Clusterin is a critical downstream mediator of stress-induced YB-1 transactivation in prostate cancer

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Abstract

Clusterin (CLU) is a stress-activated, cytoprotective chaperone that confers broad-spectrum treatment resistance in cancer. However, the molecular mechanisms mediating CLU transcription following anti-cancer treatment stress remain incompletely defined. We report that Y-box binding protein-1 (YB-1) directly binds to CLU promoter regions to transcriptionally regulate CLU expression. In response to endoplasmic reticulum (ER)-stress inducers, including paclitaxel, YB-1 is translocated to the nucleus to transactivate CLU. Furthermore, higher levels of activated YB-1 and CLU are seen in taxane-resistant, compared with parental, prostate cancer cells. Knockdown of either YB-1 or CLU sensitized prostate cancer cells to paclitaxel, while their overexpression increased resistance to taxane. CLU overexpression rescued cells from increased paclitaxel-induced apoptosis following YB-1 knockdown; in contrast, however, YB-1 overexpression did not rescue cells from increased paclitaxel-induced apoptosis following CLU knockdown. Collectively, these data indicate that YB-1 transactivation of CLU in response to stress is a critical mediator of paclitaxel resistance in prostate cancer.
Introduction

Prostate cancer is the most common noncutaneous cancer and the second leading cause of cancer-related death in men of Western countries. The incidence of prostate cancer is increasing as a result of aging populations and high-fat diet in the western world (1). While advanced prostate cancer is initially sensitive to androgen deprivation therapy (ADT), most eventually recur in a castration-resistant prostate cancer (CRPC). At present, only second-line ADT with abiraterone (2), and the taxanes docetaxel (3) and cabazitaxel (4) have been shown to delay progression and prolong survival in CRPC. However, therapeutic benefits are brief with under four month gains in survival before treatment resistance, progression, and death occurs. Many strategies used to kill cancer cells, including ADT or taxane chemotherapy, induce a treatment resistant phenotype that is the underlying basis for most cancer deaths. Improved understanding of the molecular basis underlying metastasis and resistance to ADT or chemotherapy will facilitate to design new therapeutic strategies to inhibit the emergence of this CRPC phenotype.

CRPC progression is a complex process by which cells acquire the ability to both survive and proliferate in the absence of gonadal androgens and involves mechanisms attributed to re-activation of the androgen receptor axis (5), alternative growth factor pathways (6, 7), stress-induced pro-survival genes (8–10), and cytoprotective chaperone networks (11, 12). Clusterin (CLU) is a stress-induced cytoprotective chaperone expressed in many human cancers. In prostate cancer, CLU levels increase following castration and in CRPC models (11). CLU levels are low in untreated, favorable grade tissues, but increase with higher Gleason grade (13) and within weeks after treatment stress with ADT (14) or taxane chemotherapy (15). Overexpression of
CLU in human prostate cancer LNCaP cells accelerates progression after ADT (11) or chemotherapy (16), identifying CLU as an anti-apoptotic gene upregulated by treatment-related stress that confers therapeutic resistance when overexpressed. Miyake et al. first reported that antisense treatment against CLU significantly enhanced paclitaxel sensitivity in human prostate cancer PC-3 cells (10). Moreover, CLU knockdown reduced tumor growth and sensitized to chemotherapeutic agents in various cancer cells including renal cell cancer (17), bladder cancer (18), lung cancer (19) and breast cancer (20).

Y-box binding protein-1 (YB-1) is a stress-activated transcription factor implicated in and linked to unfavorable clinical outcome in various cancers (21). In prostate cancer, YB-1 is induced by ADT (22) and its overexpression confers castration resistance (23). Moreover, YB-1 is associated with drug resistance to both cisplatin (24, 25) and paclitaxel (26, 27). YB-1 confers paclitaxel resistance in PC-3 cells (27), while its knockdown sensitized breast cancer SUM149 cells to paclitaxel (28).

