from LNCaP cells, bound proteins were pulled-down, and the presence of Ku80, Ku70, and PARP-1 was examined by Western blot analysis with specific antibodies. Ku80, Ku70, and PARP-1 interacted with C/EBPα via the C-terminal region (Fig. 3B, upper panel, C1 and C2). No interactions could be demonstrated with the N-terminal region of C/EBPα (Fig. 3B, upper panel, N).

Stable Expression of C/EBPα Increases the Sensitivity of Du145 and PC3 Cells to Radiation—It has been well known that Ku80, Ku70, and PARP-1 take part in the repair processes of DNA double-strand breaks. To determine whether the interaction of C/EBPα with Ku80, Ku70, and PARP-1 affected DNA repair function in prostate cancer cells overexpressing C/EBPα, we first examined expression of genes participating in DNA repair. Without inflicting any DNA damage onto LNCaP, Du145, or PC3 cells, the mRNA levels of Ku70, Ku80, and PARP-1 were not affected by expression of C/EBPα (data not shown). The expression of genes responsible for both homologous recombination and nonhomologous end joining was examined by microarray analysis of clones of PC3 and LNCaP cell lines expressing C/EBPα (Table 1). No significant changes were observed except for a greater than a 2-fold log ratio change of Rad50 mRNA levels in both C/EBPα-expressing lines. Next, the protein levels of Ku70, Ku80, and PARP-1 and C/EBPα were examined by Western blot analysis 72 h after exposure of the cells to radiation at 0, 4, and 8 Gy to determine whether C/EBPα affected the expression of these proteins under conditions of DNA damage, especially DNA double-strand breaks. For these experiments, clones of PC3 and Du145 cell lines were generated from cells transduced by retrovirus transformation with the full-length rat C/EBPα cDNA inserted into the retrovirus vector pLNCX or empty virus. The stable expression of C/EBPα in both cell lines was verified with Western blot analysis and clones were designated as either no (N), low (L), or high (H) expression of C/EBPα (i.e. PC3-N, PC3-L, PC3-H, Du145-N, Du145-L, and Du145-H) based on Western analysis of the clones. Interestingly, after radiation exposure, C/EBPα protein levels were distinctly increased both in the PC3-H and Du145-H cells. C/EBPα increased in PC3-H by 3.4- and 7.3-fold for radiation doses of 4 and 8 Gy, respectively (Fig. 4A, right panel, lanes 1 and 4) compared with the non-irradiated cells (Fig. 4A, right panel, lane 7). In the Du145-H cells, C/EBPα expression increased 5.5- and 5.6-fold at 4 and 8 Gy, respectively (Fig. 4A, left panel, lanes 1 and 4) compared with the non-irradiated cells (Fig. 4A, left panel, lane 7). No increase of C/EBPα expression was seen in the low C/EBPα-expressing cell clones, PC3-L and Du145-L (Fig. 4A, left and right panels, lanes 2, 5, and 8). Radiation did not affect the expression of the Ku proteins (Fig. 4A) but did affect the expression of PARP-1 in the Du145 cells but not in the PC3 cells. The relative expression of PARP-1 in Du145-N, Du145-L, and Du145-H before and after exposure to radiation is given in Table 2. In Du145-H cells the expression of PARP-1 increased by 6.5- and 4.4-fold at radiation doses of 4 and 8 Gy, respectively, compared with Du145-N (Fig. 4A, left panel, lanes 4 and 7 compared with lanes 6 and 3 and Table 2). In the absence of radiation, Du145-H expressed a similar level of PARP-1 protein as Du145-N with a relative expression ratio of 1 when corrected for tubulin expression (Fig. 4A, left panel, lane 7 compared with lane 9 and Table 2). It appeared, too, that the exposure to radiation reduced the expression of PARP-1 protein in Du145-N and Du145-L cells. In the PC3-H cells, although the expression level of PARP-1 was not changed by C/EBPα expression or radiation, the migration of PARP-1 on SDS-PAGE was slower than in PC3-N and PC3-L cells (Fig. 4A, right panel, lanes 1, 4, and 7). A clonogenic survival assay was conducted to correlate the expression of C/EBPα with the radiation sensitivity of the various C/EBPα-expressing clones. After radiation, colony formation was reduced in both the Du145 and PC3 L and H clones. The decrease was about 50% in PC3-L and PC3-H cells at 4 Gy and about 90% in PC3-L and 100% in PC3-H cells at 8 Gy compared with the PC3-N cells (Fig. 4B, bottom panel). In the Du145 cell line, the Du145-H cells exhibited a greater sensitivity to radiation than the Du145-L cells (Fig. 4B, upper panel). At 4 Gy Du145-H cells showed a 75% reduction in colony formation compared with Du145-N cells, whereas Du145-L cells did not show a significant change. At 8 Gy of exposure, colony formation was decreased by 57% in Du145-L cells and by 100% in Du145-H cells. A cell proliferation assay was performed with the Du145 N, L, and H clones after 4 and 8 Gy of radiation (Fig. 5) with a response pattern similar to that observed with the clonogenic assay. In the proliferation assay decreased proliferation was observed both of the Du145 L and H clones after 8 Gy, but only a decrease in the H clones was observed after 4 Gy (Fig. 5).
TABLE 1
Microarray analysis of RNA expression of genes responsible for nonhomologous end rejoining and homologous recombination in PC3 and LNCaP cells expressing C/EBPα

