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Original

Distinct promoters, subjected to epigenetic regulation, drive the expression of two clusterin mRNAs in prostate cancer cells / Bonacini, Martina; Coletta, Mariangela; Ramazzina, Ileana; Naponelli, Valeria; Modernelli, Alice; Davalli, Pierpaola; Bettuzzi, Saverio; Rizzi, Federica Maria Angela. - In: BIOCHIMICA ET BIOPHYSICA ACTA. - ISSN 0006-3002. - 1849:1(2015), pp. 44-54. [10.1016/j.bbagr.2014.11.003]

Availability:

This version is available at: 11381/2787332 since: 2021-10-29T11:56:06Z

Publisher:

Elsevier

Published

DOI:10.1016/j.bbagr.2014.11.003

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Distinct promoters, subjected to epigenetic regulation, drive the expression of two clusterin mRNAs in prostate cancer cellsMartina Bonacini^aMariangela Coletta^aIleana Ramazzina^{a, b, c}Valeria Naponelli^{a, b, c}Alice Modernelli^aPierpaola Davalli^dSaverio Bettuzzi^{a, b, c, *}

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Abstract

The human clusterin (CLU) gene codes for several mRNAs characterized by different sequences at their 5' end. We investigated the expression of two CLU mRNAs, called CLU 1 and CLU 2, in immortalized (PNT1a) and tumorigenic (PC3 and DU145) prostate epithelial cells, as well as in normal fetal fibroblasts (WI38) following the administration of the epigenetic drugs 5-aza-2'-deoxycytidine (AZDC) and trichostatin A (TSA) given either as single or combined treatment (AZDC-TSA).

Our experimental evidences show that:

- a) CLU 1 is the most abundant transcript variant.
- b) CLU 2 is expressed at a low level in normal fibroblasts and virtually absent in prostate cancer cells.
- c) CLU 1, and to a greater extent CLU 2 expression, increased by AZDC-TSA treatment in prostate cancer cells.
- d) Both CLU 1 and CLU 2 encode for secreted CLU.
- e) P2, a novel promoter that overlaps the CLU 2 Transcription Start Site (TSS), drives CLU 2 expression.
- f) A CpG island, methylated in prostate cancer cells and not in normal fibroblasts, is responsible for long-term heritable regulation of CLU 1 expression.
- g) ChIP assay of histone tail modifications at CLU promoters (P1 and P2) shows that treatment of prostate cancer cells with AZDC-TSA causes enrichment of Histone3(Lys9)acetylated (H3K9ac) and reduction of Histone3(Lys27)trimethylated (H3K27me3), inducing active transcription of both CLU variants.

In conclusion, we show for the first time that the expression of CLU 2 mRNA is driven by a novel promoter, P2, whose activity responds to epigenetic drugs treatment through changes in histone modifications.

Abbreviations: CLU, clusterin; TCF-1, T-cell factor-1; AP1, activating protein-1; AP2, activating protein-2; SP1, specificity protein 1; HSE, heat-shock response elements; CEBP, CCAAT-enhancer-binding protein; TSA, trichostatin A; NFI, nuclear factor I; YY1, Yin Yang 1; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; MYCN, basic helix-loop-helix protein 37; STAT, signal transducer and activator of transcription; AZDC, 5-aza-2'-deoxycytidine; H3K9ac, Histone3(Lys9)acetylated; H3K27me3, Histone3(Lys27)trimethylated; PARP, poly ADP-ribose polymerase; TBP, TATA box binding protein; SOX2, Sry-like HMG box genes 2; ARP-1, apolipoprotein repressor protein-1; APOJ, apolipoprotein J; HNF-4, Hepatocyte Nuclear Factor 4; TSS, transcription start site

Keywords: **clusterin**Clusterin; **epigenetic**Epigenetic regulation; **alternative**Alternative promoter; **prostate**Prostate cancer

1 Introduction

The human single copy clusterin (CLU) gene is located on chromosome 8 and organized into nine exons [1]. It is expressed almost ubiquitously during development and in many adult tissues, and encodes a glycosylated, secreted heterodimeric protein of approximately 75 kDa called sCLU. Under stress conditions, CLU protein may escape the canonical secretory pathway. Due to altered biogenesis, different protein forms may localize in various intracellular compartments like cytoplasm, mitochondria, microsomes, nuclei and cell membranes [2–6].

CLU is a stress-inducible gene responding to a variety of stimuli including oxidative stress, ionizing radiation and heat shock [7]. Cytokines and growth factors either promote or suppress CLU expression in vitro [8]. The human CLU gene encodes for three known mRNA variants as currently listed in the NCBI database: variant 1 (NM_001831), variant 2 (NR_038335) and variant 3 (NR_045494). All variants, identified in human colon and prostate cells [9,10], are transcribed as pre-mRNAs and comprise 9 exons and 8 introns. Exon 1 sequences are unique to each of the three mRNAs (Fig. 1). The existence of alternative first exons, here called 1a, 1b and 1c suggests the initiation of transcription from distinct transcription start sites (TSSs). Transfection of cDNAs corresponding to the three CLU transcript variants in HEK-293 cells leads to abundant expression of sCLU plus lesser amounts of unglycosylated intracellular protein forms. These rare protein forms are possibly produced by mechanisms of alternative translation extensively studied by Prochnow et al. [10]. CLU mRNA variants 1 and 2, hereafter named CLU 1 and CLU 2, are differentially regulated by androgens in prostate cells [11] and by T-cell factor-1 (TCF-1) in colonocytes [12].

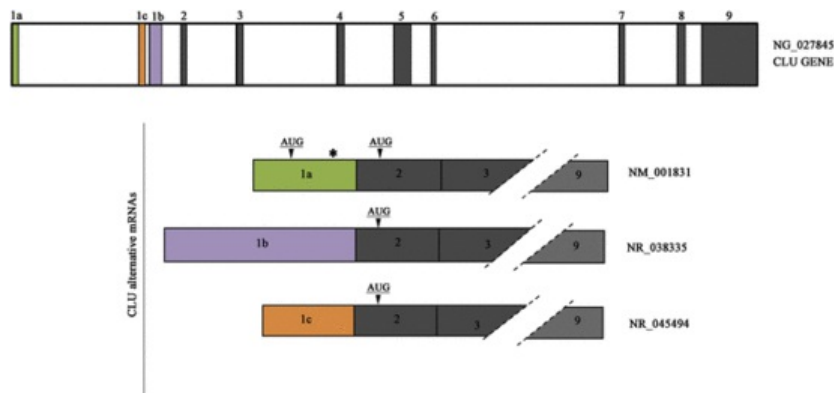


Fig. 1 The human CLU gene and its mRNA variants.

The human CLU gene maps on Chr8 (8p21-p12); it encodes and encodes three different mRNA variants listed in NCBI database as: NM_001831 (CLU 1 in the text), NR_038335 (CLU 2 in the text) and NR_045494 (not investigated in this paper). Exon 1 sequence is unique to each mRNA and is indicated as exon 1a, 1b and 1c; exons 2–9 are present in all the transcripts. All mRNAs are translated from an AUG located on exon 2 (see the arrows) encoding for a secreted glycosylated protein (sCLU). The asterisk in exon 1a indicates the position of the forward primer used to amplify CLU 1. Amplification with primers annealing upstream to the in-frame AUG of exon 1a failed to produce a result.

CLU expression in mammals has been thought to be controlled by one promoter upstream of the transcription start site of CLU 1, hereafter called P1. This regulatory sequence has a conventional TATA box element plus some potential cis-regulatory elements including activating protein-1 (AP1), activating protein-2 (AP2), specificity protein 1 (SP1) motifs [1] and a sequence with high homology to the heat-shock response element (HSE) [13].

CLU P1 was found hypermethylated in human and mouse prostate cancer cells; P1 methylation correlates with down-regulation of CLU mRNA in human prostate cancer cells and tissue specimens [14]. Differential expression of the CLU mRNAs differing in their 5' ends in tissues and cell lines leads to the hypothesis that CLU 1 and CLU 2 transcription may be directed by two distinct promoters. Alternative promoter usage for CLU was first demonstrated in the quail, where two

distinct promoters regulate the expression of two mRNAs, differing in their first exon in a coordinated fashion [13].

Considering the state of the art of CLU gene regulation studies, the aim of the present work is to investigate CLU 1 and CLU 2 expression in different type of cell lines, with a special focus on prostate cancer cells. We intend to approach the gene regulatory region upstream from the exon 1b that is, possibly, responsible for CLU 2 expression. Since CLU mRNA and protein are known to sensibly increase in human neuroblastoma [15] and tumor-conditioned endothelial cells [16] upon administration of drugs which act at the epigenetic level, we investigate the epigenetic mechanisms of CLU regulation by treating prostate cancer cell lines with TSA, a histone deacetylases (HDAC) inhibitor, and 5-aza-2'-deoxycytidine, a DNA methyltransferase (DNMT) inhibitor.

2 Materials and methods

2.1 Reagents and cell cultures

PC3, DU145 and WI38 cells were purchased from the American Tissue Culture Collection. PNT1a cells were kindly donated by Prof. N. J. Maitland (Yorkshire Cancer Center Unit, York, GB). BEAS2B, A549, MCF7 cells were a kind gift from Dr. F. Luppi (University of Modena and Reggio Emilia, Modena, IT) and HTB-125 from Dr. L. Bruni (University of Parma, Parma, IT). PNT1a, DU145, A549, MCF7 and HTB-125 cells were routinely grown in RPMI 1640; BEAS2B in LHC-9/RPMI medium, PC3 cells in Ham's F12:DMEM medium (1:1) and WI38 cells in DMEM-high glucose. The culture media were supplemented with 10% fetal bovine serum (Lonza, Basel, CH), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. All the cells lines were incubated at 37 °C under a 5% CO₂ atmosphere, and harvested by Trypsin/EDTA (Sigma-Aldrich, Steinheim, DE). PC3 and DU145, after being plated at a density of 70 × 10³ cells/mL for 24 h, underwent a treatment in which TSA (1 µM) or AZDC (10 µM) were administered for 24 and 72 h, respectively. In other experiments TSA (1 µM) was administered after AZDC for additional 24 h (AZDC-TSA treatment). In all the experiments drugs and medium were refreshed every 24 h. Both AZDC and TSA were purchased from Sigma-Aldrich.

