CLCA2 epigenetic regulation by CTBP1, HDACs, ZEB1, EP300 and miR-196b-5p impacts prostate cancer cell adhesion and EMT in metabolic syndrome disease.

Juliana Porretti[1], Guillermo N. Dalton[1,*], Cintia Massillo[1,*], Georgina D. Scalise[1], Paula L. Farré[1], Randolph Elble[2], Esther N. Gerez[3], Paula Accialini[4], Ana M. Cabanillas[5†], Kevin Gardner[6], Paola De Luca[1] & Adriana De Siervi[1,#].

[*]**Both are second authors.**

[†]Deceased

[1]Laboratorio de Oncología Molecular y Nuevos Blancos Terapéuticos, Instituto de Biología y Medicina Experimental (IBYME), CONICET; Argentina.
[2]Dept. of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois, 62794. Simmons Cancer Institute, United States of America.
[3]Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), Hospital de Clínicas, Argentina.
[4]Laboratorio de Fisiología y Biología Tumoral del Ovario, Instituto de Biología y Medicina Experimental (IBYME), CONICET; Argentina.
[5]Laboratorio de Oncología Molecular, Universidad Nacional de Córdoba, Argentina.
[6]Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY, USA

[#]**Corresponding Author:** Adriana De Siervi, Ph.D. Laboratorio de Oncología Molecular y Nuevos Blancos Terapéuticos, Instituto de Biología y Medicina Experimental (IBYME), CONICET. Vuelta de Obligado 2490, Buenos Aires, Argentina, C1428ADN. Phone: +5411-4783-2869, ext. 206; Fax: +5411-4786-2564; E-mail: adesiervi@dna.uba.ar

Key words: Metabolic syndrome; CTBP1; Prostate Cancer; CLCA2; miR-196b

Abbreviations: 3'UTR: 3' untranslated region; CDH1: E-cadherin; CDH2: N-cadherin; CLCA2: chloride channel accessory 2; ChIP: Chromatin immunoprecipitation**;** CTBP1: C-terminal binding protein 1; CTC: circulating tumor cell; CTNNB1: β-catenin; E2F1: E2F transcription factor 1; EMT: epithelial-mesenchymal transition; EP300: E1A binding protein p300; HDAC: histone deacetylase; HBB: hemoglobin subunit beta; MeS: metabolic syndrome; PEI: polyethylenimine; qPCR: quantitative polymerase chain reaction; RELA: RELA proto-oncogene, NF-kB subunit; RT: retrotranscription; SNAI: snail family transcriptional repressor; STAT3: signal transducer and activator of transcription 3; TF: transcription factors; TSA: trichostatin A;TP53: tumor protein p53; ZEB1: zinc finger E-box binding homeobox 1.

Category: Research article, Cancer Genetics and Epigenetics.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/ijc.31379

Novelty and impact: Metabolic Syndrome (MeS) is linked to increase prostate cancer (PCa) aggressiveness by unknown mechanisms. We defined a novel mechanism for PCa progression modulation associated to MeS that involved transcription regulation of *CLCA2* and miR-196b by CTBP1. This protein binds to *CLCA2* proximal promoter region and represses its transcription together with ZEB1, EP300 and HDACs. Importantly, CLCA2 functions as a PCa EMT inhibitor, since it depletion promoted circulating tumor cells (CTCs) dissemination in MeS mice.

Abstract

Prostate cancer (PCa) is the most common cancer among men. Metabolic syndrome (MeS) is associated with increased PCa aggressiveness and recurrence. Previously, we proposed C-terminal binding protein 1 (CTBP1), a transcripcional co-repressor, as a molecular link between these two conditions. Notably, CTBP1 depletion decreased PCa growth in MeS mice. The aim of this study was to investigate the molecular mechanisms that explain the link between MeS and PCa mediated by CTBP1. We found that CTBP1 repressed Chloride Channel Accessory 2 (*CLCA2*) expression in prostate xenografts developed in MeS animals. CTBP1 bound to *CLCA2* promoter and repressed its transcription and promoter activity in PCa cell lines. Furthermore, we found that CTBP1 formed a repressor complex with ZEB1, EP300 and HDACs that modulates the *CLCA2* promoter activity. CLCA2 promoted PCa cell adhesion inhibiting Epithelial-Mesenchymal Transition (EMT) and activating CTNNB1 together with epithelial markers (CDH1) induction, and mesenchymal markers (SNAI2 and TWIST1) repression. Moreover, CLCA2 depletion in PCa cells injected s.c. in MeS mice increased the Circulating Tumor Cells (CTCs) foci compared to control. A miRNA expression microarray from PCa xenografts developed in MeS mice, showed 21 miRNAs modulated by CtBP1 involved in angiogenesis, extracellular matrix organization, focal adhesion and adherents junctions, among others. We found that miR-196b-5p directly targets *CLCA2* by cloning *CLCA2* 3'UTR and performing reporter assays. Altogether, we identified a new molecular mechanism for PCa and MeS link based on CLCA2 repression by CTBP1 and miR-196b-5p molecules that might act as key factors in the progression onset of this disease.

INTRODUCTION

Prostate cancer (PCa) incidence and mortality rates have been declining since 1990. Nevertheless PCa is still the most prevalent cancer among men and the second leading cause of cancer death[1]. Age, race and family history are the established risk factors for this disease[2]; however there is increasing evidence showing that diet and lifestyle play a crucial role in PCa biology and tumorigenesis[3–5].

Metabolic syndrome (MeS) is a cluster of pathophysiological disorders that comprises increase of at least three of the following factors: abdominal obesity, triglycerides, cholesterol, blood pressure and glucose[6]. For more than one decade MeS role in PCa incidence and progression has been studied with different outcomes; nonetheless recently a meta-analysis revealed that this disease is associated with poor PCa patient prognosis, increased tumor aggressiveness and biochemical recurrence[7].

In the last years our group wondered about the mechanism whereby MeS influences PCa. We identified to C-terminal binding protein 1 (CTBP1) as a molecular link among these two conditions. CtBP1 is a transcriptional co-repressor of tumor suppressor genes that is activated with much higher affinity by NADH (>100-fold) compared to NAD^+[8]. Therefore, we generated a MeS mice model chronically feeding animals with high fat diet (HFD) and found that CTBP1 depletion in MeS mice dramatically decreased PCa growth[9,10].

Chloride Channel Accessory 2 (CLCA2) is a type I integral transmembrane protein[11]. It was proposed as a tumor suppressor in breast and colorectal cancer[12–14]. Tanikawa *et al.* found that *CLCA2* expression was remarkably decreased in prostate, breast, bladder, esophageal and lung cancers compared to normal tissues. Particularly in PCa, they established that CLCA2 expression in Prostatic Intraepithelial Neoplasia (PIN) is similar to normal prostate tissue but higher than invasive tumors[15]. However, CLCA2 role in PCa has not yet been studied.

Previously, Elble´s group showed that CLCA2 is a negative regulator of proliferation[16] and epithelial-mesenchymal transition (EMT) in breast cancer (BrCa)[17]. Recently, they reported that

CLCA2 is part of two functional adhesion complexes, one with EVA1 and ZO-1, and the other with CTNNB1, that are involved in EMT suppression[18]. Also BrCa patients with low CLCA2 expression in primary tumors have more metastasis[19]. In melanoma, Riker *et al.* demonstrated a negative correlation between a metastatic state and CLCA2 expression[20].

Few reports studied the mechanism for *CLCA2* expression regulation. So far the most important reported inductor is TP53[15,16,21]. On the other hand, epigenetic regulation came out as a key factor that represses CLCA2 expression in malignant tissues[13,15] but how this is regulated is not fully understood.