| Probe set ID | Gene symbol | PC3 C/EBPα vs control | LNCaP C/EBPα vs control | Gene expression |
|--------------|-------------|-------------------------|--------------------------|----------------|
|               |             | Log ratio | Change | Log ratio | Change |                  |
| For nonhomologous ending rejoining |             |           |        |           |        |
| 1250_at       | PRKDC       | 0.93      | I       | 0.72      | I       | Protein kinase, DNA-activated, catalytic polypeptide |
| 2012_s_at     | PRKDC       | 0.66      | I       | 0.72      | I       | Protein kinase, DNA-activated, catalytic polypeptide |
| 40129_at      | PRKDC       | 0.73      | I       | 0.56      | NC      | Protein kinase, DNA-activated, catalytic polypeptide |
| 1533_at       | RAD50       | 3.77      | I       | 1.03      | NC      | RAD50 homolog (Saccharomyces cerevisiae) |
| 39941_at      | RAD50       | 2.98      | I       | 3.03      | I       | RAD50 homolog (S. cerevisiae) |
| 32869_at      | MRE11A      | 0.16      | NC      | 0.73      | I       | MRE11 meiotic recombination 11 homolog A (S. cerevisiae) |
| 32870_g_at    | MRE11A      | 0.59      | I       | 0.28      | NC      | MRE11 meiotic recombination 11 homolog A (S. cerevisiae) |
| 160033_s_at   | XRCC1       | 0.75      | NC      | 0.85      | I       | X-ray repair complementing defective repair in Chinese hamster cells 1 |
| 2093_s_at     | XRCC5       | −0.06     | NC      | −0.02     | NC      | Ku autoantigen, 80 kDa |
| 38733_at      | XRCC5       | −0.11     | NC      | −0.06     | NC      | Ku autoantigen, 80 kDa |
| 584_s_at      | XRCC5       | 0.17      | NC      | 0.06      | NC      | Ku autoantigen, 80 kDa |
| 585_at        | XRCC5       | 0.13      | NC      | −0.03     | NC      | Ku autoantigen, 80 kDa |
| 32766_at      | G22H1       | 0.76      | I       | 0.45      | I       | Thyroid autoantigen, 70 kDa (Ku antigen) |
| 1862_s_at     | ATM         | −0.57     | NC      | −0.14     | NC      | Ataxia telangiectasia mutated |
| 35153_at      | NBS1        | −0.33     | NC      | −0.66     | D       | Nijmegen breakage syndrome 1 (nibrin) |
| For homologous recombination |             |           |        |           |        |
| 37094_at      | XRCC3       | 0.31      | NC      | −0.59     | NC      | X-ray repair complementing defective repair in Chinese hamster cells 3 |
| 41479_s_at    | RAD51C      | 0.6       | NC      | 1.59      | I       | RAD51 homolog C (S. cerevisiae) |
| 41480_at      | RAD51C      | 0.61      | I       | 1.08      | I       | RAD51 homolog (RecA homolog, E. coli) |
| 1872_at       | RAD51       | −2.44     | NC      | 1.73      | NC      | RAD51 homolog (RecA homolog, E. coli) |
| 40744_at      | RAD52       | 2.94      | NC      | −0.28     | NC      | RAD52 homolog (S. cerevisiae) |
| 1043_s_at     | RAD52       | 2.88      | NC      | 2.34      | NC      | RAD52 homolog (S. cerevisiae) |
| 1899_s_at     | RAD52       | 0.4       | NC      | 0.26      | NC      | RAD52 homolog (S. cerevisiae) |
| 966_at        | RAD54L      | 0.95      | I       | 0.06      | NC      | RAD54-like (S. cerevisiae) |
| 967_g_at      | RAD54L      | 1.02      | I       | 0.38      | NC      | RAD54-like (S. cerevisiae) |
| 37094_at      | XRCC3       | 0.31      | NC      | −0.59     | NC      | X-ray repair complementing defective repair in Chinese hamster cells 3 |