2.2 RNA extraction and cDNA preparation

All the cells lines were grown in 100 mm dishes to 80% confluence in order to extract RNA by Trizol (1 mL) (Fisher Molecular Biology, Rome, IT). Total RNA (1 µg) was heated at 100 °C for 5 min, and then immediately chilled on ice for 10 min. The synthesis of cDNA was carried out utilizing Random Primers and ImProm-II reverse Transcription System (Promega, Madison, WI) according to [manufacturer's instructions](#).

2.3 RT-PCR and quantitative real time RT-PCR (qPCR) for CLU mRNAs

The CLU mRNA variants were detected by RT-PCR using GoTaq® Flexi DNA Polymerase (Promega, Madison, WI) according to [manufacturer's instructions](#). Sequences of primers and annealing temperatures are reported in Table 1. The abundance of CLU mRNA variants was measured by qPCR, using the 2X SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, JA). The amplification was carried on a MJ Opticon 4 instrument (MJ Research, Waltham, MA). Relative quantification was calculated by the 2^{-ΔΔCT} method [17] using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeper gene for data normalization. The results are expressed as mean ± SD of four independent determinations run in duplicate.

Table 1 Sequences of the primers used in RT-PCR, qPCR, MSP and ChIP qPCR.

Technique	Primer FW5' → 3'	Primer RW5' → 3'	Ta (°C)	Cycles
<i>RT-PCR</i>				
CLU TOT	GGACATATGATGAAGACTCTGCTG	TCAGGCAGGGCTTACACTCT	57	23
CLU 1	AGGAGCGCGGGCACAG	TCAGGCAGGGCTTACACTCT	59	25
NM_001831	GCGAGCAGAGCGCTATAAAT	TCAGGCAGGGCTTACACTCT	55	38
CLU 2	CAGAGCAAGAGGACTCATCC	TCAGGCAGGGCTTACACTCT	27	38
GAPDH	AACCTGCCAAATATGATGAC	TTGAAGTCAGAGGAGACCAC	59	23
<i>qPCR</i>				
CLU TOT	TGATCCCATCACTGTGACGG	GCTTTTTGCGGTATTCCTGC	60	40
CLU 1	ACAGGGTGCCGCTGACC	CAGCAGAGTCTTCATCATGCC	60	40
NM_001831	CTCCAGTGCCACAACGC	CCACAAACAGCAGCAGAGTC	56	40
CLU 2	CCACTGCGAACCCCTCTCTAC	CCTTCACACCGAATCCATCT	60	40

GAPDH	AACCTGCCAAATATGATGAC	TTGAAGTCAGAGGAGACCAC	60	40
<i>MSP</i>				
M CLU	ATTGGGATAGATAGTCGGGTTAATC	CTCCAAAAAAAACCCATAAATACG	59	40
U CLU	TTGGGATAGATAGTTGGGTTAATTG	AACTCCAAAAAAAACCCATAAATAC	61	40
<i>ChIP qPCR</i>				
TBP P1	ATTCTTTGGGCGTGAGTCAT	GTGGCTCTGCTCAAGGGTAG	62	40
TBP P2	ATGCAACAGCCTCAGCTTCT	CCAGAGAAAGTCCCTTTGGA	60	40
H3K9ac P1	ATTCTTTGGGCGTGAGTCAT	GTGGCTCTGCTCAAGGGTAG	62	40
H3K9ac P2	TCTGGCATGACAAAGGCTCT	GGGAGAAGAGATGGGGTCAG	62	40
H3K27me3 P1	TGCTCTCTTCTCCAGCACAA	TCCAGTAGGGAAGAGCCTGA	62	40
H3K27me3 P2	GTGAGACCACAGCCTTCTCTG	GTTCCCTTCTGAAATGGT	62	40

2.4 Protein extraction, SDS-PAGE and **western-Western blot**

WI38, PNT1a, PC3 and DU145 cells were gently scraped off the surface in RIPA buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% Triton X-100) supplemented with protease inhibitors (Sigma-Aldrich, Steinheim, DE). Protein concentration was determined using the Bio-Rad DC Protein assay (Bio-Rad, Berkeley, CA). 50 µg of proteins/lane were separated on 12% SDS-PAGE gel, transferred to PVDF membranes and probed for 16 h at 4 °C by mean of the following antibodies: mouse monoclonal anti-CLU (Millipore, Billerica, MA), rabbit polyclonal anti-caspase-3, -caspase-9, -caspase-7, -poly-ADP-ribose polymerase (PARP) (Cell Signaling Technology, Danvers, MA); all antibodies were diluted 1:1000, mouse monoclonal anti-β-actin, dilution 1:200 (Santa Cruz Biotechnology, Dallas, TX). Membranes were incubated with suitable secondary IgG antibodies conjugated to horseradish peroxidase, dilution 1:5000 (Sigma-Aldrich, Steinheim, DE) and developed by the Chemiluminescence Blotting Substrate, POD (Roche, Mannheim, DE). The published results are representative of three independent experiments.

2.5 Immunocytochemical analysis

PC3 and DU145 cells, previously cultured on a glass cover slip for 24 h, were fixed and permeabilized with 1:1 methanol:acetone solution for 10 min at -20 °C. Aspecific epitopes were blocked with horse serum 3% (vol/vol) in Dulbecco's modified Phosphate Buffered Saline (D-PBS) for 20 min. Then, the cells were incubated with a mouse monoclonal anti-CLU antibody, dilution 1:50 (Millipore, Billerica, MA) in 3% bovine serum albumin (BSA) in D-PBS for 1 h. A secondary fluorescent anti-mouse Alexa Fluor™ 488 antibody (Invitrogen, Carlsbad, CA), diluted 1:200 in 3% BSA, was used for detection. Fluorescence images were acquired with Axiovert 200 inverted microscope system (Carl Zeiss, Jena, DE).

2.6 siRNA transfection and cell viability assay

The CLU siRNA sequence 5'-GCAGCAGAGUCUUCAUCAU-3' (Ambion, Austin, TX) corresponds to the human CLU translation initiation site on exon 2. A universal scrambled sequence called NC siRNA (Integrated DNA Technologies, Coralville, IA), was used as negative control. PC3 and DU145 cells were seeded at a density of 0.75×10^5 cells/mL and transfected with 100 nM CLU or NC siRNA, using the TransIT-TKO Transfection Reagent (Mirus Bio, Madison, WI). To determine the effect of CLU suppression on cell viability we used the WST-1 colorimetric assay (Roche, Lewes, UK), which contains the reagent 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt. Briefly, PC3 and DU145 cells were seeded in 96-wells and transfected with CLU or NC siRNA. 24 h after siRNA delivery, the transfection medium was replaced with standard growth medium, or medium containing treatments as specified. The cells were then incubated with WST-1 for 1 h at 37 °C. The formazan produced by metabolically active cells was quantified by measuring the absorbance at 450 nm using EnSpire® Multimode Plate Readers (PerkinEmler, Waltham, MA). The data were expressed as relative % of the viability measured in time matched CLU or NC siRNA transfected untreated cells.

2.7 CLU TSSs and promoter region prediction

The CLU geneID from Entrez-Gene was up-loaded in the MatInspector software (www.genomatix.de) that allows the identification of promoter regions based on recognition of individual Cap Analysis of Gene Expression (CAGE) verified transcripts [18]. The same software was used to perform the analysis of the most probable transcription factor binding sites of the identified promoter regions.

2.8 Plasmid construction and luciferase assay

To generate pGL4-P1 and pGL4-P2 reporter vectors, genomic DNA was extracted from PC3 cells using the QIAamp DNA mini Kit (Qiagen, Venlo, NL) according to manufacturer's protocol. The P1 and P2 sequences were PCR amplified using the following primers: XhoI-forward 5'-CTCGAGGGGACAGTGATTGC-3', HindIII-reverse 5'-AAGCTTTCATCCCTCTCTGCC-3' for P1 and XhoI-forward 5'-CTCGAGCCAGAAGTTGTTGC-3', HindIII-reverse 5'-AAGCTTGGAGAGTAGAGAGG-3' for P2 and subcloned into the pCR®2-TOPO vector (Invitrogen, Carlsbad, CA). The XhoI and HindIII restriction fragments obtained from digestion of plasmids TOPO-P1 and TOPO-P2 were then ligated into the pGL4.10 basic vector (Promega, Madison, WI) and verified through sequencing. PC3 cells were plated in 96 wells and transfected by FuGene® HD (Roche, Mannheim, DE), using 0.2 µg of pGL4-P1, pGL4-P2 and pGL4-empty vectors. Transfection efficiency was monitored by pEGFP-N1 transfection (Clontech Laboratories, Mountain View, CA). The luciferase activity was measured under basal conditions or following AZDC-TSA administration using the Britelite™ plus reactive (PerkinEmler, Waltham, MA) and the EnSpire® Multimode Plate Readers (PerkinEmler, Waltham, MA). The luciferase activities were normalized to the total protein content after checking for equal transfection efficiency. The results are representative of three independent experiments run in triplicate.

2.9 Methylation Sspecific PCR (MSP)

Genomic DNA was extracted by the QIAamp DNA mini Kit (Qiagen, Venlo, NL) from WI38, PC3 and DU145 cells. Bisulfite conversion was performed on 500 ng of genomic DNA using the EZ DNA Methylation-Gold™ Kit (Zymoresearch, Irvine, CA) according to the manufacturer's instruction. The efficiency of conversion was routinely examined by PCR before using the bisulfite-treated DNA for MSP amplification. Specific primers for MSP assay (Table 1) were designed by using the MethPrimer software [19]. PCR amplification was carried out with HotStart Taq DNA Polymerase (Promega, Madison, WI).