The aim of this work was to understand CTBP1 role linking MeS with PCa development and progression. Hence, we examined CTBP1 function in the regulation of *CLCA2* transcription and multiple miRNAs using *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Cell culture, transfections and treatments. PC3 (ATCC: CRL-1435), 22Rv1 (ATCC: CRL-2505), LNCaP (ATCC: CRL-1740), C4-2 cell lines and its stable derivatives were grown in RPMI 1640 (Invitrogen) supplemented with 10% of fetal bovine serum and antibiotics in a 5% CO_2 humidified atmosphere at 37°C. PC3.shCTBP1 and its control (PC3.pGIPZ) stable cell lines were previously described[9]. The PC3.shCLCA2 (shCLCA2) and its control PC3.pGIPZ (pGIPZ) cells lines were generated by lentiviral infection as previously described[9] using pGIPZ.shCLCA2 or pGIPZ plasmids. Stable transfected cells were selected with 2 µg/ml of puromycin (Sigma-Aldrich) for 10 days and then maintained with puromycin (1 µg/ml). Cells with CtBP1 or CLCA2 over-expression and its control were generated by transient transfection using polyethylenimine (PEI – PolySciences INC) in a PEI:DNA ratio 2:1. Trichostatin A (TSA, Sigma-Adrich) was used 1 µM for 24 h.

Plasmids. CTBP1 plasmid and its control (pcDNA3) were previously described[10]. pGL4 plasmids with different lengths of the CLCA2 promoter, pGIPZ.shCLCA2 and pGIPZ control, pLEX-CLCA2 and pLEX were previously described[16,18].

ZEB1 plasmids were previously reported[22], HDACs plasmids were kindly provided by Dr. Martin Monte (Universidad de Buenos Aires, Argentina) and EP300 plasmid was previously described[23].

The 3'UTR *CLCA2* (1,031 bp) was amplified using specific primers (Table I) and cloned in *Spe I* and *Hind III* restriction sites of pMIR-REPORT firefly luciferase vector plasmid (pMIR, Ambion, Grand Island, NY, USA). TOP FLASH plasmid was kindly provides by Jorge Filmus (Sunnybrook Health Sciences Centre, Toronto, Canada). Clone was selected by sequencing. Plasmids containing miR-196b-5p and miR-196a-5p were kindly provided by Ann-Joy Cheng (Chang Gung University, Taiwan)[24].

Luciferase reporter assay. PC3 cells were transfected in 12-well plates in triplicate using PEI and 1 µg of each plasmid. After 48 h of transfection, luciferase activity was determined by Luciferase Assay system (Promega) according to the manufacturer's instructions using GloMax® 96 Microplate Luminometer (Promega). Data were normalized to total protein determined by Bradford assay.

RNA isolation and RT-qPCR. Total RNA from cells or xenografts was isolated using TRIReagent (Molecular Research Center) followed by an isopropanol precipitation overnight at -20°C. Retrotranscription (RT) was performed using 2 µg of RNA and RevertAid First Strand kit (Fermentas, Vilnius, Lithuania). Real time PCR (qPCR) was performed using TAQ Pegasus (Productos Bio-Lógicos, Argentina) in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Primers were designed by Primer-BLAST (NIH) and purchased from Macrogen (*Supplemental Table 1*).

miRNA (100 ng) were retrotranscribed using stem-loop method as previously described[25]. Specific stem loop primer for each miRNA was used in RT. Primers for qPCR were the universal

reverse primer complementary to the stem loop, and a specific forward primer (Supplemental Table 1).

Chromatin immunoprecipitation (ChIP). ChIP was performed from PC3 cells using anti-CtBP1 or anti-GAL4 non-specific control antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), as previously described[9]. ChIP-DNA was amplified by qPCR using primers mapping at 1,165 bp upstream from *CLCA2* transcription start site (TSS), or 200 bp upstream from the *HBB* TSS (Supplemental Table 1). Fold enrichment was calculated normalizing data to input and GAL4.

Cell adhesion assay. Cell adhesion assay was performed as previously described with some modifications[26]. Briefly, after transfections, cells were attached to 96-well plate for 30 min. Media was removed and the attached cells were washed twice with PBS, fixed with methanol and stained with crystal violet for 10 min. After 2 water washes, stain was dissolved in 10% methanol: 5% acetic acid and absorbance at 620 nm was determined with ELISA Multiskan FC (Thermo Scientific).

PCa and MeS mice. Animal model was generated as previously described[9,10]. Briefly, 4 weeks old male *nu/nu* mice (N=24), were randomized into two groups and fed with CD or HFD and housed under pathogen free conditions following IBYME's animal care guidelines. Mice were fed *ad libitum* with CD (3120 kcal/kg, 5% fat, n= 22) or HFD (4520 kcal/kg, 37% fat, n=22) generated supplementing chow food with 32% of bovine fat first juice (Fatty, Buenos Aires, Argentina). After 12 weeks of diet, each group was randomly divided in two subgroups and s.c. injected with PC3.pGIPZ or PC3.shCLCA2 stable cells (4.8×10^6). Body weight was monitored once a week and tumor size was measured three times a week. Forty days after injection animals were sacrificed and tumors and soft tissues (lung and liver) were excised. Peripheral blood was collected by cardiac puncture from each mouse in a tube containing 60 µl of EDTA 0.5 M.

Circulating tumor cell (CTC) assay. To recover CTCs from peripheral blood, blood cells were obtained by centrifugation and hemolyzed by Ammonium Chloride Potassium (ACK) buffer

incubations (3 x 7 min; 1 x 5 min). Cells were washed with PBS and resuspended in RPMI medium supplemented with 10 % FBS, antibiotics and puromycin (1 µg/ml). Cells were seeded on 12-well plates and medium was replaced every 5 days. After 15 days CTCs were photographed with a Q-Color5 Digital Camera (OLYMPUS).

Microarray Analysis. mRNA microarray was previously reported[9]. RNA obtained from those tumors was hybridized with GeneChip miRNA 4.0 (Affymetrix). Data normalization and analysis was made as previously described[10]. Microarray data have been deposited in GEO database (Accession number: GSE104119). Upregulated miRNAs by CTBP1 with FC>1.5 ($p<0.05$) and signal >5 were selected. Potential miRNA target genes with at least 3 hits, Gene Ontology (GO) and pathways analysis were determined using ChemiRs database.

Statistical analysis. All results are given as mean and standard deviation (SD) of three independent experiments unless stated otherwise. Student t-tests or two-way ANOVA followed by Tukey test were performed. Shapiro–Wilk and Levene tests were used to assess normality and homogeneity of variances. *= $p< 0.05$; **= $p< 0.01$;***= $p< 0.001$.

RESULTS

CTBP1 and MeS repress *CLCA2* expression.

We previously reported a microarray analysis generated from CTBP1-depleted expression or control xenografts grown in MeS mice[9]. In this work, we focused our study on the olfactory signaling pathway obtained from the microarray Gene Set Enrichment Analysis (GSEA) (Figure 1A). We found that CTBP1 significantly repressed four genes from this pathway: *OR4C45*, *OR5P2*, *GUCA1C* and *CLCA2* (Figure 1B). Due to CLCA2 involvement in tumor suppression, we further investigated CLCA2 role in PCa and MeS. We first validated microarray results by RT-qPCR from xenografts generated with PC3.shCTBP1 or control stable cells in CD or HFD fed mice. As shown in Figure 1C, we observed *CLCA2* induction after CTBP1 depletion in MeS

conditions, validating microarrays findings. Next, we determined *CLCA2* expression in a panel of PCa cell lines (Figure 1D). We found that androgen-sensitive LNCaP cells and the androgen-insensitive PC3 cells showed higher *CLCA2* expression compared to 22Rv1 and C4-2 PCa cells lines.

CTBP1 represses *CLCA2* transcription in PC3 cell line.

To further investigate *CLCA2* expression regulation by CTBP1, reporter constructs containing different length of *CLCA2* promoter regions (-3565 bp to +1 bp from the TSS), were co-transfected with CTBP1 or control (pcDNA3) plasmids into PC3 cell line. CTBP1 significantly repressed the activity of all the different *CLCA2* promoter regions (Figure 2A). The -1182 to +1 fragment showed the highest *CLCA2* promoter activity and it was used in the subsequent experiments (Figure 2A). Accordingly to reporter results, we found that CTBP1 repressed *CLCA2* mRNA levels in PC3 cells (Figure 2B-C). Additionally, using ChIP we determined that CTBP1 bound to the *CLCA2* proximal promoter region (Figure 2D). Hence, CTBP1 associates to *CLCA2* promoter and represses its transcription in PCa cell lines.