C/EBPα Expression Interferes with NHEJ in Vivo and in Vitro—Based on the function of PARP-1 and Ku proteins in repair of DNA double-strand breaks and the increased sensitivity of C/EBPα-expressing cells to the radiation exposure, we hypothesized that the interaction of C/EBPα with Ku70, Ku80, and PARP-1 may interfere with the NHEJ process which plays a primary role in repairing DNA double-strand breaks. To test this hypothesis, the NHEJ assay was conducted with nuclear extracts from LNCaP and Du145 cells with high (LNCaP-H, Du145-H) or no expression (LNCaP-N, Du145-N) of C/EBPα. Both LNCaP-H and Du145-H cells exhibited an ~60% inhibition of end rejoining in the NHEJ assay (Fig. 6A) compared with LNCaP-N and Du145-N. Interestingly, the PC3-H cells, although expressing C/EBPα, did not show a significant decrease in end rejoining compared with PC3-N cells (data not shown). Based on the observation above that PC3-H clones exhibited a significant increase in radiation sensitivity (Fig. 4B, bottom panel), we simulated a condition that would generate DNA double-strand breaks by exposure of PC3-N, PC3-H, Du145-N, and Du145-H cells to 100 million units bleomycin plus 1 mM ferric chloride and 1 mM ferrous sulfate for 1.5 or 12 h (37). After treatment with bleomycin and iron, the end rejoining was reduced by about 3.5-fold in PC3-H cells for both the 12- and 1.5-h exposures to bleomycin and iron (Fig. 6B, lanes 2 and 4) and by 40- and 4-fold in Du145-H cells for the 12- and 1.5-h exposures, respectively (Fig. 6B, lanes 6 and 8). The reduced end joining in the PC3-H cells after bleomycin treatment indicates that in the PC3-H clones C/EBPα-related interference with NHEJ is DNA damage-dependent. This phenomenon is consistent with the previous observation that DNA damage increased binding of C/EBPα to Ku and PARP-1 protein (Fig. 2E).