2.10 Chromatin immunoprecipitation (ChIP) assay

20 × 10⁶ of treated/untreated PC3 and DU145 cells were used for chromatin preparation as previously described by Rivera-Gonzalez et al. [20]. The cells were trypsinized, re-suspended in 5 mL medium and cross-linked with 0.5% formaldehyde for 5 min at room temperature. The reaction was stopped by 0.125 M glycine for 5 min at room temperature. Cells were washed with cold PBS and re-suspended in cold swelling buffer (5 mM Pipes pH 8.0, 85 mM KCl, 0.2 % NP-40) supplemented with protease inhibitors (Sigma-Aldrich, Steinheim, DE) and incubated on ice with gentle shaking for 20 min. The suspension was centrifuged and the pellet was re-suspended in cold buffer TSE 150 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), supplemented with protease inhibitors (Sigma-Aldrich, Steinheim, Germany), then sonicated on ice. Chromatin was centrifuged at 14,000 rpm for 30 min, aliquoted at 20 µg and stored at -80 °C. A sample of the sonicated chromatin was purified and electrophoresed to check for DNA fragmentation quality. Aliquots of sheared chromatin were immunoprecipitated, recovered and purified by Magna ChIP™ A (Millipore, Billerica, MA) following the manufacturer's instruction. 1% of each chromatin aliquot was saved as input sample. ChIP was performed using the following antibodies: mouse anti-TATA box binding protein (TBP), rabbit anti-acetyl(Lys9)H3 (H3K9ac) and anti-trimethyl(Lys27)H3 (H3K27me3) (all from Millipore, Billerica, MA). Normal IgGs were used as negative controls. Linear amplification of ChIPed DNA and input sample was performed by qPCR (Table 1). ChIP qPCR data were normalized using the % input method. Results are expressed as mean ± SD of three independent determinations.

2.11 Statistical analysis

The data obtained are expressed as mean ± SD. Statistical evaluation of data was done with two-tailed Student's t test. P values < 0.05 were considered statistically significant.

3 Results

3.1 Expression of CLU 1 and CLU 2 in various cell lines

CLU 1 and CLU 2 mRNA isoforms were detected in normal fetal fibroblasts (WI38), benign immortalized prostate epithelial cells (PNT1a) and androgen independent tumorigenic prostate epithelial cells (PC3 and DU145). In addition the same transcripts were amplified in mammary epithelial cells both normal (HTB-125) and transformed (MCF7), as well as in lung epithelial cells both normal (BEAS) and transformed (A549).

Amplification was performed by RT-PCR (Fig. 2A) and qPCR (Fig. 2B and D) using primers recognizing exon 1a of CLU 1 and exon 1b of CLU 2.

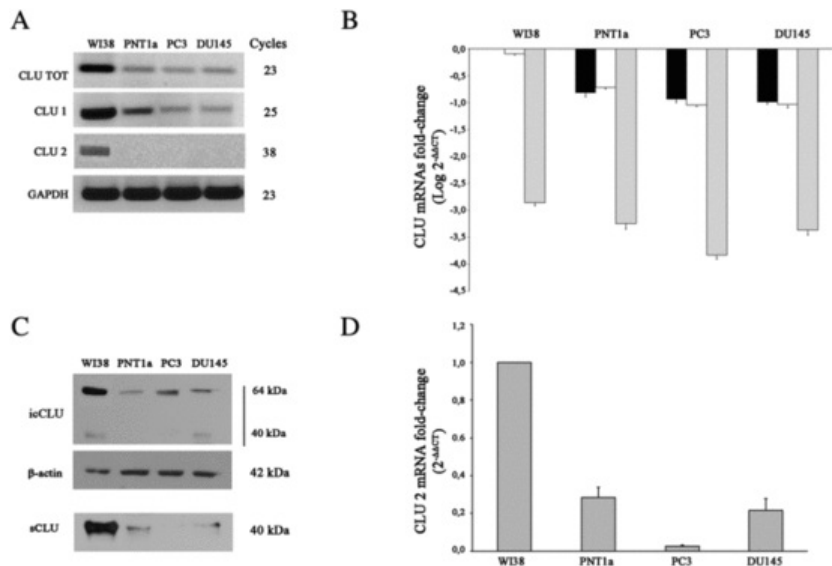


Fig. 2 CLU 1 and CLU 2 expression in prostate epithelial cells and fibroblasts.

A) Semi-quantitative RT-PCR analysis of CLU mRNAs in WI38, PNT1a, PC3 and DU145 cells. Number of amplification cycles reflects the amount of CLU mRNA variant expressed. CLU TOT was amplified using primers recognizing the common shared sequence, therefore representing the cumulative amount of all CLU mRNAs. The data shown are representative of three independent experiments. B) Quantification of CLU mRNA variants by qPCR. CLU TOT = black bars, CLU 1 = white bars, CLU 2 = grey bars. The relative expression of CLU transcripts was calculated by the $2^{-\Delta\Delta CT}$ method as fold-change of the level of CLU TOT measured in WI38 cells (reference sample fixed equal to 1). $\log_2 2^{-\Delta\Delta CT}$ values are reported on the Y axis. C) Western blot for CLU determination in WI38, PNT1a, PC3 and DU145 cells. Whole cell lysates and cell culture media were probed with an anti-CLU antibody. β -actin was used as loading control. icCLU = intracellular CLU from whole cells lysates. sCLU = secreted CLU, from cell culture media. D) Relative quantification of CLU 2 by qPCR calculated by the $2^{-\Delta\Delta CT}$ method and expressed as fold-change of the CLU 2 level measured in WI38 cells (reference sample fixed equal to 1). Error bars represent SD of at least three independent determinations. All the results are statistically significant ($P < 0.01$).

We also amplified a region common to all the CLU mRNA products (CLU TOT) using primers annealing to their shared nucleotide sequence at the 3' end (Fig. 2A). Under basal conditions, CLU 1 resulted **as** the most expressed mRNA variant among the above cell lines, accounting for the majority of total CLU mRNA content. Our attempt to amplify the region upstream the AUG element contained in exon 1a of NM_001831 failed systematically (Fig. S1). CLU 2 was expressed at **a** low, but detectable level, only in normal fetal fibroblasts (Fig. 2A).

A similar expression pattern was obtained in HTB-125, MCF7, BEAS and A549 (Fig. S2). We quantified by qPCR the relative abundance of CLU 1, CLU 2 and CLU TOT in prostate epithelial cells in comparison to WI38 cells CLU TOT content (Fig. 2B). The prostate epithelial cells expressed less CLU TOT than WI38; CLU 1 abundance roughly overlapped the CLU TOT content. PC3 cells expressed the lowest amount of CLU 2 mRNA variant (approximately less than of 1/10 of the WI38 cells CLU 2 content), while PNT1a and DU145 cells expressed more or less 5-fold less CLU 2 than normal fibroblasts (Fig. 2D).

In the same cell lines we also determined CLU protein expression by **western blot** analysis (Fig. 2C). WI38 cells produced the highest amount of sCLU, the secreted form processed from an intracellular precursor of 64 kDa and secreted in the cell culture medium. All prostate epithelial cells tested produced smaller amounts of CLU than WI38. PC3 cells secreted the lowest amount of sCLU in the culture medium.

3.2 CLU 1 and CLU 2 were up-regulated in prostate cancer cells after epigenetic drugs administration

In order to test whether CLU expression is regulated by epigenetic mechanisms, we treated PC3 and DU145 cells with TSA or AZDC-TSA. Then we measured CLU 1, CLU 2 and CLU TOT relative expression by qPCR (Fig. 3A). TSA treatment, and to a greater extent, AZDC-TSA caused both CLU mRNA variant levels to increase, in comparison to untreated cells. CLU 2 fold-change was higher than CLU 1 fold-change in all the conditions tested, showing the highest increase in DU145 cells after AZDC-TSA treatment.

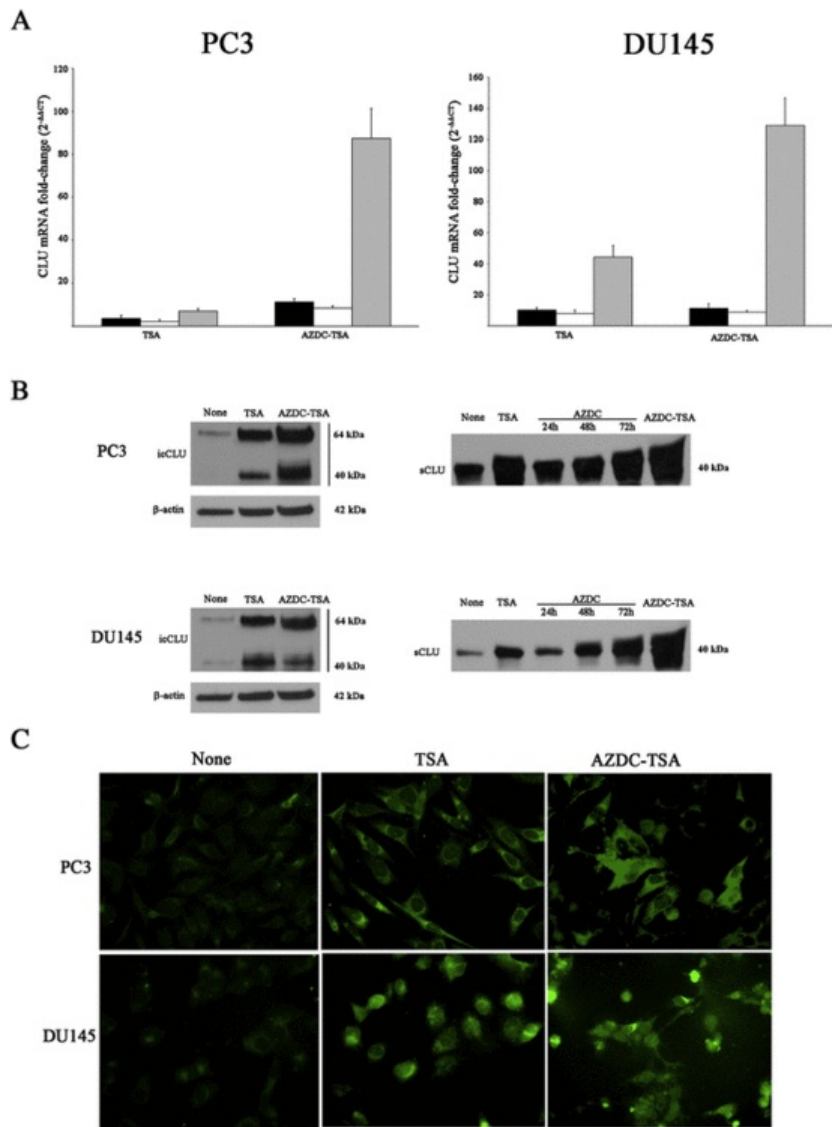


Fig. 3 Effects of epigenetic drugs on CLU mRNAs and protein production in prostate cancer cells. A) Relative quantification of CLU mRNA variants by qPCR. CLU TOT = black bars, CLU 1 = white bars, CLU 2 = grey bars. The relative expression of each CLU variant in treated cells was calculated by the $2^{-\Delta\Delta CT}$ method as fold-change of the level of the same transcript variant measured in control untreated cells (reference sample was fixed equal to 1). Error bars represent SD of four independent determinations. All results are statistically significant ($P \leq P < 0.01$). B) Western blot for CLU determination in PC3 and DU145 cells untreated (None), or treated with TSA 1 μ M for 24 h (TSA), or AZDC 10 μ M for 72 h, followed by 1 μ M TSA for 24 h (AZDC-TSA). Whole cell lysates (left panel) and cell culture media (right panel) were probed with an anti-CLU antibody. β -actin was used as loading control. icCLU = intracellular CLU, sCLU = secreted CLU. C) Subcellular localization of CLU protein (green fluorescence) in untreated (None) and treated (TSA and AZDC-TSA) PC3 and DU145 cells. The data shown are representative of three independent experiments.