***CLCA2* is epigenetically regulated by HDACs, ZEB1 and EP300.**

To determine potential transcription factors (TF) that co-regulate with CTBP1 *CLCA2* transcription, we co-transfected *CLCA2* reporter with CTBP1 expression plasmid plus a panel of TFs selected from the literature that potentially bind to *CLCA2* promoter or interacts with CtBP1 protein. We found that E2F1, STAT3, RELA and EP300 had no effect on *CLCA2* promoter activity; however TP53, BRCA1, ETS2 and ZEB1 induced its activity in PC3 cell line (Figure 3A-B and *Supplemental Figure 1*). Moreover, we observed that *CLCA2* promoter activity repression by CTBP1 was synergistically increased in the presence of EP300 and ZEB1 (Figure 3A-B).

Furthermore, we assessed HDACs role on *CLCA2* transcription regulation by CTBP1. We observed that HDAC1 and HDAC2 had no effect on *CLCA2* transcription regulation; whereas HDAC3, HDAC4 and HDAC6 repressed its activity (Figure 3C-D and *Supplemental Figure 1*). CTBP1 synergistically repressed with HDAC2 and HDAC3 the *CLCA2* promoter activity (Figure

3C-D). Notably, the HDAC inhibitor, TSA, induced *CLCA2* promoter activity and mRNA levels in PC3 cells (Figure 3E and F). Altogether these results show for the first time multiple TFs and epigenetic co-regulators that modulated *CLCA2* gene expression in PCa.

CLCA2 induces cell adhesion and epithelial phenotype.

To more deeply understand CLCA2 function in PCa, we generated PCa cells with *CLCA2* depleted or overexpressed and verified its expression (Figure 4A). We next performed cell adhesion assays in a panel of PCa cell lines including CLCA2 modulated expression cells. Interestingly, CLCA2 over-expression promoted cell adhesion in all PCa cell lines, and CLCA2 depletion significantly decreased it in PC3 cells (Figure 4B). Hence, CLCA2 plays a role increasing cell adhesion, the initial step for invasion and metastasis.

Based on previous reports[17,18], we investigated CLCA2 role in EMT and CTNNB pathways modulation in PCa. To assess the transcriptional activity of CTNNB we employed TOP FLASH reporter plasmid using PC3.shCLCA2 and control (pGIPZ) cells. Interestingly, *CLCA2* depletion significantly induced CTNNB activation (Figure 4C). In addition, *CLCA2* depletion dramatically repressed the expression of the epithelial marker *CDH1*, with concomitant induction of the mesenchymal markers expression; *SNAI2* and *TWIST1*, compared to control (Figure 4D). CDH2, *SNAI1*, *VIM*, *ZEB1* and *CTNNB* did not show expression changes in these conditions (*data not shown*).

To understand CLCA2 role in PCa associated to MeS, we injected s.c. PC3.shCLCA2 or PC3.pGIPZ control cell lines in CD or HFD fed mice. No significant differences were observed in tumor volume between *CLCA2* depleted and control groups (*Supplemental Figure 2*). However, based on CLCA2 role in PCa cell adhesion and EMT, we selected CTCs from the peripheral blood of these mice in a clonogenic assay. We found that *CLCA2* depletion increased the percentage of mice with CTC foci compared to pGIPZ control mice (Figure 4E). No sign of micro- or macro-metastasis were observed in soft tissues (lung and liver) by H&E stains from all groups (*data not shown*).

Altogether these results propose a novel mechanism for CTBP1 function linking PCa and MeS. Thus CTBP1 represses the epithelial gene promoter CLCA2, which in turn, induces mesenchymal markers and might promote metastasis.

CTBP1 regulates multiple miRNAs in PCa associated to MeS.

To increase the knowledge about the molecular mechanisms for CTBP1 role linking PCa and MeS, we assessed miRNAs regulation by CTBP1 in tumors from MeS mice. We hybridized miRNA expression microarrays with miRNAs isolated from xenografts described in Figure 1A. After data normalization, we obtained 21 miRNAs regulated by CtBP1 (Figure 5A). Interestingly, after GO and pathways analysis, we observed that these miRNAs modulated by CTBP1 are implicated in angiogenesis, extracellular matrix organization, developmental biology, cancer pathways, focal adhesion and adherents junctions among others (Figure 5B-C).

miR-196b-5p targets *CLCA2* and modulates cell adhesion.

To predict CtBP1 regulated miRNAs that potentially target *CLCA2*, we performed a supervised analysis. From the 21 miRNAs in Figure 5A, we selected the CTBP1-upregulated miRNAs that might repress CLCA2 (positive fold change) and determined the predicted target genes using ChemiRs (http://omics.biol.ntnu.edu.tw/ChemiRs/). All the miRNAs that have *CLCA2* as target by at least two databases with different algorithms were selected (Supplemental Table 2, column 1 and 2). Seven miRNAs detailed in Supplemental Table 2 (column 3 and 4) were analyzed by miRCancer (http://mircancer.ecu.edu/) database. Based on this and literature we selected miR-196b-5p as a candidate that targets *CLCA2*.

To validate whether miR-196b-5p targets *CLCA2* mRNA, we cloned *CLCA2* 3'UTR region downstream of the luciferase gene in a pMIR-REPORT plasmid. We co-transfected *CLCA2* 3´UTR and miR-196b, miR-196a or control plasmids in PC3 cells and found that miR-196a and miR-196b decreased luciferase activity demonstrating for the first time that *CLCA2* is targeted by these miRNAs in PCa (Figure 5D).

Next, we validated the miRNA microarray result by determining miR-196b-5p expression from xenografts generated in MeS or control mice after CtBP1 depletion. Accordingly, CTBP1 depletion significantly decreased miR-196b-5p expression compared to control only in HFD fed mice (Figure 5E). Finally, to understand miR-196b-5p role in PCa, we evaluated its effect on cell adhesion. Accordingly, we found that miR-196b decreased cell adhesion compared to control or miR-196a, a miRNA with similar sequence but without *CLCA2* as a target (Figure 5F). Thus, miR-196b-5p emerged as a critical miRNA regulated by CTBP1, suggesting a potential link for PCa progression in MeS.

Discussion

In this work we explored the mechanism underlying CTBP1 role in the modulation of PCa progression associated with MeS. We demonstrated that CTBP1 represses *CLCA2* in PCa xenografts developed in MeS mice. Moreover, we found that CTBP1 binds *CLCA2* proximal promoter region and represses its transcription together with ZEB1, EP300 and HDACs in PCa cell lines. It was previously reported that CTBP1 forms a repressor complex with these co-regulators[8,27]; nevertheless, we described here for the first time this co-regulation at the *CLCA2* promoter. Epigenetic repression of *CLCA2* by gene promoter hypermethylation has already been described [13,15]; however we showed here that *CLCA2* is regulated by TSA, highlighting the importance of the epigenetic modifications in the *CLCA2* expression regulation. In addition, we tested a panel of TFs that directly regulated *CLCA2*: TP53, BRCA1 and ETS2; or TFs that had no effect on CLCA2 promoter activity: E2F1, STAT3, RELA and EP300. Overall, our data demonstrated novel mechanisms for *CLCA2* transcriptional regulation in PCa.

Regarding CLCA2 function in PCa cells we demonstrated that CLCA2 is an EMT modulator. In particular, we showed that CLCA2 promoted cell adhesion, inducing epithelial markers (CDH1) and repressing mesenchymal genes (SNAI2 and TWIST1). Notably, CLCA2 depletion

significantly activated CTNNB TF function. These results are consistent with the reports in BrCa that showed CLCA2 promoted an epithelial phenotype by sequestering CTNNB in the membrane and, repressing EMT genes[17,18] (Figure 6). This is the first report showing CLCA2 as EMT inhibitor in PCa.

In this work we also assessed the CLCA2 role *in vivo.* We found that *CLCA2* depleted xenografts generated in MeS mice promoted CTCs release in a higher percentage of mice compared to *CLCA2* control expression xenografts. Although *CLCA2* expression modulation did not affect PCa growth, these results endorse the idea that CLCA2 loss promotes EMT. Moreover, all these findings propose a novel mechanism for CTBP1 oncogenic role through CLCA2 modulation.

