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Running title: CtBP1 and high fat diet regulates PCa growth.

Conflicts of Interest: No potential conflicts of interest were disclosed.

Statement of significance:

Chronic high fat diet intake induces metabolic syndrome and modulates CtBP1 expression. We reveal a novel molecular link between high fat diet and prostate tumor growth. Our results suggest that metabolic syndrome-like diseases and CtBP1 expression cooperate to induce prostate tumor growth. Targeting of CtBP1 expression might be considered for PCa management and therapy in the subset of patients with metabolic syndrome. Our findings are highly relevant because CtBP1 pathway might help to identify new molecular candidates for better prediction of PCa progression in a set of patients with metabolic syndrome.

Abstract

Purpose: Clinical and epidemiological data suggest that obesity is associated with more aggressive forms of prostate cancer (PCa), poor prognosis and increased mortality. C-terminal Binding Protein 1 (CtBP1) is a transcription repressor of tumor suppressor genes and is activated by NADH binding. High calorie intake decreases intracellular NAD+/NADH ratio. The aim of this work was to assess the effect of high fat diet (HFD) and CtBP1 expression modulation over prostate xenograft growth.

Experimental design: We developed a metabolic syndrome-like disease *in vivo* model by feeding male nude mice with HFD during 16 weeks. Control diet (CD) fed animals were maintained at same conditions. Mice were inoculated with PC3 cells stable transfected with shCtBP1 or control plasmids. Genome-wide expression profiles and Gene Set Enrichment Analysis (GSEA) was performed from PC3.shCtBP1 *versus* PC3.pGIPZ HFD fed mice tumors.

Results: No significant differences were observed in tumor growth on CD fed mice; however, we found that only 60 % of HFD fed mice inoculated with CtBP1 depleted cells developed a tumor. Moreover these tumors were significantly smaller than those generated by PC3.pGIPZ control xenografts. We found 823 genes differentially expressed in shCtBP1 tumors from HFD fed mice. GSEA from expression dataset showed that most of these genes correspond to cell adhesion, metabolic process and cell cycle.

Conclusions: Metabolic syndrome-like diseases and CtBP1 expression cooperate to induce prostate tumor growth. Hence, targeting of CtBP1 expression might be considered for PCa management and therapy in the subset of patients with metabolic syndromes.

Keywords: High Fat Diet, Prostate Cancer, CtBP1, BRCA1.

Introduction

Prostate cancer (PCa) continues to be a major health care problem worldwide (1). Epidemiological studies indicate that elevated body mass index (BMI) correlates with elevated risk of PCa-specific mortality and biochemical recurrence (2). Due to the increase of overweight and obesity incidences throughout the world, the number of men at risk for developing PCa is also on the rise. There are several molecular mechanisms that physiologically link obesity to cancer risk; however, it remains a puzzle how exactly obesity activates the transformation pathways that result in cancer.

Calorie excess intake impacts life span, the incidence of diseases and metabolic disorders through diverse mechanisms (3). For instance, nutrient excess influences NAD+/NADH ratio with the associated increase of reactive oxygen species (ROS) as a consequence of incomplete mitochondrial electron transfer during respiration. High ROS influences cell proliferation, differentiation and death, as these processes are intrinsically dependent upon the cellular redox status. In addition, ROS also contribute to increase the risk of malignant transformation by causing DNA damage (4). Calorie excess affects NAD+ availability, which in turn, modulates the activity of certain classes of mammalian proteins that utilize NAD+ or NADH as cofactors, ligands or substrates. Some of these molecules include: the Sirtuin family of Class III histone deacetylases, the PARP family of poly ADP ribosyl-transferases, and the C-terminal binding protein (CtBP) class of transcriptional repressors, which are involved in DNA damage response (4).

CtBP1 was first identified as a cellular protein that binds to the carboxy terminus of E1A (5). It is more active as a dimer and its dimerization is promoted by NAD(H) binding (6). Hence, CtBP1 is assumed as both a sensor and an effector of cellular metabolic status due to its much higher affinity (>100-fold) for NADH compared to NAD+ (7). CtBP1 interacts with a broad range of transcription factors and represses transcription of several tumor suppressor genes such as E-cadherin, BRCA1, CDKN2A, Sirtuin 1 (SIRT1) and PTEN (6, 8-11). Therefore, since the discovery of CtBP1 several efforts were made to assess the involvement of CtBP1 in cancer development (12, 13).

Previously, Di *et al* showed that NAD+/NADH levels selectively regulate BRCA1 tumor suppressor gene (8). The release of CtBP1 from the BRCA1 promoter through estrogen induction or enhanced NAD+/NADH ratio leads to HDAC1 dismissal, elevated histone acetylation and increased BRCA1 transcription, diminishing cancer risk (8).

CtBP1-mediated transcriptional repression of E-cadherin seems to be regulated by the hypoxic environment in solid tumors with poor vascularization and high metabolic activity. A hypoxic condition that increases free NADH levels has been shown to enhance recruitment of CtBP1 to the E-cadherin promoter inducing tumor cell motility. Hence, CtBP1 was suggested to participate in the epithelial to mesenchymal transition (EMT) (6).