3.3 CLU protein expression in prostate cancer cells after epigenetic drugs administration

We assessed CLU protein expression in prostate cancer cells following TSA and AZDC-TSA treatment by western-blot analysis both in cell lysates (icCLU) and cell culture media (sCLU) (Fig. 3B). Both TSA and AZDC treatment caused an increase of CLU synthesis and secretion in both PC3 and DU145 cells respect to basal levels. The highest effect on CLU protein induction was obtained with the combined treatment (AZDC-TSA) in both cell lines. Immunocytochemistry confirmed the results

obtained by ~~western blot~~Western blot in terms of CLU protein expression (Fig. 3C).

Both TSA and AZDC-TSA treatment caused an increase of CLU staining in the cytosol of both PC3 and DU145 cells compared to untreated controls. The green fluorescence signal was perinuclear and vesicular under basal conditions both in PC3 and DU145 cells, as expected for a protein committed to secretion.

After both TSA and AZDC-TSA treatment the green fluorescence increased without changing its distribution through the cell sub-compartments. DU145 cells lost their morphology after treatment and became round shaped, mostly following AZDC-TSA administration.

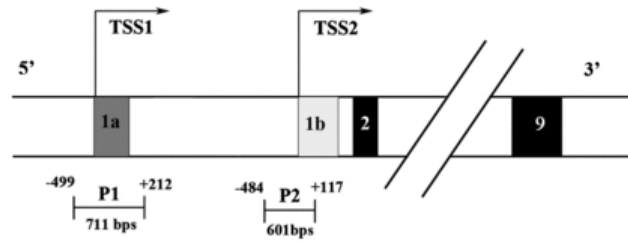
In these cells, we found evident nuclei fragmentation by DAPI staining (data not shown) and activation of caspases and PARP cleavage by ~~western blot~~Western blot analysis (Fig. S3), which are marks of apoptosis. PC3 cells were more resistant than DU145 cells to the treatment: we did not notice any change in cell morphology or activation of biochemical markers of apoptotic cell death (Fig. S3).

CLU knock out by siRNA transfection does not modify significantly the cell viability of TSA or AZDC-TSA treated cells as compared to NC siRNA transfected prostate cancer cells (Fig. S4).

3.4 In silico identification of CLU TSSs and putative promoter regions

The existence of two CLU mRNA variants differing at their 5' end, expressed at different levels in various cell lines and differently induced by epigenetic drugs administration, suggests the possibility that they could be generated by alternative transcription, i.e. starting from distinct TSSs controlled by independent promoters. To test the hypothesis, we performed an in silico analysis of the CLU gene sequence using the MatInspector software to search for highly probable mRNAs, TSSs and gene regulatory regions. We found out two TSSs in the 5' untranslated region (5' UTR) of the CLU gene corresponding to exon 1a (TSS1) and exon 1b (TSS2), surrounded by two distinct promoter regions, P1 and P2 (Fig. 4A). P1 which spans from -499 to +212 bps around TSS1 corresponds to the known CLU promoter, which contains a TATA box, the motive ten elements (MTE) and the HSE. P2, at difference, is a novel TATA-less regulatory sequence spanning from -484 to +117 bps around TSS2.

A



B

CLU P2 sequence

```

-484 TCTCTGGCATGACAAAGGCTCTGTTCTCTGCTGGAGGCATTCAGGGCTCAGTGGGCAGCT-
      SOX2                               ARP-1
-424 GGGGCAGAGCCCGTGAGACCACAGCCTTCTCTGGTGAGCCCGGTCTCCGCCCCCTACCCCA
-364 TCTCTGGGAAGGCGCTGACCCCACTCTTCTCCACGCTGCTCCCTGGCTCTTTGCGCCT
-303 GATTACTTCTCATGAGAGGCACTCCTTGTTAATGTGCTACTGAGTGTCCAGATGGGCCTGC
-242 TGGGCTGAGCGGGCTTTGGATGTGAACCAITTCAGGAAGGGGAACCCATCGTCCTGTTG
      YY1           STAT   NFKB
-182 GTTCTGTGATGGCAAATGGGTGAGCTCAGATAAGCAGTTCCTTGGGAGGGGCATGGTGGGG
      SP1
-122 GTGGAGTGCAGGGGGAGGGTTTCTGTTTATGCAACAGCCTCAGCTTCTGGGAAAGGGT
      SP1                               STAT
-62  CCATTGTGTAAGACCGGGCTATGGCCTGTGCCCGTGGCTCAGGGCAGCCAGCCAGTG
      MYCN
-2   +1
      |
      v
      GTGGCAGGAACACTGGCAGGGCAGCCTGCTGTCGGCTTAGAGGGGATGGGCAGTGTGGA
      NFI
+57  GGGCCTGGCAGAGCAAGAGGACTATCCTTCCAAAGGGACTTCTCTGGGAAGCCTGCTC
    
```

Fig. 4 In silico analysis of CLU promoter regions.

A) The TSS/promoter prediction program MatInspector predicted two alternative CLU TSSs surrounded by two distinct promoter regions. P1 = promoter 1; P2 = promoter 2. The length of the promoter region in bps is indicated. TSS1 corresponds to exon 1a, and TSS2 corresponds to exon 1b. B) Nucleotide sequence and highly probable binding sites of P2 transcription factors. Nucleotide positions are given on the left, TSS = + 1.

Firstly, we scanned P1 by an adequate MatInspector tool which utilizes a large library of matrix descriptions for transcription factor binding motifs to locate matches in a given DNA sequence. We restricted the research to highly probable results, characterized by having a core similarity of 1.0 and a matrix similarity of at least 0.80. These two parameters describe how well the retrieved binding sequences match the canonical consensus sequence. The software tool was able to identify known P1 trans-regulatory elements such as AP1, SP1 and CCAAT-enhancer-binding protein (CEBP) (Fig. S5). Some of the identified binding motives were experimentally investigated in the past and were proved to be effective by functional assays.

Therefore, encouraged by these evidences, we challenged the software scanning the new identified P2 region. The research retrieved binding sites for transcription factors that usually take part in the basic transcription machinery like SP1, nuclear factor I (NFI), and Yin Yang 1 (YY1). Other trans-regulatory element were: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), Basic helix-loop-helix protein 37 (MYCN), and Signal Transducer and Activator of Transcription (STAT) family member, which all play important roles in development, cell proliferation and survival mechanisms.

The in silico analysis revealed P2 specific transcription factors binding sites, including Sry-like HMG box genes 2 (SOX2), that is involved in pluri-potency maintenance of undifferentiated embryonic stem cells, and apolipoprotein repressor protein-1

(ARP-1), a protein that represses the apolipoprotein expression (Fig. 4B).

3.5 P2 is a functional promoter

To prove that P2 is a functional promoter, we performed a ChIP assay using an anti-TBP antibody to quantify the occupancy of this protein on the two predicted CLU promoter sequences. The assay was performed in PC3 and DU145 cells both under basal conditions and following AZDC-TSA administration (Fig. 5A).

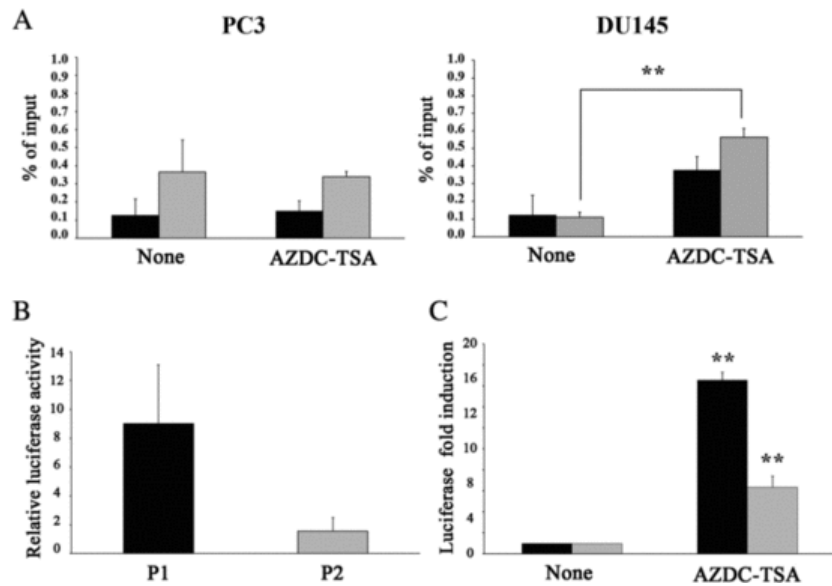


Fig. 5 TBP binding and transcriptional activity of P1 and P2.