We also described for the first time that CTBP1 modulates multiple miRNAs in PCa from MeS mice that are involved in several pathways related to cancer development and progression. The importance of miRNAs in PCa has already been widely reported[28]; however there are few reports showing miRNA modulation by CTBP1. Indeed, our group demonstrated that CTBP1 modulates several miRNAs in BrCa[10], and Chakravarthi and colleagues showed that CTBP1 modulates the miR-124 in a PCa cell line[29]. Therefore, our results provide a new insight into CTBP1 mechanisms leading to tumor progression in PCa.

Importantly, this is the first report describing miRNAs that target *CLCA2* 3´UTR in PC3. Based on bioinformatic analysis, literature and experimental validation, we selected miRNA-196b-5p as an important oncomiR up regulated in several cancers that is involved mainly in metastasis-related processes[30,31]. In addition, it was recently reported that miRNA-196b-3p overexpression promoted tumor development in castration resistant PCa mice[32]. Here, we found that the miR-196b-5p is induced by CTBP1 in tumors from MeS mice, targets CLCA2 and inhibits cell adhesion. Although miR-196a is also targeting CLCA2, this miRNA was not regulate by CTBP1 and also it did not regulated cell adhesion.

In summary, we defined a mechanism for CTBP1 oncogenic role in PCa associated with MeS, which involves CLCA2 and miR-196b-5p (*see hypothetical model Figure 6*). More studies are necessary to establish CLCA2 and miR-196b-5p as biomarker candidates for these diseases.

FIGURE TO LEGENDS

Figure 1. CtBP1 and MeS repress *CLCA2*. *A)* Gene Set Enrichment Analysis (GSEA) plot from olfactory signaling pathway. *B)* Microarray relative expression levels from *OR4C45, OR5P2*, *GUCA1C* and *CLCA2*. *C) CLCA2* RT-qPCR from xenografts with *CTBP1* depleted (shCtBP1) or control (pGIPZ) expression generated in HFD or CD fed mice. *D) CLCA2* mRNA levels in PCa cell lines measured by RT-qPCR.

Figure 2. CTBP1 represses *CLCA2* transcription in PC3 cell line. *A)* Plasmids with different length of the *CLCA2* promoter were co-transfected with CTBP1 or control (pcDNA3) plasmids in PC3 cells and Luciferase activity was determined. Bars indicate mean and SD of two independent experiments with three replicates. *B) CLCA2* expression levels by RT-qPCR from PC3 cells after CTBP1 plasmid transfection or *C)* from PC3.pGIPZ and PC3.shCTBP1 stable cell lines. *D)* ChIP analysis using CTBP1 or non-specific (GAL4) antibodies was performed. DNA-ChIP was analyzed by qPCR using primers located at the *CLCA2* proximal promoter region or a non-specific region (*HBB*). Fold enrichment was calculated normalizing data to input and GAL4.

Figure 3. *CLCA2* is epigenetically regulated by HDACs, ZEB1 and EP300. *A-D)* PC3 cells were co-transfected with the CLCA2 promoter reporter (-1182/+1) and CTBP1 or control (pcDNA3) plasmids plus ZEB1, EP300, HDAC2 or HDAC3, and Luciferase activity was determined. The mean of two independent experiments with three replicates and SD is plotted. *E)* PC3 cells were exposed to TSA (1 µM) and co-transfected with *CLCA2* promoter reporter (-1182/+1) and Luficerase activity was determined. *F)* mRNA CLCA2 levels measured by RT-qPCR of PC3 cells treated with TSA 1 µM.

Figure 4. CLCA2 induces cell adhesion and epithelial phenotype. *A) CLCA2* mRNA expression levels by RT-qPCR from PC3 cells after *CLCA2* plasmid transfection or from PC3.pGIPZ and PC3.shCLCA2 stable cell lines *B)* Cell adhesion assay in *CLCA2* depleted (shCLCA2) or overexpressed cells. *C)* PC3.pGIPZ or PC3.shCLCA2 stable cells lines were transfected with TOP FLASH plasmid and Luficerase activity was determined. The mean of a representative experiment with three replicates and SD is plotted. *D)* CDH1, SNAI2, TWIST1 gene expression from PC3.shCLCA2 cells measured by RT-qPCR. *E*) Percentage of mice with or without CTCs is shown. The mice were fed with CD or HFD and s.c. injected with PC3.shCLCA2 or control (pGIPZ) stable cell lines. Number of CTCs was calculated as the number of cells obtained from the blood with the capability to make a colony in a plate. Representative CTCs foci picture from CD or HFD peripheral blood mice after s.c. injection with PC3.shCLCA2 or control (pGIPZ) stable cell lines.

Figure 5. CLCA2 repression by CtBP1 is mediated by miR-196b-5p. *A)* miRNAs modulated by CTBP1 from xenograft generated in MeS mice. miR-196b-5c that is used in the following experiments is shadow. *B)* GO terms and *(C)* pathways for the targets of the miRNAs up regulated or down regulated by CTBP1 identified by ChemiR database. *D)* PC3 cells were co-transfected with 3' UTR CLCA2 plasmid and miR-196a, miR-196b or its control (pcDNA3). Luciferase activity was determined. *E)* miR-196b-5p expression levels from CTBP1 depleted or control xenografts generated in HFD or CD fed mice measured by miRNA RT-qPCR. *F)* Cell adhesion in PC3 cells transfected with pcDNA3 (control), miR-196a or miR-196b expression plasmids.

Figure 6. Hypothetical model. CtBP1 protein assembles to CLCA2 proximal promoter region together with p300, ZEB1, HDAC2 and HDAC3, and represses its transcription. In addition, CtBP1 induces miR-196b-5p which, in turn represses CLCA2 expression. Hence, CLCA2 promotes an epithelial phenotype by sequestering CTNNB in the membrane and repressing EMT genes.

FUNDING AND ACKNOWLEDGMENT

This research was supported by the Argentinean Agency of Science and Technology (ANPCyT PICT 2014-324; ANPCyT PICT 2013-2151; ANPCyT PICT 2015-1345). We thank the Fundación Williams (Argentina) and Fundación Rene Barón (Argentina) for their support. We also thank Dr. Martin Monte (Universidad de Buenos Aires, Argentina) and Ann-Joy Cheng (Chang Gung University, Taiwan) for providing plasmids.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:7–30.
2. Attard G, Parker C, Eeles RA, Schröder F, Tomlins SA, Tannock I, Drake CG, De Bono JS. Prostate cancer. *Lancet* 2016;387:70–82.
3. Mandair D, Rossi RE, Pericleous M, Whyand T, Caplin ME. Prostate cancer and the influence of dietary factors and supplements: a systematic review. *Nutr Metab (Lond)* 2014;11:30.
4. Bashir MN. Epidemiology of prostate cancer. *Asian Pacific J Cancer Prev* 2015;16:5137–41.
5. Nunez C, Bauman A, Egger S, Sitas F, Nair-Shalliker V. Obesity, physical activity and cancer risks: Results from the Cancer, Lifestyle and Evaluation of Risk Study (CLEAR). *Cancer Epidemiol* 2017;47:56–63.
6. NCEP -ATPIII. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Communication*

2002;106:3143–421.

7. Gacci M, Russo GI, De Nunzio C, Sebastianelli A, Salvi M, Vignozzi L, Tubaro A, Morgia G, Serni S. Meta-analysis of metabolic syndrome and prostate cancer. *Prostate Cancer Prostatic Dis* 2017;1–10.
8. Chinnadurai G. Transcriptional regulation by C-terminal binding proteins. *Int J Biochem Cell Biol* 2007;39:1593–607.
9. Moiola CP, Luca P De, Zalazar F, Cotignola J, Rodríguez-Seguí SA, Gardner K, Meiss R, Vallecorsa P, Pignataro O, Mazza O, Vazquez ES, De Siervi A. Prostate tumor growth is impaired by CtBP1 depletion in high-fat diet-fed mice. *Clin Cancer Res* 2014;20:4086–95.
10. De Luca P, Dalton GN, Scalise GD, Moiola CP, Porretti J, Massillo C, Kordon E, Gardner K, Zalazar F, Flumian C, Todaro L, Vazquez ES, et al. CtBP1 associates metabolic syndrome and breast carcinogenesis targeting multiple miRNAs. *Oncotarget* 2016;7:18798–811.
11. Elble RC, Walia V, Cheng HC, Connon CJ, Mundhenk L, Gruber AD, Pauli BU. The putative chloride channel hCLCA2 has a single C-terminal transmembrane segment. *J Biol Chem* 2006;281:29448–54.
12. Gruber AD, Pauli BU. Tumorigenicity of human breast cancer is associated with loss of the Ca2+-activated chloride channel CLCA2. *Cancer Res* 1999;59:5488–91.
13. Li X, Cowell JK, Sossey-Alaoui K. CLCA2 tumour suppressor gene in 1p31 is epigenetically regulated in breast cancer. *Oncogene* 2004;23:1474–80.
14. Liebert MA, Bustin SA, Li S, Dorudi S. Expression of the Ca 2 1 -Activated Chloride Channel Genes CLCA1 and CLCA2 Is Downregulated in Human Colorectal Cancer. 2001;20:331–8.