While the above findings functionally link YB-1 and CLU to treatment resistance in prostate cancer, the precise mechanisms mediating these effects remain undefined. Here, we define functional links between stress-induced increases in YB-1 and CLU, and analyze their biological relevance, as related to taxane resistance in prostate cancer.
Materials and Methods

Cell culture and transfection

The human prostate cancer cell lines, PC-3 and DU145, were purchased from the American Type Culture Collection (2008 and 1989, ATCC authentication by isoenzymes analysis) and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Scientific, Waltham, MA) supplemented with 5% fetal-bovine serum (FBS). LNCaP cells were kindly provided by Dr. Leland W.K. Chung (1992, MD Anderson Cancer Center, Houston, TX), tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in 2009. LNCaP cells were maintained in RPMI 1640 (Thermo Scientific) supplemented with 5% FBS. Docetaxel-resistant derivatives of LNCaP and DU145 cells (LNCaP/DTX and DU145/DTX cells) were established by long-term culture under the appropriate medium containing gradually increasing concentrations of docetaxel and maintained under media containing 3 ng/mL and 2 ng/mL of docetaxel, respectively. The LNCaP/DTX and DU145/DTX cells were found to be about 2.5-fold more resistant to docetaxel than their parental cells. Cells were transfected with the indicated siRNA or the indicated plasmid as previously described (29).

Antibodies and reagents

Antibodies against ATF4 (sc-200), Myc (9E10, sc-815), GRP78 (sc-1051) and CLU (sc-6419) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Lamin B1 antibody was purchased from Abcam (Cambridge, MA). Anti-phosphorylated YB-1 (p-YB-1, #2900), anti-cleaved PARP (#9541), anti-PARP
and anti-cleaved caspase3 (#9661) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-YB-1, anti-caspase3 and anti-β-actin antibodies were purchased from Epitomics (Burlingame, CA), BD Biosciences (San Jose, CA) and Sigma (St Louis, MO), respectively. Paclitaxel and docetaxel were obtained from Sigma. MG132 and 17-allylamino-17-demethoxy-geldanamycin (17-AAG) was purchased from Calbiochem (Gibbstown, NJ).

Plasmids

The pCMV-myc-YB-1 plasmid expressing the N-terminal, myc-tagged YB-1 protein, as well as the pCMV-YB-1-myc-nuc and pCMV-YB-1-myc-cyto plasmids expressing the C-terminal, myc-tagged YB-1 protein (with or without nuclear-localizing signaling [NLS], respectively), were constructed as described previously (23). The pRC/CMV-CLU plasmid expressing the CLU protein was kindly provided from Dr. Martin P. Tenniswood (State University of New York at Albany, Rensselaer, NY) (30).

Various lengths of the promoter and partial first exon of the wild-type CLU gene were amplified by PCR using genomic DNA and the following primer pairs: 5’-CTCGAGCATGGCAGGTAGTGAGCTCCCTG-3’ and 5’-AGATCTGTGTCCAGAGGGTTTGCT-3’ for CLU-Luc –1998/–702; 5’-AGATCTGATTTCCTAACTGGGAAGG-3’ and 5’-AAGCTTGAGCTGTGTCATCCCTCTGCCT-3’ for CLU-Luc –707/+254. The resultant PCR products were cloned and ligated into the pGL3-basic vector (Promega, Madison, WI). CLU-Luc –1998/+254 was constructed by inserting CLU-Luc –707/+254 fragment digested by BglII and HindIII into BglII and HindIII site of CLU-Luc –1998/–702. CLU-Luc –1116/–702 was constructed from CLU-Luc –1198/–702 by deletion of the
SmaI and PshAI fragments.

siRNAs

The following double-stranded 25-bp siRNA oligonucleotides were commercially generated (Invitrogen, Carlsbad, CA): 5’-UUUGCUGGUAAUUGCGUGGAGGACC-3’ for YB-1 siRNA #1; 5’-UGGAUAGCGUCUAUAAUGGUUACGG-3’ for YB-1 siRNA #2. The sequence of siRNA corresponding to the human CLU initiation site in exon II was 5’-GCAGCAGAGUCUUCAUCAU-3’ (Dharmacon Research Inc., Lafayette, CO). Stealth™ RNAi Negative Control Medium GC Duplex #2 (Invitrogen) was used as a control siRNA.

Quantitative reverse transcription (RT)-PCR

RNA extraction and RT-PCR were performed as described previously (31). Real time monitoring of PCR amplification of cDNA was performed using the following primer pairs and probes, YB-1 (Hs00898625_g1), CLU (Hs00156548_m1) and GAPDH (Hs03929097_g1) (Applied Biosystems) on ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with TaqMan Gene Expression Master Mix (Applied Biosystems). Target gene expression was normalized to GAPDH levels in respective samples as an internal control. The results are representative of at least three independent experiments.