Chromatin immunoprecipitation was performed to check for TBP occupancy on CLU promoters, P1 (black bars) and P2 (grey bars), in untreated (None) and treated (AZDC-TSA) PC3 and DU145 cells. The immunoprecipitated DNA fragments were analyzed by qPCR. TBP enrichment was calculated as % of the input sample. B) P1 and P2 were cloned upstream a luciferase reporter plasmid and transfected in PC3 cells. The luciferase activity was measured in comparison to the empty vector and normalized for the total protein content. C) P1 and P2 luciferase constructs were transfected in PC3 cells that underwent AZDC-TSA treatment. The luciferase activity of the same plasmids measured in untreated PC3 cells (None) was fixed equal to 1. Error bars represent SD of three independent determinations each performed in triplicate. ****P ≤ P < 0.01.**

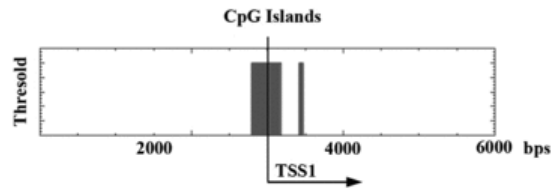
The qPCR analysis showed an enrichment of the DNA sequences corresponding to P1 and P2 in PC3 and DU145 TBP-immunoprecipitated chromatin. The specific binding of TBP on P2 in DU145 cells increased 6-fold following AZDC-TSA treatment in comparison to untreated control (**P ≤ P < 0.01**). In the same cells the TBP binding on P1 increased following AZDC-TSA, without reaching statistical significance. In PC3 cells, TBP occupancy is higher on P2 than on P1. In these cells the TBP binding is not influenced by epigenetic drugs administration.

To evaluate P1 and P2 promoter activity we cloned the two reported sequences upstream a firefly luciferase in suitable promoter less reporter plasmid. The luciferase constructs were transfected in PC3 cells and P1 and P2 promoter activity was evaluated both under basal conditions (Fig. 5B) and following AZDC-TSA treatment (Fig. 5C). In untreated PC3 cells P2 transcriptional activity is not relevant in comparison to P1 activity. When PC3 cells underwent AZDC-TSA treatment P1 and P2 transcriptional activity increased significantly in comparison to untreated condition.

3.6 P1 is methylated in prostate cancer cells

To understand whether the increase of CLU 1 and CLU 2 transcription following AZDC and AZDC-TSA treatment is related to promoter demethylation, we searched for the presence of CpG islands in the CLU gene by the free MethPrimer software, scanning a region of 6000 bps surrounding the TSS1. This genomic region includes both P1 and P2. We found a single CpG island, located in the region corresponding to P1 and mostly overlapping exon 1a of CLU 1 (Fig. 6A). By MSP-PCR we found out that the P1 is not methylated in normal fibroblasts, methylated in PC3 cells and partially methylated in DU145 cells. AZDC or AZDC-TSA treatment did not modify the P1 methylation status in prostate cancer cells (Fig. 6B).

A



B

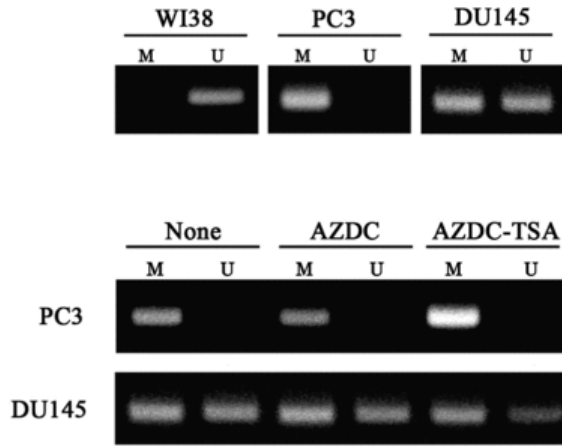


Fig. 6 CpG islands prediction in the CLU 5' genomic region and MSP analysis of the P1.

A) Graphic view of the results of CpG islands prediction at the 5' end of CLU gene performed by MethPrimer. A single CpG island surrounding TSS1, indicated by a shaded bar, was found in P1. B) MSP analysis of P1 in WI38, PC3 and DU145 cells under basal conditions (upper panel) and in PC3 and DU145 cells following epigenetic drugs treatment (lower panel). M = Methylated DNA, U = Unmethylated DNA. Data shown are representative of three independent experiments.

3.7 Changes of H3 tail post-translational modifications after epigenetic drugs treatment in prostate cancer cells

Since direct DNA methylation of CLU promoters does not seem to be the mechanism responsible for short-term induction of CLU gene transcription following treatment, we determined whether the increased CLU expression is caused by chromatin modifications. We performed a quantitative ChIP assay to evaluate the enrichment on P1 and P2 of H3K9ac and H3K27me3. Following treatment with AZDC-TSA we observed a 3-fold increase and a 7-fold increase of H3K9ac at P2 in PC3 and DU145 cells, respectively (Fig. 7A). The H3K9ac enrichment at P1 increased significantly in PC3 treated cells (Fig. 7A). The H3K27me3 enrichment at P2 was high under basal condition in both cell types. Treatment with AZDC-TSA significantly reduced H3K27me3 (by 2-fold) at both P1 and P2 in DU145 while it did not induce any significant change in PC3 cells (Fig. 7B).

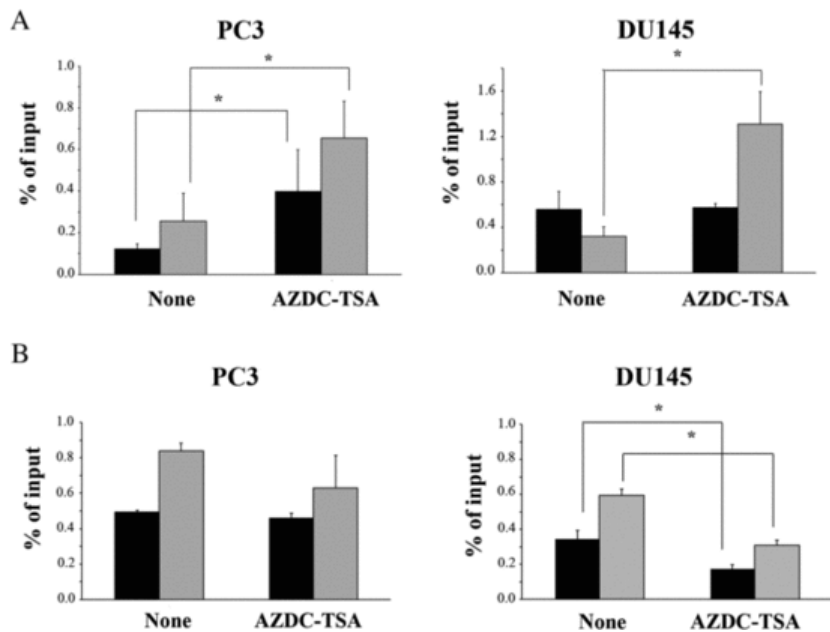


Fig. 7 H3 tail modifications at the CLU promoters after epigenetic drugs treatment.

Chromatin immunoprecipitation was performed to check for H3K9ac (A) and H3K27me3 (B) occupancy on CLU promoters, P1 (black bars) and P2 (grey bars), in untreated (None) and treated (AZDC-TSA) PC3 and DU145 cells. The immunoprecipitated DNA fragments were analyzed by qPCR. Histone modifications specific enrichment was calculated as % of the input sample. Error bars represent SD of three independent determinations each run in duplicate. * $P \leq 0.05$.

4 Discussion and Conclusions

The present work expands the understanding of the mechanisms that take part in CLU transcription regulation processes in human cells. We demonstrate that the expression of two mRNA variants, CLU 1 and CLU 2, generated by alternative transcription mechanisms, is regulated by two distinct promoters in human prostate cancer cells.

Our results demonstrate that CLU 1 and CLU 2 are both expressed at different levels 1) within the same cell line and 2) by different cell lines. CLU 1 is the most abundant mRNA variant in all the cells we tested. It is expressed at higher level in stromal cells than in epithelial cells. This observation confirms the experimental data obtained by us in primary cultures of epithelial and stromal cells derived from the same prostate gland sample [21] and by others in colorectal tissue specimens [9]. CLU 2 mRNA is expressed at detectable levels only in normal fetal lung fibroblasts; its lowest expression was in tumorigenic, androgen independent prostate cell lines (DU145 and PC3).

Some reports have indicated that CLU is up-regulated in prostate cancer, and correlates positively with low tumor differentiation [22] and androgen independence onset [23]. We, as well as others, have demonstrated that CLU mRNA and protein are consistently down-regulated in “naïve” prostate carcinoma [24–27]. Conflicting data about CLU expression were also reported for colon and lung cancer [28–30]. The existence of different mRNA variants that are expressed at various level in diverse cell populations within cancer tissue specimens, might contribute to a possible explanation of the above data inconsistency. We investigated CLU transcript variants in benign and tumorigenic epithelial cell lines, isolated from mammary or lung tissues. CLU 1 is expressed at varying levels across the entire panel of the tested cells, while CLU 2 results are undetectable, irrespective of the malignancy grade. Although our data show very low expression of CLU 2 in cancer cell lines, we cannot rule out the possibility that the occurrence might be related to a definite step in the cell differentiation process and not to the pathologic phenotype.

Recent publications have shown that CLU is down-regulated by epigenetic mechanisms in prostate cancer [14,31], in tumor conditioned endothelial cells [16], in human neural cells [15] and retinal pigment epithelial cells [32]. None of these papers evaluated the effects of epigenetic drugs administration on transcriptional regulation of the two CLU mRNA variants separately, measuring a total content of CLU mRNA or protein. Here we show, for the first time, that TSA, administered as single treatment, or combined with AZDC, induced a statistically significant fold-induction of both CLU 1 and CLU 2 in prostate cancer cells. CLU 2 induction was 10-fold higher than CLU 1 induction. We also showed that treatment of prostate cancer cells with epigenetic drugs increased translation and secretion of sCLU, the only protein form detectable at appreciable levels. CLU induction is concomitant with inhibition of cell proliferation in both cell lines, and caspases activation in DU145 cells. We showed that the cell viability of prostate cancer cells treated with epigenetic drugs is not significantly different when CLU expression is abrogated by siRNA transfection in comparison to NC siRNA-transfected cells. Therefore we conclude that CLU is not directly involved in the mechanism of cell death induced by TSA or AZDC-TSA treatment.