15. Tanikawa C, Nakagawa H, Furukawa Y, Nakamura Y, Matsuda K. CLCA2 as a p53-inducible senescence mediator. *Neoplasia* 2012;14:141–9.

16. Walia V, Ding M, Kumar S, Nie D, Premkumar L, Elble C. hCLCA2 is a p53-inducible inhibitor of breast cancer cell proliferation. *Cancer Res* 2009;69:6624–32.

17. Walia V, Yu Y, Cao D, Sun M, McLean JR, Hollier BG, Cheng J, Mani S a, Rao K, Premkumar L, Elble RC. Loss of breast epithelial marker hCLCA2 promotes epithelial-to-mesenchymal transition and indicates higher risk of metastasis. *Oncogene* 2012;31:2237–46.

18. Ramena G, Yin Y, Yu Y, Walia V, Elble RC. CLCA2 interactor EVA1 is required for mammary epithelial cell differentiation. *PLoS One* 2016;11:1–21.

19. Walia V, Yu Y, Cao D, Sun M, McLean JR, Hollier BG, Cheng J, Mani SA, Krishna Rao Ç, Premkumar L, Elble RC. Loss of breast epithelial marker hCLCA2 promotes epithelial to mesenchymal transition and indicates higher risk of metastasis. *Oncogene* 2012;57:742–68.

20. Riker AI, Enkemann SA, Fodstad O, Liu S, Ren S, Morris C, Xi Y, Howell P, Metge B, Samant RS, Shevde LA, Li W, et al. The Gene Expression Profiles of Primary and Metastatic Melanoma Yields a Transition Point of Tumor Progression and Metastasis. *BMC Med Genomics* 2008;1:13.

21. Sasaki Y, Koyama R, Maruyama R, Hirano T, Tamura M, Sugisaka J, Suzuki H, Idogawa M, Shinomura Y, Tokino T. CLCA2, a target of the p53 family, negatively regulates cancer cell migration and invasion. *Cancer Biol Ther* 2012;13:1512–21.

22. Llorens MC, Lorenzatti G, Cavallo NL, Vaglienti M V., Perrone AP, Carenbauer AL, Darling DS, Cabanillas AM. Phosphorylation Regulates Functions of ZEB1

Transcription Factor. *J Cell Physiol* 2016;231:2205–17.

23. Smith JL, Freebern WJ, Collins I, De Siervi A, Montano I, Haggerty CM, McNutt MC, Butscher WG, Dzekunova I, Petersen DW, Kawasaki E, Merchant JL, et al. Kinetic profiles of p300 occupancy in vivo predict common features of promoter structure and coactivator recruitment. *Proc Natl Acad Sci U S A* 2004;101:11554–9.
24. Lu Y-C, Chang JT, Liao C-T, Kang C-J, Huang S-F, Chen I-H, Huang C-C, Huang Y-C, Chen W-H, Tsai C-Y, Wang H-M, Yen T-C, et al. OncomiR-196 promotes an invasive phenotype in oral cancer through the NME4-JNK-TIMP1-MMP signaling pathway. *Mol Cancer* 2014;13:218.
25. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 2005;33:e179.
26. Fernández NB, Lorenzo D, Picco ME, Barbero G, Dergan-Dylon LS, Marks MP, García-Rivello H, Gimenez L, Labovsky V, Grumolato L, Lopez-Bergami P. ROR1 contributes to melanoma cell growth and migration by regulating N-cadherin expression via the PI3K/Akt pathway. *Mol Carcinog* 2016;55:1772–85.
27. Shi Y, Sawada J, Sui G, Affar EB, Whetstine JR, Lan F, Ogawa H, Luke MP-S, Nakatani Y, Shi Y. Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* 2003;422:735–8.
28. Massillo C, Dalton GN, Farré PL, De Luca P, De Siervi A. Implications of microRNA dysregulation in the development of prostate cancer. *Reproduction* 2017;154:R81–97.
29. Chakravarthi BVSK, Pathi SS, Goswami MT, Cieslik M, Zheng H, Nallasivam S,

Arekapudi SR, Jing X, Siddiqui J, Athanikar J, Carskadon SL, Lonigro RJ, et al. The miR-124-prolyl hydroxylase P4HA1-MMP1 axis plays a critical role in prostate cancer progression. *Oncotarget* 2014;5:6654–69.

30. Lu YC, Joseph T Chang, Chan EC, Chao YK, Yeh T Sen, Chen JS, Cheng AJ. miR-196, an emerging cancer biomarker for digestive tract cancers. *J Cancer* 2016;7:650–5.

31. Chen C, Zhang Y, Zhang L, Weakley SM, Yao Q. MicroRNA-196: critical roles and clinical applications in development and cancer. *J Cell Mol Med* 2011;15:14–23.

32. Jeong J-H, Park S-J, Dickinson SI, Luo J-L. A Constitutive Intrinsic Inflammatory Signaling Circuit Composed of miR-196b, Meis2, PPP3CC, and p65 Drives Prostate Cancer Castration Resistance. *Mol Cell* 2016;65:154–67.