In summary, literature fosters a strong role for CtBP1 as a negative regulator of several important tumor suppressors. This function suggests that CtBP1 might be crucial in tumor initiation and progression. In addition, the regulation of CtBP1 by NAD+/NADH places this factor in the category of molecules that link transcription to cellular metabolism. Nevertheless, the importance of CtBP1 as a sensor of nuclear redox state *in vivo* has yet to be determined. Our hypothesis was that enhanced CtBP1 activity in prostate tissues with low NAD+/NADH ratios, as a consequence of high calorie intake, contributes to increase prostate tumor development. Here, we explored CtBP1 role in prostate malignant transformation. Using an *in vivo* model of reduced NAD+/NADH ratio, we investigated the effect of the high fat diet (HFD) on prostate tumor growth through CtBP1 modulation. We focused our studies in the molecular pathways regulated by CtBP1 and the interplay with sexual hormones in PCa.

Methods

Cell culture, transfections and treatments.

PC3 (ATCC: CRL-1435), 22Rv1 (ATCC: CRL-2505), LNCaP (ATCC: CRL-1740) and C4-2 (14) PCa cells were grown in RPMI 1640 (GIBCO, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified atmosphere at 37°C.

PC3 stable cell line (PC3.CtBP1) was generated by transfecting with pcDNA3.CtBP1 plasmid (15 µg, Origene) and lipofectamine 2000 (Invitrogen, Sao Pablo, Brazil) as previously described (15). PC3 CtBP1 depleted cells (PC3.shCtBP1) lentiviral infection was performed as described in Supplemental Methods.

PC3 cells were exposed to testosterone undecanoate (10 µM) or DMSO as vehicle in phenol red free RPMI 1640 medium (GIBCO) supplemented with 10% charcoaled FBS. Letrozole (Femara®-Novartis) was prepared in ethanol and PC3 cells were exposed for 1h (5 µM) previously to testosterone stimulation and incubated for 24 h. PC3 cells were transfected with lipofectamine 2000 and 1 µg of AR5 vector (gently gifted by Dr. G. Jenster, Erasmus University Rotterdam, Netherlands).

Chromatin immunoprecipitation (ChIP), RT-qPCR, Western blot and IHC.

ChIP, RT-qPCR, WB and IHC were performed as described in Supplemental Methods (15-17).

Focus formation and clonogenic assays.

NIH3T3 cells were transfected with 5 µg pcDNA3.CtBP1 or control (pcDNA3.β-Gal) plasmids by calcium-phosphate method as previously described (18). For clonogenic assays, 10^3 cells were seeded in a 100 mm plate and incubated for 2 weeks. In both methods, cells were methanol-fixed and stained with crystal violet. Foci number and area were quantified using GelPro Analizer v4.0 software. Photographs were acquired with Phosphorimager (Fuji Photo Film Co. Ltd.).

PC3-high fat xenograft model.

All animal experiments followed the institutional guidelines for animal welfare. Four week old athymic male Swiss *nu/nu* mice were fed for 16 weeks with control diet (CD) (n=18) or HFD (n=18). Chow food was supplemented with bovine fat in a 2:1 proportion (w/w) to generate HFD. CD and HFD had 3 kcal or 5 kcal per gram of food, respectively. After 12 weeks of diet, each CD and HFD fed mice group were randomly divided and *s.c.* injected with PC3.pGIPZ or PC3.shCtBP1 cells (4.8×10^6). Body weight was determined three times a week. Tumor size was measured for 4 to 6 weeks and tumor volume was calculated as described (19). At necropsy, blood was drawn from all mice by direct heart puncture; serum was separated and tumors were excised. Tissues were formalin fixed and paraffin-embedded. Histopathology and IHC studies were performed in 5 µm tissue sections using hematoxylin and eosin (H&E) or specific antibodies: anti-CtBP1 (BD Biosciences); anti-BRCA1 (ARP33338_P050, Aviva System Biology, San Diego, CA, USA), anti-E-cadherin (Clone HECD-1, Zymed Laboratories Inc, San Francisco, CA, USA); and anti-Cyclin D1 (H295, Santa Cruz Biotechnologies).

Serum cholesterol, triglycerides, glycemia, NAD+/NADH levels, testosterone and estradiol determinations were performed as described in Supplemental Methods.

Microarrays

Microarrays were performed as described in Supplemental Methods. (20)

Statistical Analysis

All results are given as mean ± standard deviation of three independent experiments. Students´ t tests were used to ascertain statistical significance with a threshold of $P < 0.05$. For *in vivo* experiments two-way ANOVA followed by Dunnett test were performed. Shapiro-Wilk and Levene test were used to test normality and homogeneity of variances. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Results

CtBP1 is overexpressed in human prostate tumors

We analyzed CtBP1 expression on a small group of 20 radical prostatectomies specimens and we found that normal, benign and carcinomatous epitheliums showed different positive CtBP1 immunoreactivity patterns (Fig. 1A). Normal glands columnar epithelia cells and some basal cells showed a mild positive nuclear stain (Fig. 1A), while in benign prostatic hyperplasia, the columnar epithelium showed a moderate positive nuclear immunostain (Fig. 1A). Remarkably, all the cells in the epithelia of the carcinomatous glands showed intense nuclear and moderate cytoplasmatic immunostaining (Fig. 1A). In accordance with these findings showing that CtBP1 expression is increased in human PCa samples, recently, Wang et al. (12) observed CtBP1 overexpression and mislocalization in human metastatic PCa.