We failed to detect the full NM_001831 sequence by RT-PCR in all cell lines tested, confirming the results obtained by two research groups by 5' rapid amplification of cDNA ends (5' RACE) in different cell lines [10,12]. We propose that CLU 1 is shorter at its 5' than the NM_001831 RefSeq published in NCBI. This finding is relevant because the longest sequence of variant 1, overlapping NM_001831, was predicted to have a translational start site in exon 1a, that enables the production of a putative nuclear protein. On the contrary, both CLU transcripts reported here present an untranslated first exon. Therefore the protein product of CLU 1 and CLU 2 is identical and corresponds to the well-characterized secreted protein sCLU.

Alternative transcription altering the 5' UTR of the mRNA, but not the protein-coding sequence, may allow tissue-specific expression with differing translation efficiency and is often associated with the existence of different promoters responding to specific stimuli [33].

We observed that CLU 1 has a short untranslated first exon, **whilst** while CLU 2 has a long (276 nucleotides) 5' UTR, corresponding to the untranslated, unique exon 1b, which is thought to contain extensive secondary structure and may act as negative regulator of translation.

CLU gene sequence inspection, by the MatInspector software, resulted in the identification of two highly probable TSSs, corresponding to each of the two CAGE verified mRNA variants. The software identified, also, two putative promoter regions surrounding each TSS. The first regulatory region P1, corresponds to the canonical CLU promoter first identified by Wong et al. [1] and drives CLU 1 expression. Recently Shiota et al. found that Y-box binding protein-1, directly binds P1 inducing CLU expression following endoplasmic reticulum stress [34]. Also, P1 and the genomic region upstream P1 binds Twist-1 following transforming growth factor- β or insulin growth factor-1 stimulation [35,36]. P1 was indicated to bind the hypoxia induced factor 1-alpha (HIF-1 α) by EMSA and luciferase assay [37]. Many other authors contributed to the understanding of CLU transcription regulation (Table 2 for summary and references).

Table 2 State of the art of the research on CLU transcriptional regulation.

Reference	Transcription factor_investigated	DNA binding element	Methods	Genomic region_involved
Park J et al. [37]	HIF-1a	HREs	Luciferase assay, EMSA, ChIP	P1
Takeuchi A et al. [36]	Twist 1	E-box	Luciferase assay, ChIP	Upstream P1
Corvetta D at al. [55]	MYCN	Non canonical E-box	Luciferase assay, EMSA, ChIP	P1
Shiota M et al. [35]	Twist 1	E-box	Luciferase assay, ChIP	P1
Klokov D et al. [56]	TGF- β dependent factor	Not Available	Luciferase assay	P1
Shiota M et al. [34]	YB-1	E-box	Luciferase assay, ChIP	P1
Kim G et al. [57]	SREBP-1c	E-box	Luciferase assay, ChIP	P2
Cervellera M et al. [58]	B-Myb	Myb consensus sequence	Luciferase assay, EMSA	P1

In the present paper we described the new promoter region, P2, identified downstream of P1 and surrounding TSS2, which functions as a weaker promoter than P1 and accounts for CLU 2 expression. It is a TATA-less, GC rich promoter, characterized by two binding sites for SP1, a frequent enhancer of transcription in TATA-less promoters [38]. A putative YY1 binding site may significantly contribute to the promoter transactivation/repression of small heat shock proteins, as previously seen for the metastasis suppressor HLJ1 [39]. We noticed two putative STAT binding sites, a canonical E-box predicted to bind the protooncogene MYCN, and a NF- κ B binding site, all factors known to be involved in the regulation of CLU expression [8]. We also identified a putative binding site for the transcription factor NFI, which has been implicated in several growth-related processes, including p21Waf1/Cip1 transcriptional repression in growing fibroblasts [40]. It is a matter of fact that CLU belongs to the family of apolipoproteins and was once called apolipoprotein J (APOJ) [41]. We identified a P2 specific binding sequence for the Hepatocyte Nuclear Factor 4 (HNF-4) on the minus strand, and ARP-1 on the plus strand, two transcription factors typically present on apolipoprotein promoters where they can activate or repress gene transcription [42]. Immediately upstream from the promoter region which accounts for binding of general core promoter transcription factors, we recognized a putative SOX2 binding site, which is exclusive for P2. SOX factors play critical roles in establishing and maintaining cell types during development and in the adult [43]. Many authors reported that CLU promotes cell differentiation of neuronal [44–46] and vascular smooth muscle cells [47,48], is implicated in the differentiation and morphogenesis of epithelia [49], and promotes terminal differentiation in different human hematopoietic cells [50]. One may speculate that CLU 2, normally repressed, might be specifically up-regulated by SOX2 in response to cell- or tissue-specific signaling able to promote cell differentiation. All these putative binding sites will need extensive experimental evaluation and confirmation by binding assay, gel shift assay and luciferase assay.

To demonstrate that P2 is a genuine promoter we evaluated its capacity of TBP binding. TBP binds the DNA minor groove independently of the presence of a TATA box and is essential for transcription, even in a promoter lacking

canonical TATA sequence [51]. A wide genome chromatin immune-precipitation (ChIP)-on-chip assay proved that novel unknown TBP-binding sites are part of genomic fragments not yet annotated as promoters [52].

We found out a significant TBP binding enrichment both on P1 and P2 in prostate cancer cells, supporting the hypothesis that P2 is a CLU regulatory region as well as P1. By luciferase assay we demonstrated that P1 is able to direct luciferase transcription under basal conditions and at higher extent following AZDC-TSA stimulation. P2 is unable to drive active transcription under basal condition but its activity increased 6-fold after AZDC-TSA administration. Altogether, the data support the hypothesis that P2 is a genuine promoter, which remains silent in prostate cancer cells under basal conditions and become active following administration of epigenetic drugs.

We deepen our understanding further by studying the mechanism by which TSA and AZDC, or a combination of both, induced CLU 1 and CLU 2 expression. Chromatin structure is essential to regulation of gene expression. The best known epigenetic mechanisms are DNA methylation of CpG islands and post-translational modifications of the histone tails. We found that P1 contains a CpG island which is methylated in prostate cancer cells and unmethylated in normal fibroblasts. This may explain, at least in part, lower expression of both CLU 1 mRNA and CLU protein in these cells than in normal WI38 cells. Treatment with AZDC did not change the P1 methylation status, even when used in combination with TSA. We conclude that direct methylation of P1 promoter may be a long-term heritable mechanism of CLU partial repression.

Histone post-translational modification is a dynamic and flexible epigenetic mechanism that may cause changes in chromatin configuration, thereby regulating the accessibility of chromatin to transcription regulatory proteins [53].

H3K9ac is one of the most predictive histone modifications in estimating the promoter usage by RNAP II enzyme [54]. Also it is well known that H3K27me3 strongly associates with transcription repression.

We found that AZDC-TSA treatment of prostate cancer cells strongly increased the H3K9ac modification, while reducing H3K27me3 at CLU regulatory regions. Both these modifications caused a switch in the acetylation/methylation state of H3 at CLU promoters, induced chromatin relaxation and active transcription of CLU 1 and CLU 2. It is noteworthy that the highest fold induction was reached by CLU 2 in DU145 cells. In these cells, concomitantly, we measured the highest inverse variation of H3K9ac and H3K27me3 and the highest TBP recruitment, indicating that these H3 post-translational modifications are directly involved in the mechanism by which P2 regulates transcription of CLU 2 in prostate cancer cells.

In conclusion, our work identified for the first time a second promoter of CLU, P2, that is directly responsible for CLU 2 transcription. CLU 2 is repressed in prostate cancer cells, and its expression may be up-regulated after treatment with epigenetic drugs. Both direct promoter methylation and histone post-translational modifications contribute to regulation of CLU expression, even if with a different significance and extent. Promoter methylation is a long-term heritable mechanism that occurs only on P1, resulting in partial repression of CLU 1 expression in prostate cancer cells. Dynamic modeling of histone tails is a reversible mechanism directly implicated in CLU 1 and especially CLU 2 derepression after administration of epigenetic drugs.

Further studies will be necessary to elucidate the role of CLU 2 in different cellular settings and/or clinical conditions, also searching for specificity in tissues distribution and transcription factors that binds P2 contributing to CLU 2 differential expression in pathological conditions. In particular, the possible link of CLU 2 expression with prostate epithelial cell transformation should be explored.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagr.2014.11.003>.

Funding

[This study is funded by the](#) Italian Ministry of Education and Scientific Research, 'Futuro in Ricerca' (RBF100CEJ_002); University of Parma (FIL 2013 to S.B. and F.R.).

Acknowledgments

We acknowledge Professor Giorgio Dieci for suggesting the TBP ChIP assay and Mr. Paul T. Wegener for language editing.

References

[1]

P. Wong, D. Taillefer, J. Lakins, J. Pineault, G. Chader and M. Tenniswood, Molecular characterization of human TRPM-2/clusterin, a gene associated with sperm maturation, apoptosis and neurodegeneration, *Eur. J. Biochem.* **221**, 1994, 917–925.

[2]

S. Bettuzzi and F. Rizzi, Chapter 5: Nuclear CLU (nCLU) and the fate of the cell, *Adv. Cancer Res.* **104**, 2009, 59–88.

[3]

J. O'Sullivan, L. Whyte, J. Drake and M. Tenniswood, Alterations in the post-translational modification and intracellular trafficking of clusterin in MCF-7 cells during apoptosis, *Cell Death Differ.* **10**, 2003, 914–927.

[4]

S.W. Kang, S.Y. Yoon, J.Y. Park and D.H. Kim, Unglycosylated clusterin variant accumulates in the endoplasmic reticulum and induces cytotoxicity, *Int. J. Biochem. Cell Biol.* **45**, 2013, 221–231.

[5]

P. Nizard, S. Tetley, Y. Le Drean, T. Watrin, P. Le Goff, M.R. Wilson and D. Michel, Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol, *Traffic* **8**, 2007, 554–565.

[6]

N. Li, A. Zoubeidi, E. Beraldi and M.E. Gleave, GRP78 regulates clusterin stability, retrotranslocation and mitochondrial localization under ER stress in prostate cancer, *Oncogene* **32**, 2013, 1933–1942.

[7]

I.P. Trougakos, J.Y. Djeu, E.S. Gonos and D.A. Boothman, Advances and challenges in basic and translational research on clusterin, *Cancer Res.* **69**, 2009, 403–406.