Western blot analysis

Whole-cell extracts were obtained by lysis of cells in an appropriate volume of ice-cold RIPA buffer composed of 50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 0.5%
sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) containing 1 mmol/L Na$_3$VO$_4$, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail tablets (Complete, Roche Applied Science, Indianapolis, IN). Nuclear and cytoplasmic extracts were obtained using CelLytic™ NuCLEAR™ Extraction Kit (Sigma) according to manufacturer’s protocol. Cellular extracts were clarified by centrifugation at 13000 x $g$ for 10 min and protein concentrations of the extracts determined by a BCA protein assay kit (Thermo Scientific). Thirty micrograms of the extracts were boiled for 5 min in SDS sample buffer and separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane following standard methods. Membranes were probed with dilutions of primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, proteins were visualized by a chemiluminescent detection system (GE Healthcare, Buckinghamshire, UK).

**Luciferase reporter assay**

Prostate cancer cells were transfected with the indicated reporter plasmid, expression plasmid, or siRNA, and 0.05 μg of pRL-TK as an internal control. After 48 h, the luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and a microplate luminometer (EG&G Berthold). The Firefly luciferase activities were corrected by the corresponding Renilla luciferase activities. The results are representative of at least three independent experiments.

**Chromatin immunoprecipitation assay (ChIP assay)**

Prostate cancer cells were treated with paraformaldehyde and digested with
micrococcal nuclease to achieve a DNA smear of 200–1000 bp. ChIP assay on the CLU and RPL30 genes was performed using SimpleChIP™ Enzymatic Chromatin IP Kit (Agarose Beads) according to the manufacturer’s protocol (Cell Signaling Technology). The quantitative RT-PCR assay was performed using ABI PRISM 7900 HT Sequence Detection System with 2 μL of 20 μL DNA extraction, the primer pairs below and RT² Real-Time™ SYBR Green/Rox PCR master mix (Qiagen, Valencia, CA). The results are representative of at least three independent experiments. The primer pairs for CLU–02 (GPH025704(−)02A) targeting around −1403 bp, CLU–01 (GPH025704(−)01A) targeting around −417 bp and CLU+07 (GPH025704(+)+07A) targeting around +6603 bp were obtained from Qiagen. The primer pairs for RPL30 gene (exon 3) were included in SimpleChIP™ Enzymatic Chromatin IP Kit.

**Immunofluorescence**

Prostate cancer cells were grown on coverslips and treated with 10 nmol/L of paclitaxel for 1 h. After media were refreshed, cells were recovered for 1 h, and fixed in paraformaldehyde for 10 min at room temperature. Immunofluorescence was performed as described previously (32) using YB-1 (1:100) antibody.

**Cell Growth assay**

Prostate cancer cells transfected with the indicated amount of siRNA and expression plasmid were plated in 96-well plates and treated with paclitaxel at indicated concentration. After incubation for 48 h, cell growth was measured using the crystal violet assay as described previously (31). The results are representative of at least three independent experiments.
Flow cytometry analysis

Prostate cancer cells transfected with the indicated amount of siRNA and expression plasmid were plated in 6-well plates and cells were treated with 10 nmol/L of paclitaxel. After incubation for 48 h, cell-cycle fraction was analyzed as described previously (33). The results are representative of at least three independent experiments.

Statistical analysis

All data were assessed using the Student’s t-test. Levels of statistical significance were set at P < 0.05.
Results

YB-1 regulates CLU expression in prostate cancer cells

YB-1 is known to regulate the expression of various genes through transcriptional and translational mechanisms. To investigate the functional link between YB-1 and CLU, we examined CLU expression levels after YB-1 knockdown. CLU expression, both at transcript and protein levels, decreased after YB-1 silencing in prostate cancer LNCaP and PC-3 cells (Figs. 1A and 1B). Conversely, YB-1 overexpression increased CLU mRNA and protein levels in both LNCaP and PC-3 cells (Figs. 1C and 1D).

YB-1 binds to CLU promoter region and regulates CLU transcription

Since YB-1 has been reported to act both transcriptionally (21) and translationally (34), to begin defining mechanisms by which YB-1 regulates CLU expression we searched for putative YB-1 binding sites (Y-boxes) in the CLU promoter region between –2000 bp and +500 bp from transcription start site. Ten Y-boxes were identified in CLU promoter regions (Fig. 2A). Subsequently, we cloned the CLU promoter region and constructed various lengths of CLU reporter plasmids (CLU-Luc –1998/+254, –1998/–702, –1116/–702 and –707/+254) as shown in Fig. 2A, and then performed reporter assays with YB-1 overexpression. The results indicate increased luciferase activity with CLU-Luc –1998/–702 and CLU-Luc –702/+254, but not CLU-Luc –1116/–702, in response to YB-1 overexpression in both LNCaP and PC-3 cells (Fig. 2B). These findings suggest that the cis-element of CLU promoter region containing –702/+254 bp and –1998/–1116 bp was activated by YB-1. Conversely, YB-1 knockdown reduced CLU transcriptional activity in the CLU-Luc –1998/+254 constructs.
in both LNCaP and PC-3 cells (Fig. 2C). These data indicate that YB-1 transcriptionally activates CLU expression.