However, because the C/EBPα-expressing cells were clonally selected, we could not exclude that decreased NHEJ in C/EBPα-expressing cells was caused by clonal selection, allowing for factors other than direct interaction of C/EBPα with DNA repair proteins to interfere with DNA repair. To address this question, we performed the NHEJ assay using purified C/EBPα protein expressed in E. coli strain BL21-condon plus-RIL. The addition of purified C/EBPα to the nuclear extracts from LNCaP, PC3, and Du145 cells resulted in a 33% decrease of end rejoining in LNCaP cells and a 75% decrease in Du145 cells (Fig. 7A). The NHEJ assay using PC3 cell lysates demonstrated no change in end rejoining, similar to the observation that in intact PC3 cells expression of C/EBPα interfered with NHEJ only under conditions of DNA damage. To determine the regions of C/EBPα necessary for interference with DNA repair, we observed the effect of C-terminal- and N-terminal-deleted C/EBPα peptides on end rejoining (Fig. 7B). Neither the C/EBPα polypeptides with a C-terminal or N-terminal deletion affected end rejoining, suggesting that the intact protein is needed for function in C/EBPα-mediated inhibition of the NHEJ repair process.

DISCUSSION

The transcription factor C/EBPα is instrumental in promoting cell differentiation and decreased cell proliferation in many tissues. C/EBPα is known to be expressed in normal prostate epithelium; however, we have observed that overexpression of C/EBPα in prostate cancer cell
C/EBPα Interacts with Ku70, Ku80, and PARP-1

FIGURE 4. Expression of Ku70, Ku80, PARP-1, and C/EBPα and clonogenic survival of Du145 and PC3 cells after the exposure to radiation. A, Western blots showing the expression of Ku70, Ku80, PARP-1, and C/EBPα after the exposure of cells to radiation. Clones of Du145 and PC3 cells stably expressing C/EBPα were developed by retroviral transfection of the full-length rat C/EBPα cDNA in the retrovirus vector pLNCX. The amount of C/EBPα in the clones was established by Western blot analysis, and clones were designated either as high producers (H), no producers (N), or low producers (L) of C/EBPα. These clones were then subjected either to 0, 4, or 8 Gy as described under “Experimental Procedures,” and the amount of Ku70, Ku80, PARP-1, and C/EBPα was determined by Western blot analysis at 72 h. The Western blots for Du145 or PC3 are shown in the left or right panel with lanes 1, 4, and 9 representing H clones, lanes 2, 5, and 8 representing L clones, and lanes 3, 6, and 9 representing N clones. B, clonogenic survival assay. The effect of radiation on clonogenic survival of C/EBPα-overexpressing cells was assayed by colony formation as detailed under “Experimental Procedures,” with the number of colonies enumerated at 10 days after radiation. Shown are the means ± S.D. of the colonies of three separate experiments with Du145 cells in the top panel and PC3 cells in the bottom panel. The open bars represent the N clones, the black bars represent the L clones, and the line bars represent the H clones. Data were analyzed with a two-sample unpaired Student’s t test. Double stars indicate statistical significance at p < 0.01 compared with cells expressing no exogenous C/EBPα.

TABLE 2

The relative expression of PARP-1 in Du145-N, Du145-L, and Du145-H cells before and after radiation was obtained by densitometric scanning of the Western blots shown in Fig. 4A corrected for the amount of β-tubulin.

| Radiation  | 0 Gy | 4 Gy | 8 Gy |
|-----------|------|------|------|
| Du145 cell |      |      |      |
| N          | 1.0  | 0.48 | 1.07 |
| L          | 0.23 | 0.5  | 1.48 |
| H          | 0.39 | 0.40 | 1.73 |

The data are expressed relative to the expression level of PARP-1 in Du145-N in the absence of radiation.