[8]

A. Sala, S. Bettuzzi, S. Pucci, O. Chayka, M. Dews and A. Thomas-Tikhonenko, Regulation of CLU gene expression by oncogenes and epigenetic factors implications for tumorigenesis, *Adv. Cancer Res.* **105**, 2009, 115–132.

[9]

C.L. Andersen, T. Schepeler, K. Thorsen, K. Birkenkamp-Demtroder, F. Mansilla, L.A. Aaltonen, S. Laurberg and T.F. Orntoft, Clusterin expression in normal mucosa and colorectal cancer, *Mol. Cell. Proteomics* **6**, 2007, 1039–1048.

[10]

H. Prochnow, R. Gollan, P. Rohne, M. Hassemer, C. Koch-Brandt and M. Baiersdorfer, Non-secreted clusterin isoforms are translated in rare amounts from distinct human mRNA variants and do not affect Bax-mediated apoptosis or the NF-kappaB signaling pathway, *PLoS One* **8**, 2013, e75303.

[11]

D.R. Cochrane, Z. Wang, M. Muramaki, M.E. Gleave and C.C. Nelson, Differential regulation of clusterin and its isoforms by androgens in prostate cells, *J. Biol. Chem.* **282**, 2007, 2278–2287.

[12]

T. Schepeler, F. Mansilla, L.L. Christensen, T.F. Orntoft and C.L. Andersen, Clusterin expression can be modulated by changes in TCF1-mediated Wnt signaling, *J. Mol. Signal.* **2**, 2007, 6.

[13]

D. Michel, G. Chatelain, Y. Herault and G. Brun, The expression of the avian clusterin gene can be driven by two alternative promoters with distinct regulatory elements, *Eur. J. Biochem.* **229**, 1995, 215–223.

[14]

H.E. Rauhala, K.P. Porkka, O.R. Saramaki, T.L. Tammela and T. Visakorpi, Clusterin is epigenetically regulated in prostate cancer, *Int. J. Cancer* **123**, 2008, 1601–1609.

[15]

T. Nuutinen, T. Suuronen, A. Kauppinen and A. Salminen, Valproic acid stimulates clusterin expression in human astrocytes: implications for Alzheimer's disease, *Neurosci. Lett.* **475**, 2010, 64–68.

[16]

D.M.E.I. Hellebrekers, V. Melotte, E. Vire, E. Langenkamp, G. Molema, F. Fuks, J.G. Herman, W. Van Criekinge, A.W. Griffioen and M. van Engeland, Identification of epigenetically silenced genes in tumor endothelial cells, *Cancer Res.* **67**, 2007, 4138–4148.

[17]

K.J. Livak and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods* **25**, 2001, 402–408.

[18]

K. Cartharius, K. Frech, K. Grote, B. Klocke, M. Haltmeier, A. Klingenhoff, M. Frisch, M. Bayerlein and T. Werner, MatInspector and beyond: promoter analysis based on transcription factor binding sites, *Bioinformatics* **21**, 2005, 2933–2942.

[19]

L.C. Li and R. Dahiya, MethPrimer: designing primers for methylation PCRs, *Bioinformatics* **18**, 2002, 1427–1431.

[20]

G.C. Rivera-Gonzalez, A.P. Droop, H.J. Rippon, K. Tiemann, D. Pellacani, L.J. Georgopoulos and N.J. Maitland, Retinoic acid and androgen receptors combine to achieve tissue specific control of human prostatic transglutaminase expression: a novel regulatory network with broader significance, *Nucleic Acids Res.* **40**, 2012, 4825–4840.

[21]

M. Scaltriti, M. Brausi, A. Amorosi, A. Caporali, D. D'Arca, S. Astancolle, A. Corti and S. Bettuzzi, Clusterin (SGP-2, ApoJ) expression is downregulated in low- and high-grade human prostate cancer, *Int. J. Cancer* **108**, 2004, 23–30.

[22]

J. Steinberg, R. Oyasu, S. Lang, S. Sintich, A. Rademaker, C. Lee, J.M. Kozlowski and J.A. Sensibar, Intracellular levels of SGP-2 (Clusterin) correlate with tumor grade in prostate cancer, *Clin. Cancer Res.* **3**, 1997, 1707–1711.

[23]

H. Miyake, C. Nelson, P.S. Rennie and M.E. Gleave, Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene testosterone-repressed prostate message-2 in prostate cancer xenograft models, *Cancer Res.* **60**, 2000, 2547–2554.

[24]

F. Rizzi, L. Belloni, P. Crafa, M. Lazzaretti, D. Remondini, S. Ferretti, P. Cortellini, A. Corti and S. Bettuzzi, A novel gene signature for molecular diagnosis of human prostate cancer by RT-qPCR, *PLoS One* **3**, 2008, e3617.

[25]

S. Bettuzzi, P. Davalli, S. Astancolle, C. Carani, B. Madeo, A. Tampieri and A. Corti, Tumor progression is accompanied by significant changes in the levels of expression of polyamine metabolism regulatory genes and clusterin (sulfated glycoprotein 2) in human prostate cancer specimens, *Cancer Res.* **60**, 2000, 28–34.

[26]

F. Rizzi and S. Bettuzzi, The clusterin paradigm in prostate and breast carcinogenesis, *Endocr. Relat. Cancer* **17**, 2010, R1–R17.

[27]

L. Varisli, Identification of new genes downregulated in prostate cancer and investigation of their effects on prognosis, *Genet. Test. Mol. Biomarkers* **17**, 2013, 562–566.

[28]

F. Panico, F. Rizzi, L.M. Fabbri, S. Bettuzzi and F. Luppi, Clusterin (CLU) and lung cancer, *Adv. Cancer Res.* **105**, 2009, 63–76.

[29]

T. Chen, J. Turner, S. McCarthy, M. Scaltriti, S. Bettuzzi and T.J. Yeatman, Clusterin-mediated apoptosis is regulated by adenomatous polyposis coli and is p21 dependent but p53 independent, *Cancer Res.* **64**, 2004, 7412–7419.

[30]

A.M. Rodriguez-Pineiro, A. Garcia-Lorenzo, S. Blanco-Prieto, P. Alvarez-Chaver, F.J. Rodriguez-Berrocal, M.P. Cadena and V.S. Martinez-Zorzano, Secreted clusterin in colon tumor cell models and its potential as diagnostic marker for colorectal cancer, *Cancer Invest.* **30**, 2012, 72–78.

[31]

C. Wang, Z. Liu, C.W. Woo, Z. Li, L. Wang, J.S. Wei, V.E. Marquez, S.E. Bates, Q. Jin, J. Khan, K. Ge and C.J. Thiele, EZH2 Mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3, and NGFR, *Cancer Res.* **72**, 2012, 315–324.

[32]

T. Suuronen, T. Nuutinen, T. Ryhanen, K. Kaamiranta and A. Salminen, Epigenetic regulation of clusterin/apolipoprotein J expression in retinal pigment epithelial cells, *Biochem. Biophys. Res. Commun.* **357**, 2007, 397–401.

[33]

B. Tolner, K. Roy and F.M. Sirotnak, Structural analysis of the human RFC-1 gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5' end heterogeneity, *Gene* **211**, 1998, 331–341.

[34]

M. Shiota, A. Zoubeidi, M. Kumano, E. Beraldi, S. Naito, C.C. Nelson, P.H. Sorensen and M.E. Gleave, Clusterin is a critical downstream mediator of stress-induced YB-1 transactivation in prostate cancer, *Mol. Cancer Res.* **9**, 2011, 1755–1766.

[35]

M. Shiota, A. Zardan, A. Takeuchi, M. Kumano, E. Beraldi, S. Naito, A. Zoubeidi and M.E. Gleave, Clusterin mediates TGF-beta-induced epithelial-mesenchymal transition and metastasis via Twist1 in prostate cancer cells, *Cancer Res.* **72**, 2012, 5261–5272.

[36]

A. Takeuchi, M. Shiota, E. Beraldi, D. Thaper, K. Takahara, N. Ibuki, M. Pollak, M.E. Cox, S. Naito, M.E. Gleave and A. Zoubeidi, Insulin-like growth factor-I induces CLU expression through Twist1 to promote prostate cancer growth, *Mol. Cell. Endocrinol.* **384**, 2014, 117–125.

[37]

J. Park, S.Y. Park, E. Shin, S.H. Lee, Y.S. Kim, D.H. Lee, G.S. Roh, H.J. Kim, S.S. Kang, G.J. Cho, B.Y. Jeong, H. Kim and W.S. Choi, Hypoxia inducible factor-1alpha directly regulates nuclear clusterin transcription by interacting with hypoxia response elements in the clusterin promoter, *Mol. Cell* **37**, 2014, 178–186.

[38]

C. Yang, E. Bolotin, T. Jiang, F.M. Sladek and E. Martinez, Prevalence of the initiator over the TATA box in human and yeast genes and identification of DNA motifs enriched in human TATA-less core promoters, *Gene* **389**, 2007, 52–65.

[39]

C.C. Wang, M.F. Tsai, T.M. Hong, G.C. Chang, C.Y. Chen, W.M. Yang, J.J. Chen and P.C. Yang, The transcriptional factor YY1 upregulates the novel invasion suppressor HLJ1 expression and inhibits cancer cell invasion, *Oncogene* **24**, 2005, 4081–4093.

[40]

S. Ouellet, F. Vigneault, M. Lessard, S. Leclerc, R. Drouin and S.L. Guerin, Transcriptional regulation of the cyclin-dependent kinase inhibitor 1A (p21) gene by NFI in proliferating human cells, *Nucleic Acids Res.* **34**, 2006, 6472–6487.

[41]

S.E. Jones and C. Jomary, Clusterin, *Int. J. Biochem. Cell Biol.* **34**, 2002, 427–431.

[42]

J.A. Ladias, M. Hadzopoulou-Cladaras, D. Kardassis, P. Cardot, J. Cheng, V. Zannis and C. Cladaras, Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3, *J. Biol. Chem.* **267**, 1992, 15849–15860.

[43]

Y. Kamachi and H. Kondoh, Sox proteins: regulators of cell fate specification and differentiation, *Development* **140**, 2013, 4129–4144.