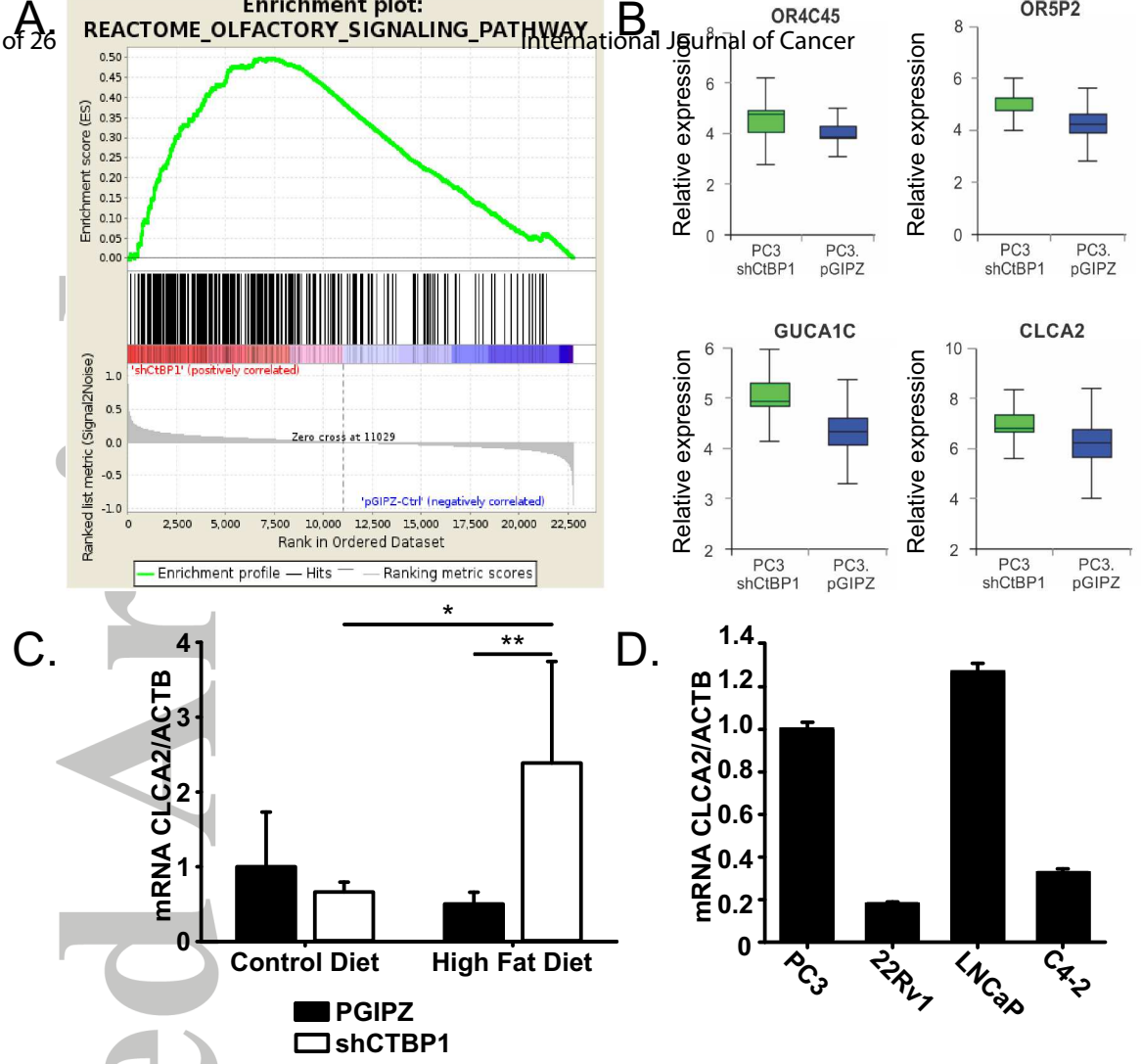


Figure 1

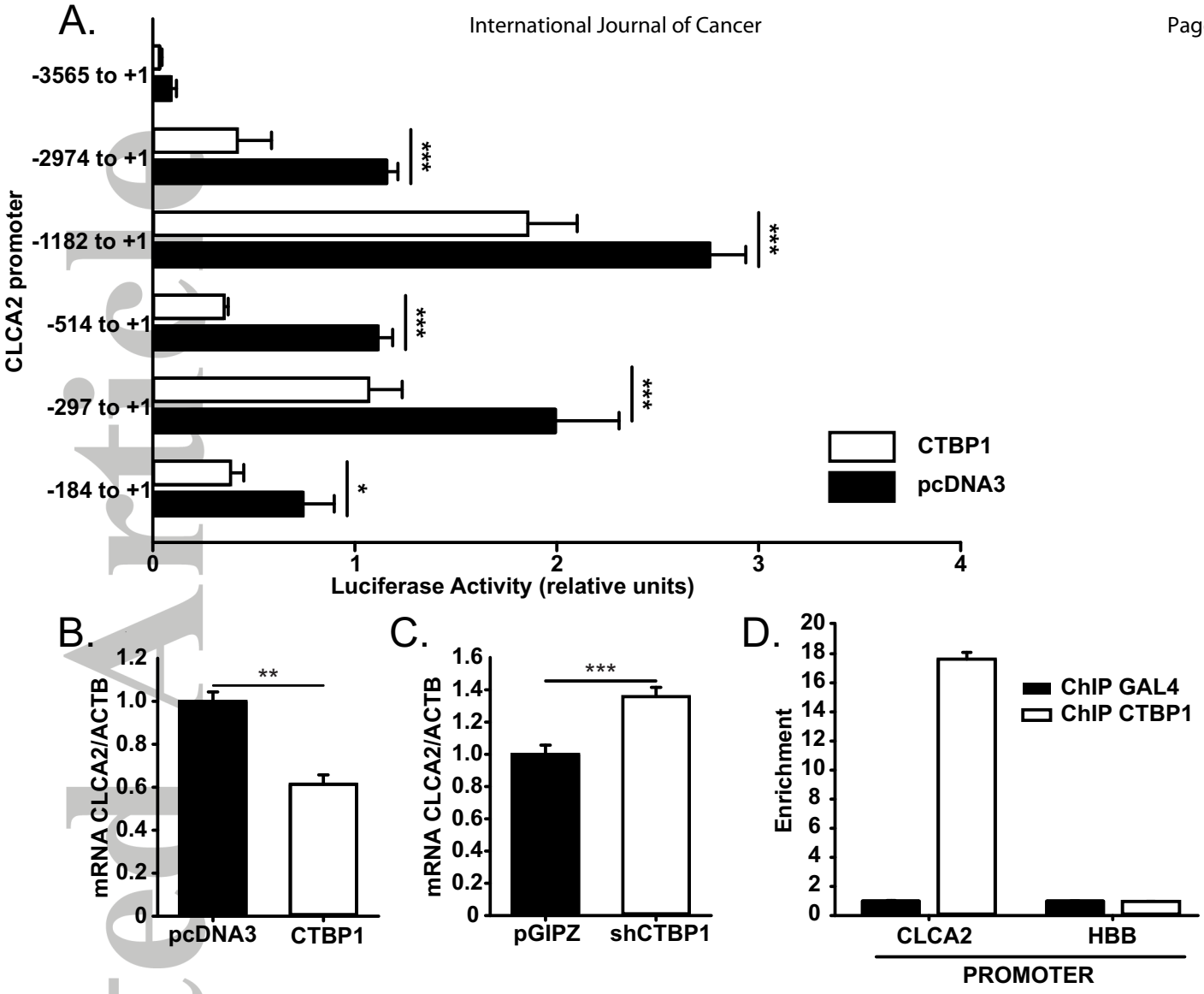


Figure 2

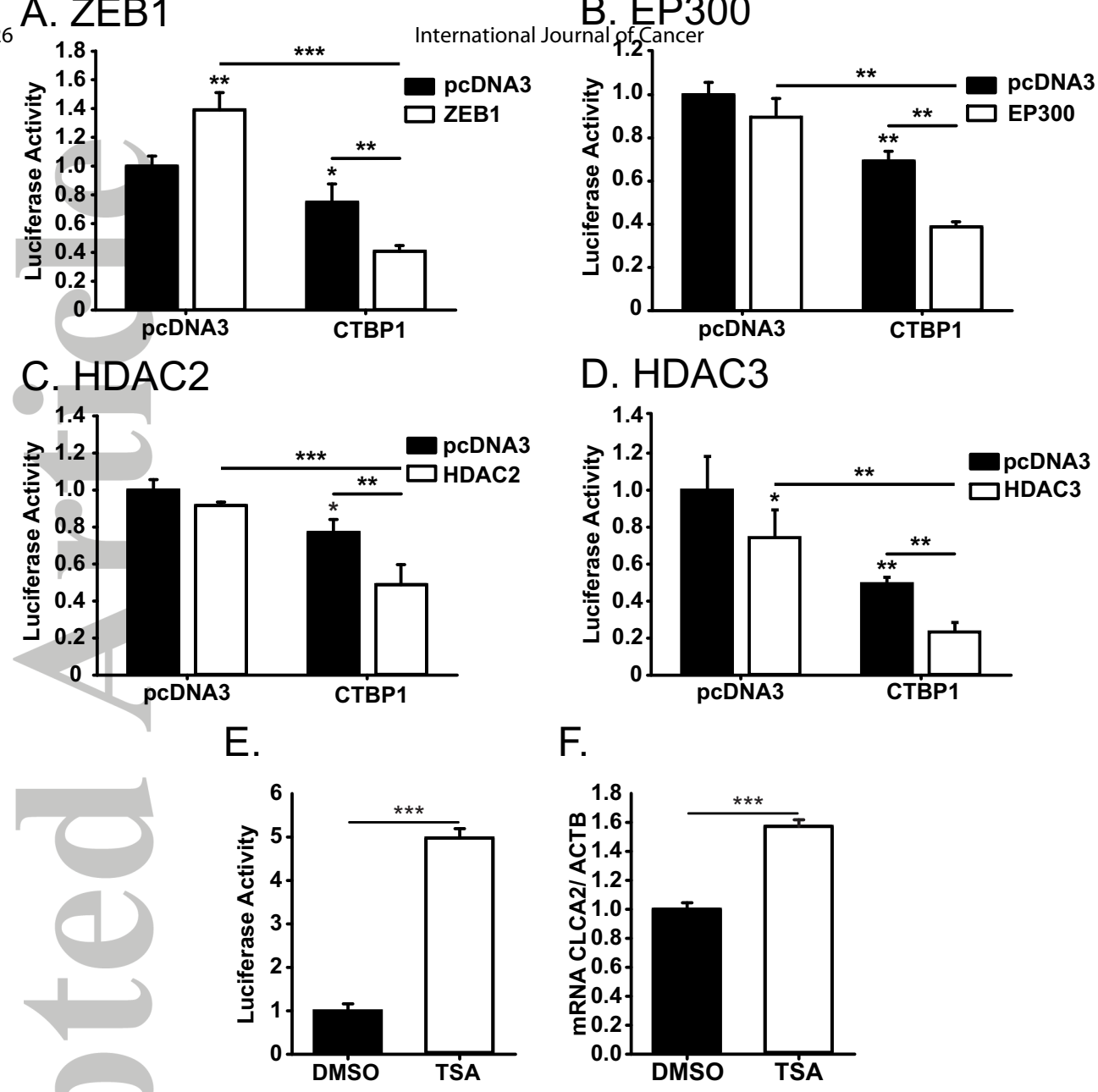
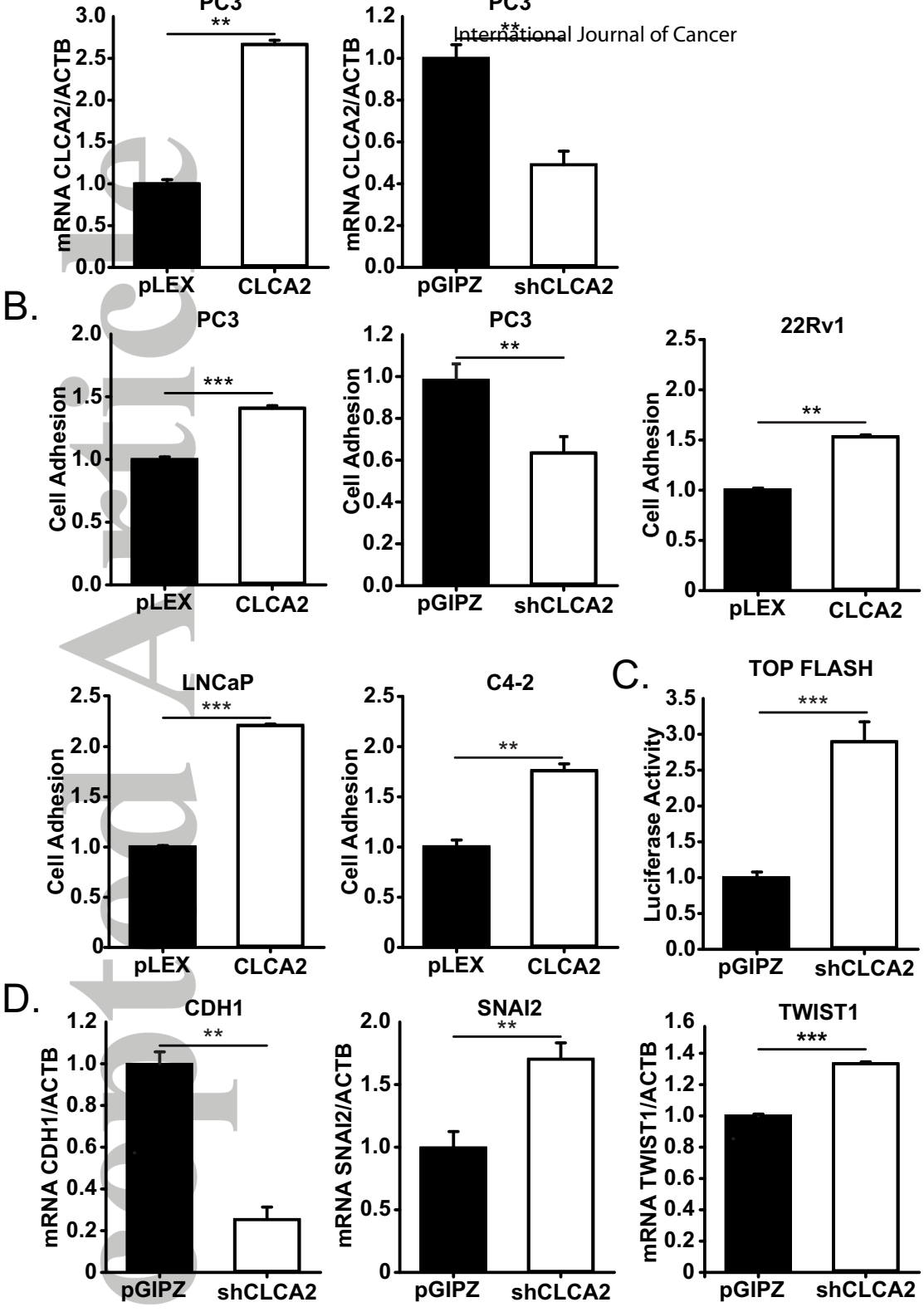


Figure 3



E.