Du145 and PC3 prostate cancer cell lines as prey we identified with MALDI-TOF-MS analysis three proteins, Ku80, PARP-1, and HSC, as putative partners of C/EBPα. Further analysis using GST-C/EBPα and purified proteins confirmed Ku80 and PARP-1 as binding to C/EBPα. In addition, because Ku70 and Ku80 form heterodimers, purified Ku70 was also examined in the GST-pull down assay and was confirmed as a C/EBPα partner. The initial experiments searching for protein partners of C/EBPα used C/EBPα that had been synthesized in prokaryotic cells. It is possible that recombinant C/EBPα synthesized in bacteria may not have the same folding as in mammalian cells, and therefore, either some partners might be missed or others observed that would not interact with C/EBPα in eukaryotic systems. In addition, the strategy used might
C/EBPα Interacts with Ku70, Ku80, and PARP-1

**FIGURE 7. The addition of C/EBPα protein into the NHEJ reaction inhibits DNA end rejoining.** A, the NHEJ assay was conducted as in Fig. 6 using nuclear extracts prepared from LNCaP, PC3, and Du145 cells not transfected with C/EBPα. Purified His-tagged C/EBPα was added to the NHEJ reaction (lanes 3, 5, and 7). Lane 1, pBlueK5 plasmid alone; lanes 2, 4, and 6, NHEJ reaction without adding C/EBPα. The percentage of rejoining (calculated by the sum of monomer, dimer, and multimer divided by the sum of monomer, dimer, and multimer) is shown with each lane. B, top panel, the NHEJ reaction was performed with nuclear extract from non-C/EBPα-expressing DU145 cells as above with the His-tagged C/EBPα fragments (1 μg), as described in Fig 3, added to the reaction. The percent rejoining is shown below each lane. C, control, no C/EBPα added; F, full-length C/EBPα; N, N-terminal fragment of C/EBPα; C1, truncated fragment of C/EBPα. **Bottom left panel,** shown is a Western blot analysis of the purified His-tagged C/EBPα peptides used in the NHEJ reaction. **Bottom right panel,** shown is the percent rejoining in the presence of F, N, and C1 compared with C (means ± S.E. for three independent experiments). Comparisons of NHEJ with the addition of full-length C/EBPα to the other conditions were statistically significant at a level of *p* < 0.01 (double diamond, F versus control; double star, F versus N) and *p* < 0.02 (single circle, F versus C1) by Student’s t test.

not detect either weak interactions or the interaction with low concentration of proteins. We were able to exclude the potential artifact imposed by improper folding by demonstrating that Ku70, Ku80, and PARP-1 interacted with C/EBPα that was 1) overexpressed in prostate cancer cell lines, 2) endogenously expressed in LNCaP cells, and 3) endogenously expressed in human prostate cancer. Furthermore, that the interactions could be demonstrated to occur with a discrete region of C/EBPα, namely the C-terminal basic leucine zipper domain, suggests that mis-folding of C/EBPα with expression in prokaryotes did not play a role in the detection of protein partners.

The Ku proteins were originally identified as autoantibodies in patients with rheumatic disorders (38). The two Ku proteins have been well demonstrated to dimerize and to function in repair of DNA double-strand breaks, DNA telomere length maintenance, transcription regulation, and V(D)J recombination (39–43). Recently, Ku proteins expressed on the cell surface were found to be associated with the extracellular matrix and to interact specifically with matrix metalloprotease 9, suggesting that the Ku proteins may be involved in tumor invasion (44, 45). PARP-1 is a founding member of poly(ADP-ribose) polymerases whose superfamily consists of 18 proteins (46–48). PARP-1 is a DNA damage-activated protein and a sensor for DNA breaks that binds to DNA breaks, catalyzes NAD⁺ hydrolysis, produces a polymer of ADP-ribose, and transfers the polymer to histones and other nuclear proteins. The poly ADP-ribosylation of histones and nuclear proteins facilitates the access of DNA repair enzymes to relaxed chromatin. In response to DNA damage, both PARP-1 and Ku proteins participate in the non-homologous end joining pathway by direct binding to the broken ends of damaged DNA. The close association of Ku proteins and PARP-1 has been demonstrated with co-immunoprecipitation (49), although recently the importance of PARP-1 in DNA double-strand break repair has been questioned (50, 51).