[44]

S.W. Kang, Y.J. Shin, Y.J. Shim, S.Y. Jeong, I.S. Park and B.H. Min, Clusterin interacts with SCLIP (SCG10-like protein) and promotes neurite outgrowth of PC12 cells, *Exp. Cell Res.* **309**, 2005, 305–315.

[45]

J. Pahnke, E. Mix, R. Knoblich, J. Muller, M. Zschiesche, B. Schubert, D. Koczan, P. Bauer, T. Bottcher, H.-J. Thiesen, L. Lazarov, A. Wree and A. Rolfs, Overexpression of glial cell line-derived neurotrophic factor induces genes regulating migration and differentiation of neuronal progenitor cells, *Exp. Cell Res.* **297**, 2004, 484–494.

[46]

O. Cordero-Llana, S.A. Scott, S.L. Maslen, J.M. Anderson, J. Boyle, R.R. Chowdhury, P. Tyers, R.A. Barker, C.M. Kelly, A.E. Rosser, E. Stephens, S. Chandran and M.A. Caldwell, Clusterin secreted by astrocytes enhances neuronal differentiation from human neural precursor cells, *Cell Death Differ.* **18**, 2011, 907–913.

[47]

A.J. Millis, M. Luciani, H.M. McCue, M.E. Rosenberg and C.L. Moulson, Clusterin regulates vascular smooth muscle cell nodule formation and migration, *J. Cell. Physiol.* **186**, 2001, 210–219.

[48]

Y. Miwa, F. Takahashi-Yanaga, S. Morimoto and T. Sasaguri, Involvement of clusterin in 15-deoxy-delta12,14-prostaglandin J2-induced vascular smooth muscle cell differentiation, *Biochem. Biophys. Res. Commun.* **319**, 2004, 163–168.

[49]

L.E. French, A. Chonn, D. Ducrest, B. Baumann, D. Belin, A. Wohlwend, J.Z. Kiss, A.P. Sappino, J. Tschopp and J.A. Schifferli, Murine clusterin: molecular cloning and mRNA localization of a gene associated with epithelial differentiation processes during embryogenesis, *J. Cell Biol.* **122**, 1993, 1119–1130.

[50]

I.P. Trougakos, G. Pawelec, C. Tzavelas, T. Ntourophi and E.S. Gonos, Clusterin/Apolipoprotein J up-regulation after zinc exposure, replicative senescence or differentiation of human haematopoietic cells, *Biogerontology* **7**, 2006, 375–382.

[51]

B.F. Pugh and R. Tjian, Transcription from a TATA-less promoter requires a multisubunit TFIID complex, *Genes Dev.* **5**, 1991, 1935–1945.

[52]

S. Denissov, M. van Driel, R. Voit, M. Hekkelman, T. Hulsen, N. Hernandez, I. Grummt, R. Wehrens and H. Stunnenberg, Identification of novel functional TBP-binding sites and general factor repertoires, *Embo J.* **26**, 2007, 944–954.

[53]

T. Jenuwein and C.D. Allis, Translating the histone code, *Science* **293**, 2001, 1074–1080.

[54]

Y. Chen, M. Jorgensen, R. Kolde, X. Zhao, B. Parker, E. Valen, J. Wen and A. Sandelin, Prediction of RNA Polymerase II recruitment, elongation and stalling from histone modification data, *BMC Genomics* **12**, 2011, 544.

[55]

D. Corvetta, O. Chayka, S. Gherardi, C.W. D'Acunto, S. Cantilena, E. Valli, I. Piotrowska, G. Perini and A. Sala, Physical interaction between MYCN oncogene and polycomb repressive complex 2 (PRC2) in neuroblastoma: functional and therapeutic implications, *J. Biol. Chem.* **288**, 2013, 8332–8341.

[56]

D. Klokov, K. Leskov, S. Araki, Y. Zou, E.M. Goetz, X. Luo, D. Willson and D.A. Boothman, Low dose IR-induced IGF-1-sCLU expression: a p53-repressed expression cascade that interferes with TGFbeta1 signaling to confer a pro-survival bystander effect, *Oncogene* **32**, 2013, 479–490.

[57]

G. Kim, G.H. Kim, G.S. Oh, J. Yoon, H.W. Kim, M.S. Kim and S.W. Kim, SREBP-1c regulates glucose-stimulated hepatic clusterin expression, *Biochem. Biophys. Res. Commun.* **408**, 2011, 720–725.

[58]

M. Cervellera, G. Raschella, G. Santilli, B. Tanno, A. Ventura, C. Mancini, C. Seignani, B. Calabretta and A. Sala, Direct transactivation of the anti-apoptotic gene apolipoprotein J (clusterin) by B-MYB, *J. Biol. Chem.* **275**, 2000, 21055–21060.

▼ E-Extra

We also amplified a region common to all the CLU mRNA products (CLU TOT) using primers annealing to their shared nucleotide sequence at the 3' end (Fig. 2A). Under basal conditions, CLU 1 resulted as the most expressed mRNA variant among the above cell lines, accounting for the majority of total CLU mRNA content. Our attempt to amplify the region upstream the AUG element contained in exon 1a of NM_001831 failed systematically (Fig. S1). CLU 2 was expressed

at a low, but detectable level, only in normal fetal fibroblasts (Fig. 2A).

A similar expression pattern was obtained in HTB-125, MCF7, BEAS and A549 (Fig. S2). We quantified by qPCR the relative abundance of CLU 1, CLU 2 and CLU TOT in prostate epithelial cells in comparison to WI38 cells CLU TOT content (Fig. 2B). The prostate epithelial cells expressed less CLU TOT than WI38; CLU 1 abundance roughly overlapped the CLU TOT content. PC3 cells expressed the lowest amount of CLU 2 mRNA variant (approximately less than of 1/10 of the WI38 cells CLU 2 content), while PNT1a and DU145 cells expressed more or less 5-fold less CLU 2 than normal fibroblasts (Fig. 2D).

In these cells, we found evident nuclei fragmentation by DAPI staining (data not shown) and activation of caspases and PARP cleavage by ~~western blot~~Western blot analysis (Fig. S3), which are marks of apoptosis. PC3 cells were more resistant than DU145 cells to the treatment: we did not notice any change in cell morphology or activation of biochemical markers of apoptotic cell death (Fig. S3).

CLU knock out by siRNA transfection does not modify significantly the cell viability of TSA or AZDC-TSA treated cells as compared to NC siRNA transfected prostate cancer cells (Fig. S4).

Firstly, we scanned P1 by an adequate MatInspector tool which utilizes a large library of matrix descriptions for transcription factor binding motifs to locate matches in a given DNA sequence. We restricted the research to highly probable results, characterized by having a core similarity of 1.0 and a matrix similarity of at least 0.80. These two parameters describe how well the retrieved binding sequences match the canonical consensus sequence. The software tool was able to identify known P1 trans-regulatory elements such as AP1, SP1 and CCAAT-enhancer-binding protein (CEBP) (Fig. S5). Some of the identified binding motive were experimentally investigated in the past and were proved to be effective by functional assays.

▼ E-component

The following are the supplementary data related to this article.

[Multimedia Component 1](#)

Fig. S1 Amplification of NM_001831 in fibroblasts and prostate epithelial cells.

Amplification by RT-PCR of NM_001831 in WI38, PNT1a, PC3 and DU145 cells upstream the AUG element of exon 1a.

[Multimedia Component 2](#)

Fig. S2 Expression of CLU 1 and CLU 2 in normal and transformed cells from lung and mammary epithelium.

RT-PCR analysis of CLU mRNA variants in normal lung (BEAS2B) and mammary (HTB-125) cells and in transformed lung (A549) and mammary (MCF7) epithelial cells. The different number of amplification cycles performed reflects the variable amount of CLU mRNA variant expressed.

[Multimedia Component 3](#)

Fig. S3 Induction of apoptosis in prostate cancer cells treated with epigenetic drugs.

Western blot for PARP, caspase-3 and -7 in PC3 and DU145 cells untreated (None), or treated with TSA 1 μ M for 24 h (TSA), or AZDC 10 μ M for 72 h, followed by 1 μ M TSA for 24 h (AZDC-TSA). β -actin was used as loading control.

[Multimedia Component 4](#)

Fig. S4 Effect of CLU silencing on cell viability of prostate cancer cells treated with epigenetic drugs.

A) Relative quantification of CLU TOT mRNA by qPCR in CLU or NC siRNA-transfected PC3 cells. Non-transfected cells = black bars, NC siRNA = white bars, CLU siRNA = grey bars. The relative expression of CLU TOT mRNA was calculated by $2^{-\Delta\Delta C_t}$ method as fold-change of the level of CLU TOT measured in non-transfected time matched PC3 cells (reference sample fixed equal to 1). The data shown are representative of three independent determinations. B) Western blot for CLU determination in CLU or NC siRNA-transfected or non-transfected (C) cells. β -actin was used as loading control. C) Cell viability was detected by WST-1 assay in CLU or NC siRNA-transfected prostate cancer cells treated with TSA or AZDC-TSA. The data were expressed as % of the viability of CLU or NC siRNA-transfected untreated time matched cells. Error bars represent SD of three independent determinations. NC siRNA = white bars, CLU siRNA = grey bars.

[Multimedia Component 5](#)

Fig. S5 CLU P1 sequence.

Nucleotide sequence of P1. Nucleotide positions are given on the left. TSS1 = + 1. Core promoter elements and transcription factor binding sites are underlined.

Highlights

- CLU 2 is a low abundance mRNA of the CLU gene.
 - Epigenetic drugs up-regulate CLU 2 expression in prostate cancer cells.
 - We identify a new promoter of CLU, P2, that drives CLU 2 expression.
 - Histone-3 tail modifications at P2 promote chromatin relaxation and CLU 2 transcription.
-

Queries and Answers

Query:

Please check if the change made retained the intended meaning of the sentence.

Answer: No, the change made modify the intended meaning of the sentence. Please see changes introduced in the text.

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Please confirm that given names and surnames have been identified correctly.

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Query:

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Answer: No, the change do not retain the intended meaning. Please replace "run in duplicate" with "each performed in duplicate"

Query:

Please check if the change made retained the intended meaning of the sentence.

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