CTCs	mice (%)			
	Control Diet		High Fat Diet	
	pGIPZ2	shCLCA2	pGIPZ2	shCLCA2
Absence	60	25	100	60
Presence	40	75	0	40

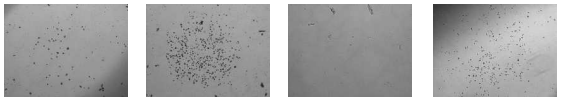
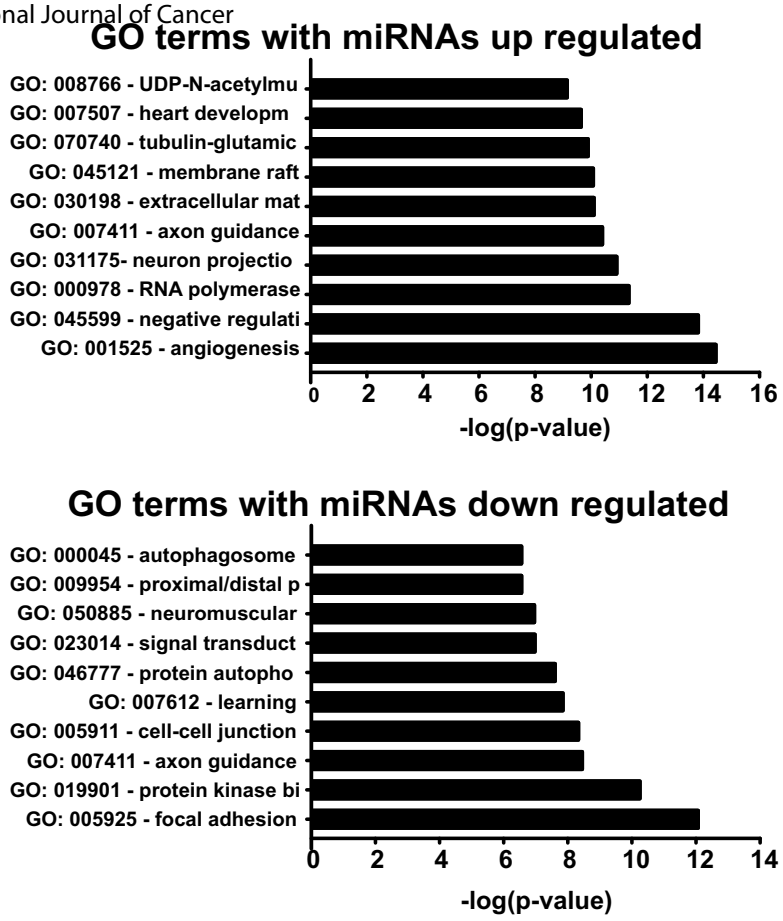