Next, we set out to determine if YB-1 binds to CLU promoter using ChIP assay and the primer pairs against CLU gene. As shown in Fig. 2D, YB-1 bound to CLU gene regions around −1400 bp and −400 bp from transcription start site represented by CLU−02 and CLU−01, respectively, in both LNCaP and PC-3 cells. In contrast, YB-1 did not bind to the CLU gene around +6600 bp region from transcription start site or PRL30 gene in either cell lines (Fig. 2D).

Nuclear YB-1 regulates CLU transcription

To further confirm transcriptional regulation of CLU by YB-1, we employed different YB-1 expression plasmid that expresses YB-1 proteins with or without a NLS (23). As shown in Fig. 3A and in corroboration with data in Fig. 2, reporter gene assays indicate that YB-1-NLS⁺ plasmids increased CLU-Luc luciferase activity more effectively than YB-1-NLS⁻ in both LNCaP and PC-3 cells (Fig. 3A). Similarly, CLU transcription was induced more effectively by YB-1-NLS⁺ plasmids compared to YB-1-NLS⁻ in both LNCaP and PC-3 cells (Fig. 3B), finding also confirmed at the protein level (Fig. 3C).

Endoplasmic reticulum (ER)-stress inducers increase CLU expression through YB-1

CLU expression is induced by various stresses including proteasome inhibitors like MG132 (35), Hsp90 inhibitors (31) and chemotherapeutics like paclitaxel (15). We next investigated whether these stressors induced CLU expression via YB-1 in prostate cancer cells.
MG132 treatment induced time-dependent expression of ER-stress markers ATF4 and GRP78 in both LNCaP and PC-3 cells (Supplementary Figs. 1A and 1B); similar time-dependent increases in CLU mRNA and protein expression were also observed, while YB-1 was not altered at either mRNA or protein levels. Based on the functional links between YB-1 and CLU expression (Figs. 2 and 3), we next measured changes in CLU expression following ER stress with MG132 when YB-1 was silenced with siRNA. YB-1 knockdown attenuated CLU transcript (Supplementary Fig. 1C) and protein (Supplementary Fig. 1D) induction after MG132 treatment compared with control siRNA. Interestingly, induction of the ER stress markers ATF4 and GRP78 were not affected by YB-1 silencing, suggesting that YB-1 was not a general upstream activator of these markers of ER stress, but rather specifically upregulated CLU.

Similarly, ER stress induction using the Hsp90 inhibitor, 17-AAG, also increased CLU protein and mRNA expression concurrently with ER stress markers ATF4 and GRP78 in both LNCaP and PC-3 cells; again YB-1 expression was not affected (Supplementary Figs. 2A and 2B). When YB-1 was silenced, however, CLU transcript and protein induction by 17-AAG was attenuated compared with control siRNA; again, induction of the ER stress markers ATF4 and GRP78 were not affected by YB-1 silencing (Supplementary Figs. 2C and 2D).

Similar patterns of ER stress and CLU induction, without changes in YB-1 levels, were seen following treatment with paclitaxel in both LNCaP and PC-3 cells (Figs. 4A and 4B); however, YB-1 silencing again attenuated CLU transcript and protein induction by paclitaxel, without affecting induction of ER stress markers ATF4 and GRP78 (Figs. 4C and 4D). Collectively, these data indicate that CLU induction following ER stress is mediated, in part, by YB-1.
Paclitaxel treatment induces YB-1 nuclear translocation and transactivation

Because treatment stress did not increase expression levels of YB-1, we next examined the effects of treatment stress with paclitaxel on YB-1 intracellular localization and transactivation. We examined phosphorylation status of YB-1 after paclitaxel treatment. As shown in Fig. 5A, phosphorylated YB-1 was increased by paclitaxel treatment. Next, we investigated YB-1 localization with or without paclitaxel treatment. Then, the translocation into nucleus was revealed by immunofluorescence against endogenous YB-1 protein and using western blot analysis after LNCaP or PC-3 cell fractionation (Fig. 5B). To confirm that paclitaxel treatment leads to transactivation of YB-1 and CLU expression, we performed ChIP assay after paclitaxel stress. As shown in Fig. 5C, YB-1 binding to CLU promoters (CLU–02 and CLU–01) was enhanced after paclitaxel treatment in both LNCaP and PC-3 cells. These data confirm that paclitaxel-induced stress leads to nuclear translocation of YB-1 with binding to CLU promoter.