CtBP1 increases transformation and proliferation *in vitro*

To investigate whether CtBP1 induces transformation *in vitro*, we performed the focus formation assay transfecting NIH 3T3 cells with pcDNA3.CtBP1 or control vectors. We found that CtBP1 transfected cells showed larger and higher number of foci compared to control (Fig. S1A).

Next, we determined CtBP1 expression in PCa cell lines (Fig. 1B). We found that androgen sensitive LNCaP cells showed higher CtBP1 expression compared to the other PCa cells lines. Notably, androgen-insensitive PC3 cells showed five times lower CtBP1 expression than LNCaP cells (Fig. 1B). We generated stable transfected PC3 cells with enhanced (PC3.CtBP1) or depleted (PC3.shCtBP1) CtBP1 expression, as confirmed by WB and RT-qPCR compared to PC3.pGIPZ control cells (Fig. 1C and S1B).

In order to determine CtBP1 role on cell proliferation, we assessed stable transfected PC3 cells clonogenic capacity. PC3.shCtBP1 cells showed significantly lower colony number compared to control (Fig. 1D). On the other hand, CtBP1 overexpression increased not only the colony number but also the colony area (Fig. 1D). Altogether, these results indicate that CtBP1 induces transformation of NIH 3T3 cells and proliferation of prostate tumor PC3 cells *in vitro.*

HFD induces metabolic syndrome-like disease in nude mice

Based on high NADH levels induce CtBP1 dimerization and activation (7), we investigated whether HFD influences NAD+/NADH ratio and, in turn, tumor growth using a murine xenograft experimental model. Males *nu/nu* mice fed with HFD or CD during 12 weeks were *s.c.* inoculated with PC3.pGIPZ or PC3.shCtBP1 stable cell lines. After 4 to 6 weeks from cell inoculation mice were sacrificed. Despite HFD fed mice showed a trend towards gaining body weight, no significant differences were detected among mice fed with HFD or CD along the experiment (Fig. 2A). Nevertheless, animals fed with HFD presented hypercholesterolemia at the end of the experiment (Fig. 2B), with no significant changes in serum triglycerides and glycemia compared to control mice (Fig. S2A-B).

As a consequence of a prolonged HFD intake, animals developed a metabolic syndrome-like disease, evidenced by hormonal imbalance and kidney and liver histological architecture disorganization. We found significant decreased testosterone serum levels in HFD mice (Fig. 2C) with no changes in estradiol serum levels (Fig. S2C).

Furthermore, kidneys from HFD fed mice showed glomeruli significantly enlarged by mesangial hypercellularity and edema at the epithelium of the collecting duct (Fig. S2D), while liver displayed altered architecture of hepatic lobules and a pronounced vacuolization of hepatic cells as a consequence of steatosis (Fig. S2E).

CtBP1 depletion decreases tumor growth in HFD fed mice

We assessed tumor growth in a xenograft model injected *s.c.* with PC3.shCtBP1 or PC3.pGIPZ cells. As shown in Fig. 3A, non-significant differences were observed in the tumor growth curves from PC3.shCtBP1 and PC3.pGIPZ cells in the CD group. Interestingly, in the HFD group, CtBP1 depleted PC3 cells developed significantly smaller tumors than animals inoculated with PC3 control cells (Fig. 3B). In addition, we could not detect tumor growth in 40 % of mice injected with PC3.shCtBP1 cells and fed with a HFD while all the animals of the other 3 groups developed tumors.

Remarkably, tumors from HFD fed mice showed significantly reduced NAD+/NADH ratio which strongly support our hypothesis that HFD induces NADH levels (Fig. 3C). Moreover, estradiol levels in HFD xenografts were increased but no significant changes in intratumoral testosterone levels were observed (Fig. S3A-B).

Histological characterization revealed that all tumors were poorly differentiated adenocarcinoma (Fig. 3D). Moreover, CtBP1 depletion was confirmed in PC3.shCtBP1 xenografts by IHC and RT-qPCR compared to PC3.pGIPZ control tumors (Fig. 3D-S3C).

CtBP1 modulates cell adhesion, cell cycle and steroid hormone response pathways

Since only HFD fed animals inoculated with PC3.shCtBP1 cells showed tumor growth impairment, we next investigated the molecular pathways deregulated by CtBP1 depletion that might decrease tumor development *in vivo*. We performed a whole genome expression array (Affymetrix HuGene 1.0 ST) from PC3.shCtBP1 and PC3.pGIPZ tumor xenografts growing in HFD fed mice. These arrays interrogate 28,869 annotated genes in the human genome. Three independent samples for each condition were used. Our results showed 823 genes differentially expressed (fold change > 1.5; $p < 0.05$) comparing PC3.pGIPZ with PC3.shCtBP1 xenografts from HFD fed mice. Functional gene ontology (GO) analysis revealed that most of these genes correspond to metabolic processes (44.1%), cell communication (30.7%) and cell adhesion (11.7%) among other biological functions (Fig 4A).

Furthermore, Gene Set Enrichment Analysis (GSEA) revealed enhancement of genesets associated with cell cycle progression and proliferation (Supplementary Table 1). In addition, PC3.shCtBP1 samples showed enrichment of genesets associated with cell adhesion, hormone metabolism and BRCA1 targets gene regulation (Supplementary Table 2).

CtBP1 depletion in xenografts from HFD fed mice induces E-cadherin related pathways.

GSEA further allowed the identification of a large number of genes associated with cell adhesion genesets (Fig S4). Enrichment plot and heatmap from a selected cell adhesion geneset were shown.