Because Ku70, Ku80, and PARP-1 are expressed universally, we asked if the interaction of C/EBPα with Ku70, Ku80, and PARP-1 occurs in all cells or only in prostate cancer cells. Co-immunoprecipitation using C/EBPα-transfected H358 cells, a lung cancer cell line, demonstrated that Ku70 and Ku80 were co-precipitated by antibodies to C/EBPα, suggesting that the interaction between C/EBPα and Ku proteins and PARP-1 is not unique to prostate cancer cells. The association of Ku80 and PARP-1 with C/EBPα has not been previously described and may in our studies with prostate cancer cells have
C/EBPα Interacts with Ku70, Ku80, and PARP-1

been the result of the strategy and methods used for screening with a fortuitous ratio of bait to prey that allowed visualization of the co-precipitating proteins. Both the Ku proteins and PARP-1 are abundantly expressed and, therefore, could be visualized by protein staining of the gel. Using a similar strategy, Ku70 and Ku80 have been shown to interact with the androgen receptor in prostate cells (52). The failure to identify Ku70 on the initial gels was the mischaracterization of the Ku70 (which migrates slightly slower than HSC) as an E. coli protein. Detection of the complexes may have been enhanced by the relatively strong binding of PARP-1 to GST-C/EBPα in the GST-pull-down assay. In addition, cell-specific patterns of protein–protein interactions could also have enhanced detection. We repeated the pull-down assay by incubation of GST-C/EBPα fusion protein with whole cell lysate from the prostate cancer cells, LNCaP, Du145, and PC3 and the erythroleukemia cell line, K562. Although Ku80 could be directly immunoprecipitated in all the cells lines, only in the prostate cell lines did C/EBPα also result in a dominant pull-down band of Ku80 in stained SDS-PAGE. In the K562 cell line GST-C/EBPα pulled down a distinct 50-kDa protein that was not present in the pull-down assay with the prostate cells and which was identified by MALDI-TOF-MS as elongation factor γ (data not shown). On the other hand, in the LNCaP, Du145, and PC3 C/EBPα overexpressing cell lines, no interaction could be observed of C/EBPα with E2F, CDK2, and CDK4. When the Ku proteins were removed from LNCaP cell lysates with antibodies to Ku70 and Ku80, binding of C/EBPα to E2F was seen in the pull-down assay (data not shown), supporting our hypothesis that the spectrum of proteins interacting with C/EBPα depends on the cell type and the relative concentrations of C/EBPα and the C/EBPα-interacting proteins.

In general, prostate cancer cells are more resistant to radiation-induced killing compared with other cancer cells. The death of prostate cancer cells after exposure to radiation may be predominantly by mechanisms other than apoptosis. Indeed, we have observed that after radiation of various prostate cell lines, apoptosis occurred in less than 5% of cells as measured with DNA electrophoresis, 4,6-diamidino-2-phenylindole staining, and labeling with annexin V-fluorescein isothiocyanate (data not shown). There are multiple reasons why a particular cell type might exhibit resistance to radiation including the ability to repair radiation-induced DNA double-strand breaks. The repair of DNA double-strand breaks occurs preferentially through NHEJ rather than through homologous recombination (53). Having demonstrated that C/EBPα expressed in prostate cancer cell lines can associate with Ku70, Ku80, and PARP-1, which are important participants in the repair of DNA double strand breaks, we next wanted to examine if the interactions in C/EBPα-expressing cells affected radiation sensitivity and DNA repair ability. In keratinocytes, UVB radiation exposure increases expression of C/EBPα both at the RNA and protein levels via a p53-mediated pathway, and knockdown of C/EBPα diminished the DNA damage G (1) checkpoint activity and increased sensitivity to UVB-induced apoptosis (54). In our experimental system exposure to radiation increased the protein level of C/EBPα by severalfold in PC3 and Du145 clones with the high C/EBPα expression. Although we have not yet determined the mechanism for the increased expression, as the transcription of C/EBPα in these cells was driven by the strong cytomegalovirus promoter, it is most likely that the increased expression reflects increased translation or decreased degradation. Because the C/EBPα expressing PC3 and Du145 cell lines demonstrated increased radiation sensitivity by both the clonogenic survival and cell proliferation assays, it was of interest to see if the interaction of C/EBPα with the DNA repair proteins, Ku70, Ku80, and PARP-1, could interfere with the repair of DNA double-strand breaks. This possibility was verified by a C/EBPα-mediated decreased NHEJ in LNCaP and Du145 cell lines assayed both in cell lysates and intact cells. The inhibition of NHEJ by C/EBPα was dependent on a full-length C/EBPα protein. In addition, decreased NHEJ was seen in the PC3-H cells only when double-strand DNA breaks were induced by exposing cells to bleomycin plus iron. These observations support our hypothesis that overexpression of C/EBPα in prostate cancer cells causes increased sensitivity to double-strand DNA breaks via decreased DNA repair.