YB-1 and CLU are augmented in docetaxel-resistant cells, and involved in paclitaxel resistance

We next examined the expression levels of YB-1 and CLU in prostate cancer cells resistant to docetaxel (LNCaP/DTX and DU145/DTX). As shown in Fig. 6, higher expression levels of both YB-1 and CLU mRNA (Fig. 6A) and protein (Fig. 6B) were seen in both docetaxel-resistant cell lines.

To determine the biological significance of the functional link between YB-1 and CLU, we evaluated the effects of YB-1/CLU knockdown and overexpression on stress
induced prostate cancer cell death. As shown in Supplementary Fig. 3, YB-1 and CLU siRNAs potentially and specifically silenced their target gene in intact and overexpressed cell lines. As expected, YB-1 overexpression led to higher CLU levels, which could be silenced by CLU siRNA; in contrast, CLU overexpression did not affect YB-1 levels. As shown in Fig. 7A, both YB-1 and CLU knockdown sensitized LNCaP and PC-3 cells to paclitaxel, while both YB-1 and CLU overexpression conferred paclitaxel resistance to both cell lines. Importantly, when CLU was silenced, YB-1 overexpression did not significantly rescue cell survival, suggesting that CLU is a critical downstream mediator of stress-induced YB-1 transactivation. Consistent with this view, CLU overexpression almost completely rescued cell survival following YB-1 knockdown. These findings were confirmed by other methods shown in Fig. 7B, where knockdown of either YB-1 or CLU plus paclitaxel stress led to activation of caspase3 and increased cleavage of Poly (ADP-ribose) polymerase (PARP). CLU overexpression with YB-1 knockdown attenuated caspase3 activation and PARP cleavage while YB-1 overexpression with CLU knockdown did not. Similarly, paclitaxel induction of apoptosis, as measured by subG0/G1 fraction using flow cytometry, increased after either YB-1 or CLU knockdown, an effect that was rescued only by overexpression of CLU, but not by YB-1 (Fig. 7C). Collectively, these data confirm that CLU is a critical downstream mediator of stress-induced YB-1 transactivation.
Discussion

YB-1 and CLU are both stress-activated survival factors functionally associated with anti-cancer treatment resistance (21, 36). In prostate cancer, both are induced by castration and confer castration resistance when overexpressed (10, 22, 23). Additionally, both YB-1 and CLU are linked to cytotoxic resistance including cisplatin (18, 24, 25) and taxanes (16, 19, 20, 26–28). YB-1 is a transcription factor that binds to inverted CCAAT “Y” boxes present in the promoter region of a variety of genes, including MHC class II gene, EGFR, PCNA, DNA polymerase a, topoisomerase Ila, MDR-1, and MMP-2. The cascades of gene expression regulated by YB-1 have been linked to genotoxic stress, drug resistance, and metastatic invasion (21).

While YB-1 and CLU are functionally linked to treatment resistance in cancer, the role of YB-1 in stress-induced CLU transactivation and cytoprotection is undefined. Promoter regions of CLU gene are conserved during evolution, and include stress-associated sites like activator-protein-1 (AP-1), AP-2, SP-1 (stimulatory element), HSE (heat shock element), CRE (cAMP response element), and a “CLU-specific element” (CLE) recognized by HSF-1/HSF-2 heterocomplexes (37) as well as steroid response elements including glucocorticoid and androgen response element sites (38). Our initial analysis of the CLU promoter region identified 10 putative YB-1 binding sites (Y-boxes), which led us to investigate the role of YB-1 in stress-induced CLU transactivation.