Genes listed alongside heatmap were up-regulated in PC3.shCtBP1 compared to control PC3.pGIPZ tumors. Notably, a crucial cell adhesion gene over-represented in these genesets was CDH1 (E-cadherin) (Fig. 4B and S4). We validated this finding by RT-qPCR from tumor xenografts using CDH1 specific primers and found that CDH1 was overexpressed in PC3.shCtBP1 tumors (Fig S4B). IHC CDH1 analysis showed that PC3.shCtBP1 xenografts exhibited moderate to high E-cadherin staining at single cells, mainly at the cellular membrane of necrobiosis foci, while in PC3.pGIPZ tumors, E-cadherin staining was low to moderate, diffuse and located at single cells in necrobiosis foci (Fig 4C).

CtBP1 depletion in xenografts from HFD fed mice induces aromatase related pathways.

We found several steroid hormone metabolism related genes over-represented and over-expressed in PC3.shCtBP1 xenografts compared to control PC3.pGIPZ as indicated in heatmap and enrichment plots in Fig. 4D and S4C. From these analyses we found that one of the genes that was over-represented in the genesets related to hormone biosynthesis and metabolism was CYP19A1 (aromatase enzyme), involved in the estradiol synthesis by conversion from testosterone. We validated this finding by RT-qPCR from tumor xenografts using specific primers for CYP19A1. As shown in Fig 4E, CYP19A1 transcription was significantly induced in PC3.shCtBP1 compared to pGIPZ control.

These results are consistent with the metabolic syndrome-like disease since HFD fed mice showed hormone imbalance. Nevertheless, the fact that CtBP1 depletion induces aromatase expression is a novel finding that reinforces the role of hormones and fat in tumor growth.

CtBP1 depletion in xenografts from HFD fed mice decreases Cyclin D1 expression.

PC3.pGIPZ xenografts also showed a marked enrichment of genesets related to cell cycle regulation and proliferation (Supplementary Table 1 and Fig. S5). Genes in these categories included several cyclin proteins (CCND1, CCND2 and CCND3) and BRCA1, among others. We focused our studies particularly on CCND1 (cyclin D1). We validated the expression of this gene by RT-qPCR in tumors from HFD fed animals and we found that CCND1 is significantly diminished in PC3.shCtBP1 xenografts compared to

control (Fig. 5A). In addition, by IHC we found that PC3.pGIPZ tumors presented very high CCND1 immunoreactivity, while CtBP1 depletion completely abolished CCND1 staining (Fig. 5B).

CtBP1 and steroid hormones impair prostate tumor growth probably through BRCA1 regulation.

It was previously reported that CtBP1 represses BRCA1 transcription in breast cancer cells (8). Here, we found by GSEA that PC3.shCtBP1 xenografts showed an enrichment of genes regulated by BRCA1 (Fig 5C). Moreover, IHC analysis determined that CtBP1 depletion increased BRCA1 positive immunostaining compared to control xenografts from HFD fed animals (Fig. 5D).

Based on several factors might be involved in BRCA1 transcription regulation in PCa, such as steroid hormones and CtBP1, we first determined BRCA1 transcription in the stable CtBP1 PC3 cell lines. We found that CtBP1 repressed BRCA1 transcription *in vitro*, while CtBP1 depletion highly increased BRCA1 expression compared to control PC3.pGIPZ cells (Fig. 6A). Then we exposed three PCa cell lines which differ in AR expression and androgen sensitivity to different testosterone concentrations. We found that BRCA1 mRNA levels significantly increased in PC3 cells exposed to testosterone, even after transfecting with the constitutive active AR vector (AR5) (Fig 6B). Similar results were found using 22Rv1 cells that express AR but are androgen insensitive (Fig.S6A). However, testosterone decreased BRCA1 mRNA levels in LNCaP cells (AR+/+ and androgen sensitive) (Fig. S6B).

Moreover, by ChIP we determined that CtBP1 was associated to BRCA1 proximal promoter region, and testosterone released CtBP1 from this region (Fig. 6C). Altogether these results indicate that BRCA1 transcription is regulated in PCa cell lines by CtBP1 and testosterone.

It was previously reported that BRCA1 transcription is controlled by estradiol in breast cancer cell lines (8). To investigate whether BRCA1 transcription regulation in PCa is due to testosterone or estrogen, as a consequence of testosterone conversion to estradiol by the aromatase enzyme, we assessed BRCA1 transcription in the presence of testosterone and an aromatase inhibitor (letrozole) that inhibits this conversion. We found that letrozole abolished BRCA1 induction by testosterone in both PC3 (Fig 6D) and

22Rv1 (Fig S6C) cells, however, letrozole have no effect over BRCA1 transcription in LNCaP cells exposed to testosterone (Fig. S6D).

In summary, these observations indicate that BRCA1 transcription regulation by testosterone in PCa cells is modulated by aromatase-derived estrogen. Hence, CtBP1 and steroid hormone imbalance induced by the metabolic syndrome disease, govern BRCA1 transcription, which in turn, influence tumor suppression impeding prostate tumor growth.