Figure 4

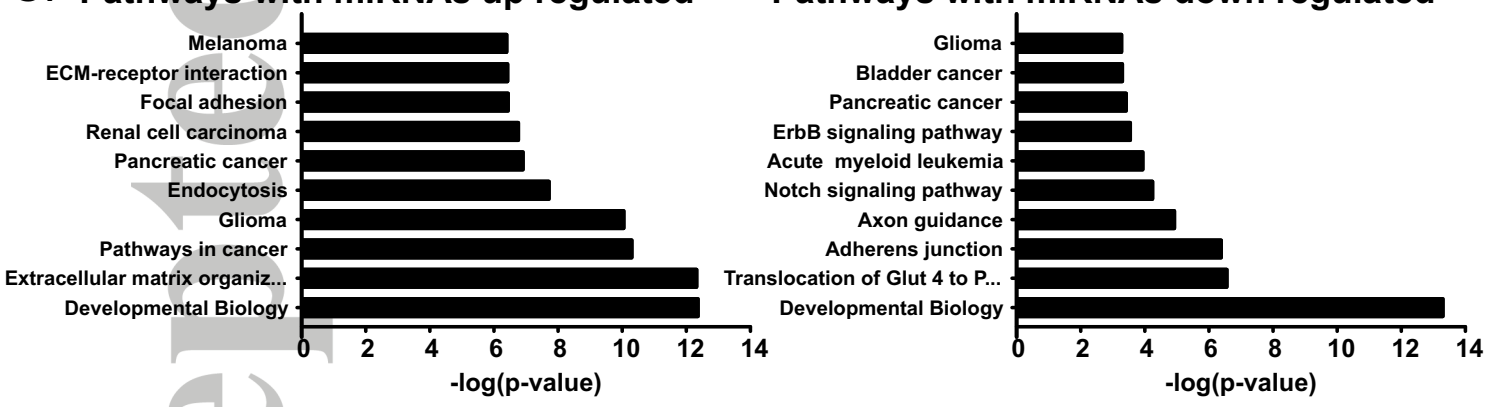
A.

Transcript ID	Accession	FC	ANOVA p-value
hsa-miR-205-5p	MIMAT0000266	-31,61	0,030686
hsa-miR-214-3p	MIMAT0000271	-2,42	0,014958
hsa-miR-423-5p	MIMAT0004748	-2,39	0,012982
hsa-miR-23a-5p	MIMAT0004496	-1,74	0,03824
hsa-miR-744-5p	MIMAT0004945	-1,62	0,037815
hsa-miR-25-5p	MIMAT0004498	-1,55	0,012352
hsa-miR-3135b	MIMAT0018985	1,51	0,042012
hsa-miR-29a-3p	MIMAT0000086	1,54	0,034618
hsa-miR-130a-3p	MIMAT0000425	1,58	0,008581
hsa-miR-30c-5p	MIMAT0000244	1,58	0,041018
hsa-let-7g-5p	MIMAT0000414	1,62	0,014808
hsa-miR-374b-5p	MIMAT0004955	1,64	0,033057
hsa-miR-196b-5p	MIMAT0001080	1,67	0,046507
hsa-miR-128-3p	MIMAT0000424	1,78	0,011086
hsa-miR-6790-5p	MIMAT0027480	1,98	0,012939
hsa-miR-30b-5p	MIMAT0000420	2,07	0,012015
hsa-miR-19b-3p	MIMAT0000074	2,17	0,045632
hsa-miR-335-5p	MIMAT0000765	2,2	0,047453
hsa-miR-31-3p	MIMAT0004504	2,29	0,03893
hsa-miR-8063	MIMAT0030990	2,3	0,01417
hsa-miR-140-5p	MIMAT0000431	6,78	0,032651

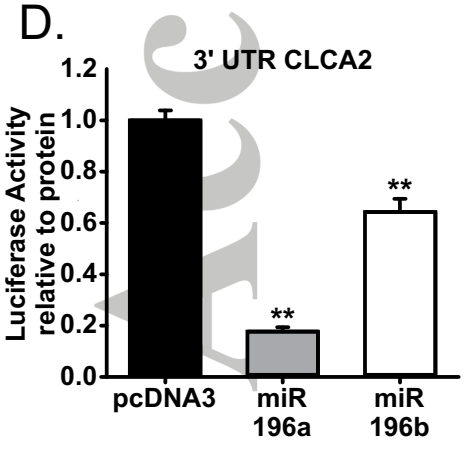
B.



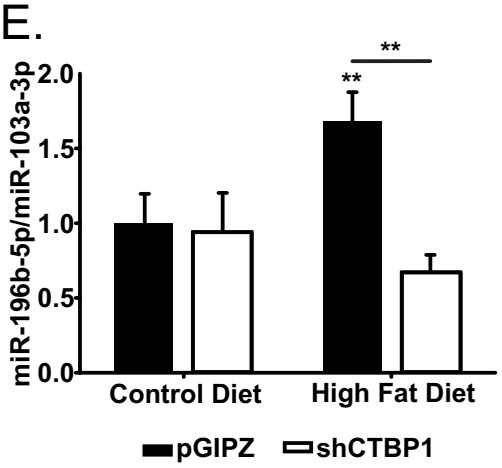
C.



D.



E.



F.

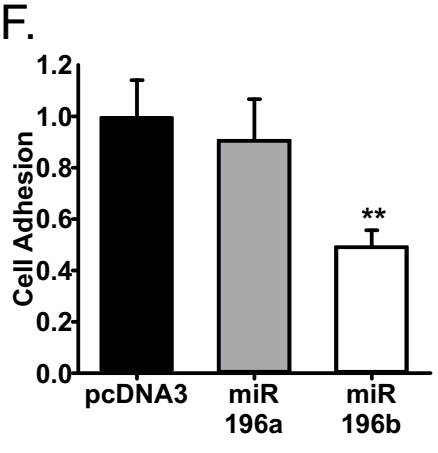


Figure 5

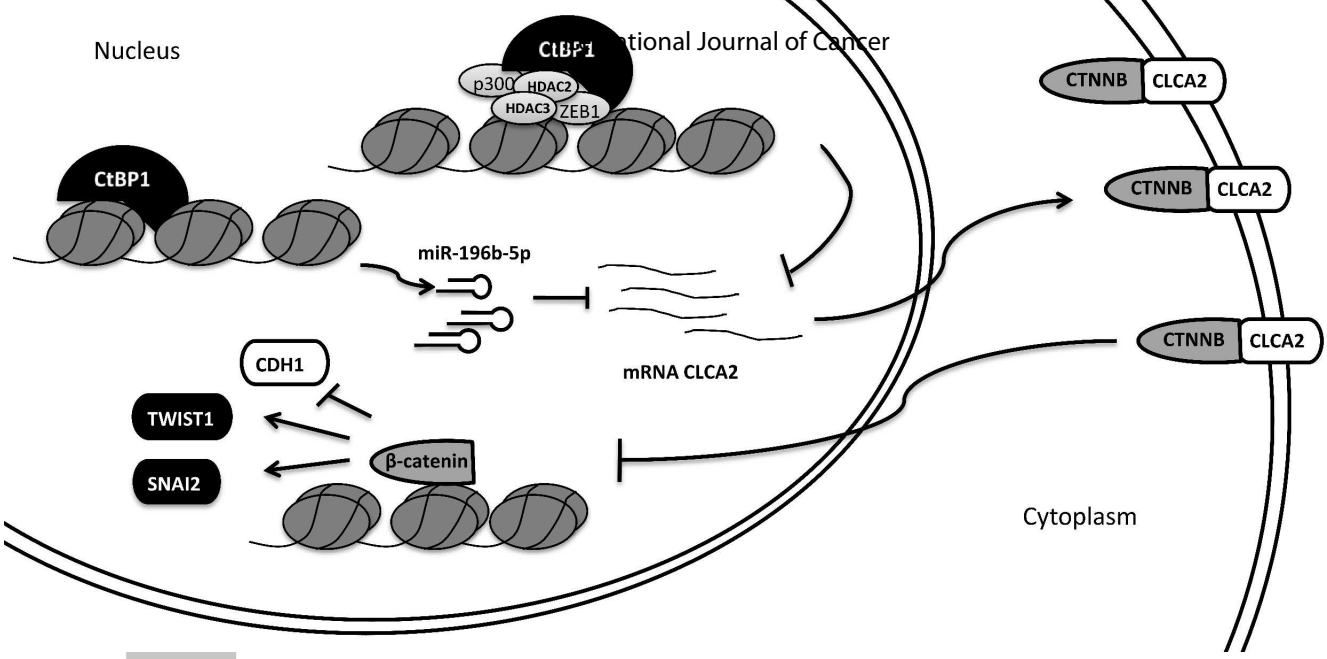


Figure 6