YB-1 is known to be activated by many varied stressors via phosphorylation by AKT (39) and p90RSK (40), stimulating its translocation to the nucleus. Stresses linked to activation of YB-1 include genotoxic stress with radiation (41, 42) and cisplatin (43),
as well as paclitaxel (26, 28). In this study, we show that ER stress associated with paclitaxel, proteasome inhibition, or Hsp90 inhibition, led to YB-1 nuclear translocation. Because CLU is a potent inhibitor of protein aggregation and functions like small heat shock proteins to chaperone and stabilize proteins under stress, we used these ER stress inducers to define the role of YB-1 in regulation of CLU transcription. We further focused on paclitaxel activation of YB-1 and CLU since taxane-based chemotherapy is the standard first-line therapy for men with metastatic CRPC (3).

Although it was previously reported that CLU is overexpressed in taxane-resistant cells (44, 45), the status of YB-1 in taxane-resistant cells was unknown. This study revealed that YB-1 is overexpressed in taxane-resistant prostate cancer cells. Furthermore, we show that both YB-1 and CLU are functionally involved in paclitaxel resistance, findings compatible with previous reports (16, 26, 27). For example, since YB-1 regulates P-glycoprotein (P-gp) expression, and paclitaxel is a substrate of P-gp, it is reasonable to speculate that YB-1 may affect paclitaxel sensitivity via P-gp. However, prostate cancer cell lines like LNCaP and PC-3 cells express P-gp at undetectable levels (46), and hence it is likely that YB-1 affects paclitaxel sensitivity through factors other than P-gp. CLU is an extracellular chaperone which directly interacts with paclitaxel and confers paclitaxel resistance (47). Our studies indicate that CLU acts a critical mediator of stress-induced YB-1 activity and paclitaxel resistance. CLU overexpression reversed increases in paclitaxel-induced apoptosis following YB-1 knockdown; in contrast, however, YB-1 overexpression did not rescue cells from increased paclitaxel-induced apoptosis following CLU knockdown. Additionally, these data indicate that both YB-1 and CLU confer taxane resistance irrespective of p53 and androgen receptor (AR) expression status because LNCaP cells express wild-type p53.
as well as AR while PC-3 cells express neither p53 nor AR.

Many strategies used to kill cancer cells induce stress-responses that promote the emergence of a treatment resistant phenotype and novel therapeutic approaches targeting these stress responses are being developed. With respect to YB-1, a small molecular inhibitor of integrin-linked kinase (ILK), QLT0267, was found to inhibit YB-1 with suppression of breast cancer cell growth (48). Alternatively, a molecular decoy to YB-1 also inhibited both breast cancer and prostate cancer cell growth without affecting normal immortalized breast epithelial cells and primary breast epithelial cells (49). CLU is being targeted using the antisense oligonucleotide (OGX-011) now in phase III clinical trials in CRPC after randomized Phase II studies reported a significant survival benefit when OGX-011 was added to docetaxel (50). Previously, we reported that CLU downregulation using OGX-011 resensitized docetaxel-resistant prostate cancer cells to docetaxel and mitoxantrone, and sensitized breast cancer cells to paclitaxel (20). While inhibition of either YB-1 or CLU sensitizes prostate cancer cells to paclitaxel, CLU overexpression can reverse increases in paclitaxel-induced apoptosis following YB-1 knockdown; in contrast, however, YB-1 overexpression did not rescue cells from increased paclitaxel-induced apoptosis following CLU knockdown, suggesting that CLU may be a more relevant therapeutic target.

In summary, YB-1 transcriptionally enhances CLU expression following ER-stress in prostate cancer cells, resulting in inhibition of treatment-induced apoptosis and taxane resistance. While inhibition of either YB-1 or CLU sensitized prostate cancer cells to paclitaxel, CLU appears to play a more dominant role. Collectively, these data indicate that YB-1 transactivation of CLU in response to stress is a critical mediator of paclitaxel resistance in prostate cancer.
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Figure legends

Figure 1 YB-1 regulates CLU expression in prostate cancer cells. A and B. LNCaP (A) and PC-3 (B) cells were transfected with 40 nmol/L of the indicated siRNA. At 48 h after transfection, quantitative RT-PCR was performed using the primer pairs and probes for YB-1, CLU and GAPDH. Each transcript level from cells transfected with control siRNA was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with cells transfected with control siRNA). Whole-cell extracts were analyzed by SDS–PAGE and western blot analysis with specific antibodies. C and D. LNCaP (C) and PC-3 (D) cells were transfected with 1.0 μg/mL of the indicated expression plasmid. At 72 h after transfection, quantitative RT-PCR was performed using the primer pairs and probes for CLU and GAPDH. Each transcript level from cells transfected with Myc expression plasmid was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with cells transfected with Myc expression plasmid). Whole-cell extracts were analyzed by SDS–PAGE and western blot analysis with specific antibodies.