Discussion

CtBP1 is a transcriptional co-repressor that binds to histone modifiers and recruits repressive complexes to tumor suppressor promoters in order to inhibit their expression. It is becoming increasingly clear that dysregulation of CtBP1 transcriptional repression plays a crucial role in tumorigenesis (4, 8, 12). We present new evidence for CtBP1-mediated oncogenesis in PCa. Our studies demonstrated that gene transcription regulation by CtBP1 provides an important molecular link among caloric intake, CtBP1 function and tumor growth. We demonstrated that CtBP1 overexpression induces cell transformation and proliferation.

We developed a metabolic syndrome-like disease in nude mice adding high fat content to their diet to modulate CtBP1 activity in a reduced NAD+/NADH ratio cellular context. These mice showed a clear hormone imbalance, hypercholesterolemia and histology alterations in their liver and kidneys. Surprisingly, CtBP1 knockdown delays tumor growth in these mice, suggesting that tumor growth in HFD fed animals is CtBP1-dependent.

Several reports showed that HFD induced tumor growth in PCa xenografts using AR+/+ cell lines (22-25). Here, we found that HFD did not significantly influence tumor growth using the AR-/- cells (PC3) xenograft model. In addition, Wang *et al.* demonstrated that CtBP1 depletion decreased DU145 tumor growth in mice on normal chow diet (12). Here, CtBP1 depletion markedly diminished tumor growth

only in HFD fed mice. We believe that intratumor hormone and CtBP1 levels, and probably AR-cell status are crucial "collaborating" factors that are involved in PCa growth.

Takayama *et al* recently demonstrated that CtBP1 regulates AR signaling (26), showing that CtBP1 exerts tumor suppressive effects in AR+/+ PCa cells. In contrast, Wang *et al* (12) described that CtBP1 is upregulated in metastatic PCa and CtBP1 exerts growth stimulatory effects in tumor cells. We demonstrated here that testosterone downregulates CtBP1 mediated transcription targets in AR+/+ cells; meanwhile in AR-/- cells testosterone upregulates CtBP1 transcription targets. We believe that one of the reasons for the inconsistency in CtBP1 functions may be due to impairments in the AR status in the different tumor cell lines. Further experiments will be necessary to understand the mechanism of regulation mediated by CtBP1 in AR-positive or AR-negative cells.

Here, we found that CtBP1 represses BRCA1 transcription in PCa. We showed for the first time that testosterone regulates BRCA1 transcription, probably via its conversion to estrogen by the aromatase enzyme. The fact that several genesets for steroid hormone secretion and biosynthesis were enriched in CtBP1 depleted tumors in addition to an enhance aromatase expression is a novel finding that reinforced hormone and fat role in tumor growth.

The role of NADH in regulating CtBP1 activity is also an important finding that increases researchers' interest. CtBP1 has been proposed to act as a metabolic sensor, responding to changes in NADH levels and modulating gene expression (7). The combination of high NADH content in tumor cells and high fat rich diets are factors that might impact on carcinogenesis. Our work links reliably HFD and high NADH levels with CtBP1 dependent tumorigenesis. Even though, this novel molecular link, might be further explored to understand the potential implications of these pathways in PCa risk.

Expression array profiles from these xenografts showed that the major biological processes affected by CtBP1 depletion are cell adhesion and communication, metabolism and cell cycle. Finally, we were able to determine the potential role of CtBP1 as a gene transcriptional regulator and its implication in tumor development in PCa. Both approaches, GSEA and *in vivo* xenograft model, allowed us to demonstrate that CtBP1 depletion dramatically decreased tumor growth and cell proliferation. Accordingly, Deng *et al*

generated CtBP1 transgenic mice (K5.CtBP1) that showed epidermis hyperproliferation by downregulation of p21 and BRCA1, and loss of E-cadherin expression (21).

We also found that CtBP1 depletion had implication in regulation of genes involved in cell adhesion such as CDH1. However, other important players were also deregulated by CtBP1 depletion and still have to be studied, such as Integrin family (ITGB7, ITGAL, etc), cadherins (CDH3, CDH15, etc) and cell adhesion molecules (ICAM2, NCAM, CADM3, etc). Furthermore, we found several genes involved in cell communication and signaling also altered by CtBP1 depletion; probably having a great influence over PCa tumor signaling and growth.

Obesity, type 2 diabetes and metabolic syndrome are placing an increasing burden on the healthcare systems. Many resources are currently being devoted to the identification of novel therapeutic targets that could alleviate or reverse the progress of these disorders. CtBP1 protein might be a critical target on these diseases. Recent studies have identified CtBP1 as a key transcriptional co-regulator in adipose tissue (27). CtBP proteins interact with several different partners to regulate the development of both white and brown adipocytes. Manipulation of CtBP1 function may provide a mechanism by which energy storage in white adipose tissue can be limited and energy expenditure by brown adipose tissue can be increased.

Overall, our results reveal that CtBP1 govern crucial molecular pathways in prostate tumors in the presence of metabolic syndrome. Thus, it will be important to determine CtBP1 expression level status in the tumors from patients with metabolic syndrome to improve prognosis and disease management. In the future, it will be also important to correlate high fat consumption/weight gain/ body fat/BMI with CtBP1 expression to better understand this role. Finally, CtBP1 pathway might help to identify new molecular candidates for a better prediction of the disease outcome.