It was of interest that radiation induced different clonogenic survival assay responses in the PC3 and Du145 cell lines. In Du145 cell line, increased radiation sensitivity was more related to expression level of C/EBPα than in the PC3 cell line. In addition, the NHEJ assay showed no altered end rejoining in PC3-H cells without DNA damage, and the addition of purified C/EBPα protein into NHEJ reaction with nuclear extract from non-treated PC3 cells did not decrease the end rejoining. Even with bleomycin-iron induction of DNA damage, NHEJ in the PC3 cell line was weaker than that seen with the Du145 cells. Because the levels of Ku70 and Ku80 were similar in the two cell lines, it was of interest to examine the role of PARP-1 in mediating the different responses to radiation. Radiation induced a significant increase of PARP-1 expression in the Du145-H cells. Although PARP-1 is important for repair of DNA double-strand breaks, increased expression of PARP-1 will enhance cell death via activation of apoptosis-inducing factor released from the mitochondria with subsequent activation of caspase-independent apoptosis (53, 54). Hence, the net result of PARP-1 activation will be the balance between DNA repair and the induction of apoptosis. In the PC3 cell line, slow migration of PARP-1 in the PC3-H cells raises the possibility that protein modification of PARP-1 could affect PARP-1 function. PARP-1 has previously been demonstrated to be phosphorylated by DNA-dependent protein kinase, although it is not certain if the phosphorylation inhibited PARP-1 activity or if the binding of the kinase to PARP-1 was responsible for decreased PARP-1 activity (49). The nature of the modification induced by overexpression of C/EBPα and how this modification affects PARP-1 activity in PC3 cells needs to be investigated. Additionally, a PARP-1-dependent double-strand break end-joining activity may exist as an alternative route to non-homologous end-joining repair (55). Perhaps one or another repair route is favored in one cell type or under a particular condition. Also, the differences in DNA repair between the PC3-H and Du145-H cells may reflect even more complicated protein interactions than what we have found.

In addition to the apparent interference of C/EBPα with Ku- and PARP-1-mediated DNA repair, it is possible that the binding of Ku70, Ku80, and PARP-1 to C/EBPα could alter C/EBPα function. To address this possibility, we examined the effect of forced expression of Ku70, Ku80, and PARP-1 on transcription regulation by C/EBPα of the PSA promoter. In LNCaP cells, co-transfection of C/EBPα with Ku70, Ku80, or PARP-1 had no effect on the inhibition of the PSA promoter by C/EBPα (data not shown). It seems then that under our experimental conditions the interaction of C/EBPα with the Ku proteins and PARP-1 does not affect transcription regulation by C/EBPα.

In summary, we report newly identified protein partners of C/EBPα and that these associated proteins contribute to increasing radiosensitivity and block NHEJ in prostate cancer cell lines, especially in the Du145 prostate cell line. These findings suggest a new avenue for prostate cancer therapy.

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