Figure 2 YB-1 binds to CLU promoter region and regulates CLU transcription. A. Schematic representation of the promoter region and 5’ end of the CLU gene. Double bars, Y-boxes (5’-ATTG-3’); gray box, CLE; white box, AP-1-binding site; rail, primer pairs used in (D). CLU-Luc plasmids (−1998/+254, −1998/−702, −1116/−702 and −707/+254) used in (B) and (C) are shown. B. LNCaP and PC-3 cells were cotransfected with 0.5 μg/mL of the various CLU-Luc plasmids, 0.5 μg/mL of myc or myc-YB-1 expression plasmid and 0.05 μg/mL of pRL-TK. The luciferase activity of CLU-Luc −1998/+254 alone was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05.
(compared with cells transfected with myc expression plasmid). C. LNCaP and PC-3 cells were cotransfected with 0.5 μg/mL of the various CLU-Luc plasmids, 20 nmol/L of the indicated siRNA and 0.05 μg/mL of pRL-TK. The luciferase activity of CLU-Luc –1998/+254 alone was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with cells transfected with control siRNA). D. ChIP assays were performed on nuclear extracts from LNCaP and PC-3 cells using 2.0 μg of the indicated antibodies and 20 μL of Protein G agarose. The quantitative RT-PCR was performed using immunoprecipitated DNAs, soluble chromatin and specific primer pairs for the CLU and RPL30 genes. The results of immunoprecipitated samples were corrected for the results of the corresponding soluble chromatin samples. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with immunoprecipitants by IgG).

Figure 3 Nuclear YB-1 regulates CLU transcription. A. LNCaP and PC-3 cells were cotransfected with 0.5 μg/mL of CLU-Luc –1998/+254 plasmid, 0.5 μg/mL of the indicated expression plasmid and 0.05 μg/mL of pRL-TK. The luciferase activity of myc-nuc expression plasmid was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with cells transfected with myc expression plasmid). B. LNCaP and PC-3 cells were transfected with 1.0 μg/mL of the indicated expression plasmid. At 48 h after transfection, quantitative RT-PCR was performed using the primer pairs and probes for CLU and GAPDH. Each transcript level from cells transfected with myc-nuc was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with cells transfected with myc expression plasmid). C. LNCaP and PC-3 cells were transfected with 1.0 μg/mL of the indicated expression plasmid. At 48 h after transfection, whole-cell extracts were analyzed for by SDS–PAGE and western blot analysis with specific antibodies.
Figure 4 Paclitaxel treatment increases CLU expression through YB-1. A. LNCaP and PC-3 cells were cultured with 10 nmol/L of paclitaxel for 1 h, then changed into fresh media, and further incubated for the indicated duration. After the cells were harvested, whole-cell extracts were analyzed by SDS–PAGE and western blot analysis with specific antibodies. B. LNCaP and PC-3 cells cultured as described in (A). After the cells were harvested, quantitative RT-PCR was performed using the primer pairs and probes for YB-1, CLU and GAPDH. Each transcript level from cells treated with vehicle was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with cells treated with vehicle). C. LNCaP and PC-3 cells were transfected with 40 nmol/L of the indicated siRNA and incubated for 48 h. Then, cells were cultured with cultured with 10 nmol/L of paclitaxel for 1 h, then changed into fresh media, and further incubated for the indicated duration. After the cells were harvested, quantitative RT-PCR was performed using the primer pairs and probes for YB-1, CLU and GAPDH. Each transcript level from cells transfected with control siRNA and treated with vehicle was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with cells treated with vehicle). D. LNCaP and PC-3 cells were transfected and cultured as described in (C). After the cells were harvested, whole-cell extracts were analyzed by SDS–PAGE and western blot analysis with specific antibodies.