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Legend to figures

Figure 1. CtBP1 induces cell transformation and proliferation. *(A)* CtBP1 IHC for radical prostatectomies from different stages of PCa pathogenesis (normal gland to highly undifferentiated adenocarcinoma). An increment in positive immunostain was observed from normal to carcinomatous tissue. Similar positive nuclei stain in muscular and connective stromal fibers were seen in all tissues. Magnification 250x. *(B)* CtBP1 RT-qPCR from PCa cell lines. Fold induction was calculated normalizing data to Actin β and control. Bars represent the average and standard deviation of one representative experiment. ** $p<0.01$ *(C)* Western blot, from PC3.CtBP1, PC3.shCtBP1 and PC3.pGIPZ stable cells using specific CtBP1 and Actin β antibodies. Quantification was obtained by normalization to Actin β as indicated under the bands. *(D)* Clonogenic assay from PC3.CtBP1, PC3.shCtBP1 and PC3.pGIPZ stable transfected cells. A representative photograph for each group, histograms for area distribution and colony number and area analyses are shown (boxes).

Figure 2. HFD consumption for 16 weeks induced metabolic syndrome-like disease in mice. *(A)* Weekly average body weight from nu/nu mice fed with CD or HFD was plotted normalizing data to initial body weight. Box plots for *(B)* cholesterol and *(C)* total testosterone levels for CD or HFD fed mice are shown. Boxes represent the interquartile range, the horizontal line within each box represents the median, and the upper and lower whiskers represent the standard deviation of one independent experiment. **$p<0.01$

Figure 3. CtBP1 depletion decreases tumor growth in HFD fed mice. Tumor growth curves from *(A)* CD or *(B)* HFD male nu/nu mice inoculated with PC3.pGIPZ or PC3.shCtBP1 cells after 12 weeks of diet. Each data point represents tumor volume average and standard deviation from each group of animals. *(C)* Tumors removed were used to determine NAD+/NADH ratio. Plotted boxes represent the interquartile range, the horizontal line within each of the boxes represents the median, and the upper and lower whiskers represent the standard deviation. * $p < 0.05$ *(D)* H&E and CtBP1 IHC staining from tumor

sections generated as xenografts in CD or HFD fed mice (Magnifications 250x). HFD PC3.shCtBP1 tumors showed irregular and hyperchromatic nuclei with several necrobiosis and leukocyte foci.

Figure 4. Microarray analysis and functional genomics of xenografts. *(A)* Functional GO annotations from 823 genes differentially expressed in PC3.pGIPZ and PC3.shCtBP1 HFD xenografts using Panther software after Hugene 1.0 ST array hybridization and normalization. *(B)* GSEA from microarray data from HFD tumor xenografts. Enrichment plot and heatmap from a selected geneset were shown. Only genes that significantly contribute to core enrichment were shown in heatmap. *(C)* CDH1 (e-cadherin) IHC from PC3.pGIPZ and PC3.shCtBP1 xenografts HFD fed mice (Magnifications 400x). *(D)* GSEA from microarray data from HFD tumor xenografts showing enrichment plot and heatmap from steroid hormone biosynthesis. *(E)* RT-qPCR from the HFD fed mice tumors using specific primers for CYP19A1. Fold induction was calculated normalizing data to Actin β and control. Bars represent the average and standard deviation from 3 mice. * $p < 0.05$

Figure 5. Differentially expressed genes identified by whole genome expression array. *(A)* CCND1 RT-qPCR from the HFD fed mice tumors. Fold induction was calculated normalizing data to Actin β and control. Bars represent the average and standard deviation from 3 mice. * $p < 0.05$ *(B)* CCND1 IHC from PC3.pGIPZ and PC3.shCtBP1 xenografts HFD fed mice (Magnifications 400x). *(C)* GSEA from microarray data from HFD tumor xenografts. BRCA1 targets enrichment plot and heatmap geneset were shown. Only genes included in the core enrichment were shown at heatmap. *(D)* BRCA1 IHC staining from tumor sections generated as xenografts in HFD fed mice (Magnifications 400x).

Figure 6. CtBP1 and testosterone regulates BRCA1 transcription. *(A)* BRCA1 RT-qPCR from PC3 stable cell lines. Fold induction was calculated normalizing data to Actin β and control PC3.pGIPZ cells. Bars represent the average and standard deviation of one representative experiment. *(B)* BRCA1 RT-qPCR from PC3 cells transiently transfected with pcDNA3 or AR5 expressing vectors, grew in charcoal FBS-

medium and exposed to testosterone or vehicle for 24h. Fold induction was calculated normalizing data to Actin β and control. Bars represent the average and standard deviation of one representative experiment. *(C)* CtBP1 specific ChIP-scanning from PC3 cells incubated in charcoal FBS medium and exposed to testosterone or vehicle for 24hs. Specific primers scanning BRCA1 promoter at the indicated sites were used. Enrichment was calculated normalizing data to INPUT and non-specific IP antibody. *(D)* BRCA1 RT-qPCR from PC3 cells, grown as indicated above and pretreated with letrozole (1h) and then exposed to testosterone or vehicle (24h). Fold induction was calculated normalizing data to Actin β and control. Data represents the average and standard deviation of one representative experiment. * $p < 0.05$; ** $p < 0.01$