Figure 5 Paclitaxel treatment induces YB-1 nuclear translocation and transactivation. A. LNCaP and PC-3 cells were cultured with 10 nmol/L of paclitaxel for 1 h, then changed into fresh media, and further incubated for the indicated duration. After the cells were harvested, whole-cell extracts were analyzed by SDS–PAGE and western blot analysis.
with specific antibodies. **B.** LNCaP and PC-3 cells cultured with 10 nmol/L of paclitaxel for 1 h, then changed into fresh media, and further incubated for 1 h. After the cells were fixed, immunofluorescence was performed for YB-1 (green). Then, cells were stained with DAPI (blue) and observed by fluorescence microscopy. Representative images of high-magnification fields (400×) are shown. Bars, 10 μm. For western blotting, after cells were harvested and fractioned into nuclear and cytoplasmic extracts, extracts were analyzed by SDS–PAGE and western blot analysis with specific antibodies. **C.** ChIP assays were performed on nuclear extracts from LNCaP and PC-3 cells cultured as described in (B) using 2.0 μg of the indicated antibodies and 20 μL of Protein G agarose. The quantitative RT-PCR was performed using immunoprecipitated DNAs, soluble chromatin and specific primer pairs for the CLU gene. Results of immunoprecipitated samples were corrected for the results of the corresponding soluble chromatin samples. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with immunoprecipitants from cell treated with vehicle).

**Figure 6** YB-1 and CLU are increased in docetaxel-resistant cells. **A.** Quantitative RT-PCR using cDNAs from LNCaP, LNCaP/DTX, DU145 and DU145/DTX cells was performed using the primer pairs and probes for YB-1, CLU and GAPDH. Each transcript level from parental cells was set as 1. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with parental cells). **B.** Whole-cell extracts were analyzed by SDS–PAGE and western blot analysis with specific antibodies.

**Figure 7** YB-1 and CLU are involved in paclitaxel resistance. **A.** LNCaP and PC-3 cells transfected with 20 nmol/L of the indicated siRNA and 0.5 μg/mL of the indicated
expression plasmid were seeded into 96-well plates. The following day, 10 nmol/L of paclitaxel were applied. After incubation for 48 h, cell survival was analyzed by a cell growth assay. Cell growth in the absence of paclitaxel corresponds to 1. Boxes, mean; bars, ± s.d. *P < 0.05 (compared with cells transfected with mock expression plasmid).

B. Whole-cell extracts from LNCaP and PC-3 cells were transfected as described in (A) and treated with 10 nmol/L of paclitaxel for 48 h were analyzed by SDS–PAGE and western blot analysis with specific antibodies. C. LNCaP and PC-3 cells transfected as described in (A) were seeded into 6-well plates. The following day, 10 nmol/L of paclitaxel were applied. After incubation for 48 h, the cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of sub G0/G1 fraction is shown. Boxes, mean; bars, ± s.d. *P < 0.05 (compared with cells transfected with mock expression plasmid).
**Figure 1 Shiota et al.**

**A**

![Graph showing mRNA expression levels in LNCaP cells with different siRNA treatments.](image)

**B**

![Graph showing mRNA expression levels in PC-3 cells with different siRNA treatments.](image)

**C**

![Graph showing mRNA expression levels in LNCaP cells with Myc and Myc-YB-1 expression.](image)

**D**

![Graph showing mRNA expression levels in PC-3 cells with Myc-YB-1 expression.](image)
Figure 3 Shiota et al.

A

B

C

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Figure 4 Shiota et al.

A

\[ \text{LNCaP} \]

\[ \text{PC-3} \]

- 6 12 24 Paclitaxel (h)

CLU

YB-1

ATF4

GRP78

\( \beta \)-actin

B

\[ \text{LNCaP} \]

\[ \text{PC-3} \]

Paclitaxel

Relative mRNA expression

Vehicle 6 h 12 h 24 h

* * *

C

\[ \text{LNCaP} \]

\[ \text{PC-3} \]

Control siRNA

YB-1 siRNA #1

Relative mRNA expression

Vehicle 6 h 12 h 24 h

* * *

D

\[ \text{LNCaP} \]

\[ \text{PC-3} \]

Control siRNA

YB-1 siRNA #1

Paclitaxel (h)

CLU

YB-1

ATF4

GRP78

\( \beta \)-actin
Figure 6 Shiota et al.

A

B
**Figure 7 Shiota et al.**

A. Graphs showing relative cell viability of LNCaP and PC-3 cells treated with control siRNA or siRNA targeting CLU and YB-1, respectively.

B. Western blots showing cleaved PARP, PARP, cleaved caspase3, caspase3, and β-actin in LNCaP and PC-3 cells treated with control siRNA or siRNA targeting CLU and YB-1, respectively.

C. Histograms showing the sub G0/G1 fraction (%) of LNCaP and PC-3 cells treated with control siRNA or siRNA targeting CLU and YB-1, respectively.
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Clusterin is a critical downstream mediator of stress-induced YB-1 transactivation in prostate cancer

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