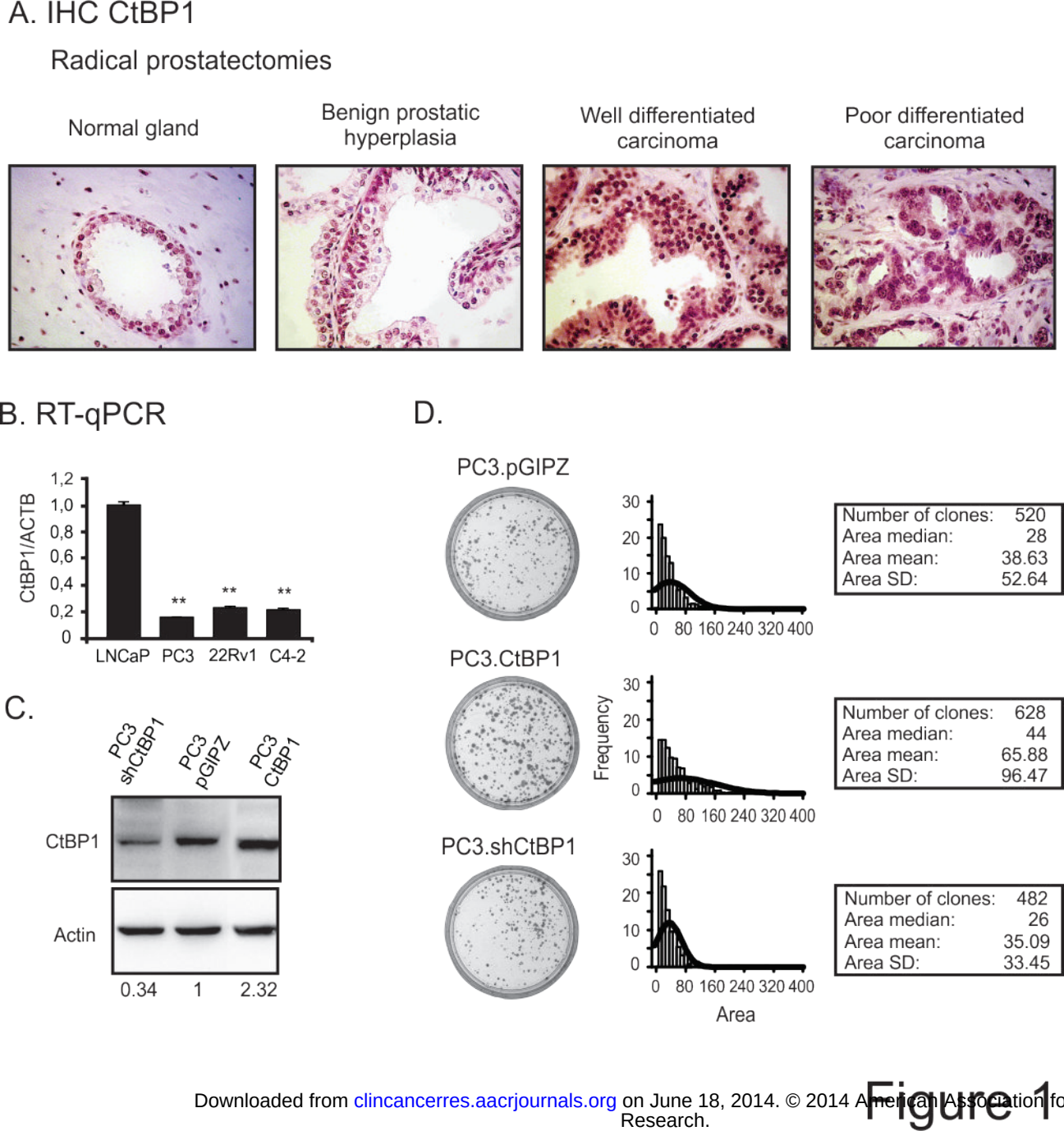


Figure 1

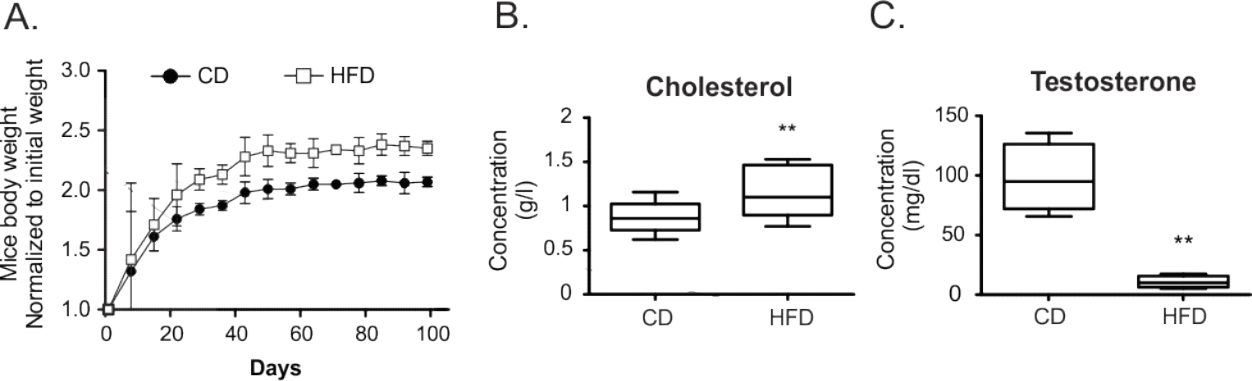


Figure 2

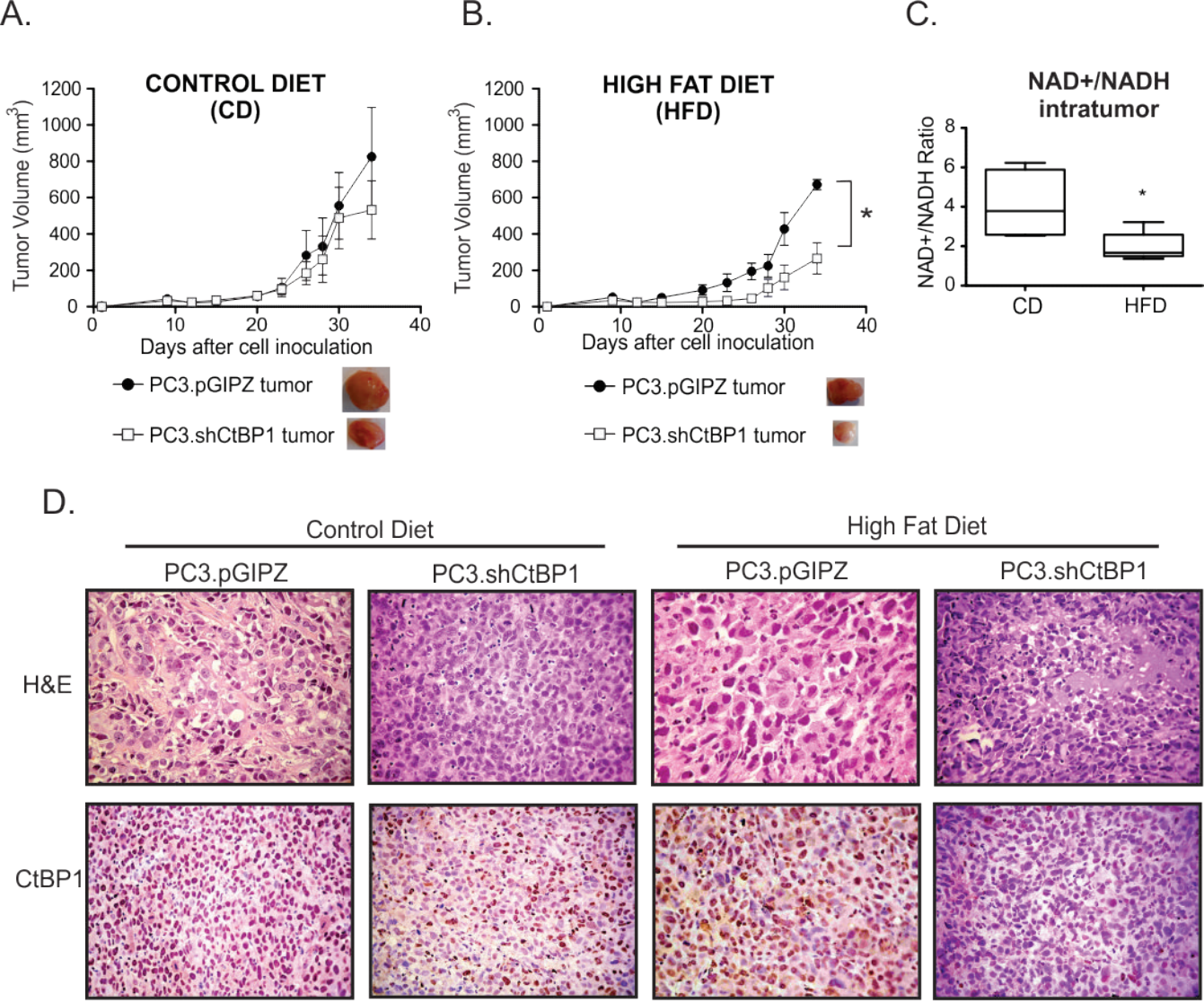
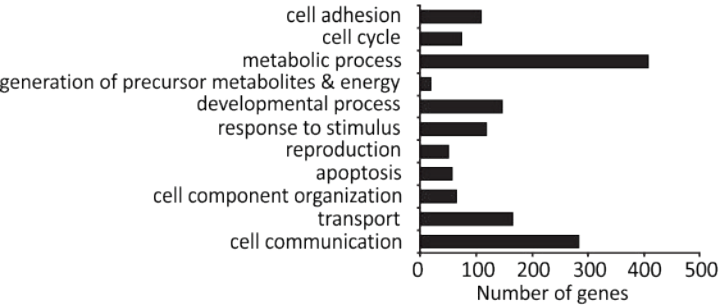


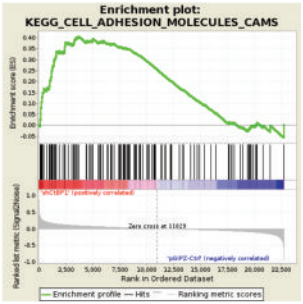
Figure 3

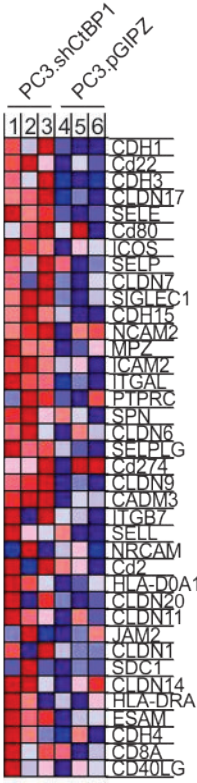
A. Functional GO - Biological Process



B. GSEA

KEGG cell adhesion molecules cams 31





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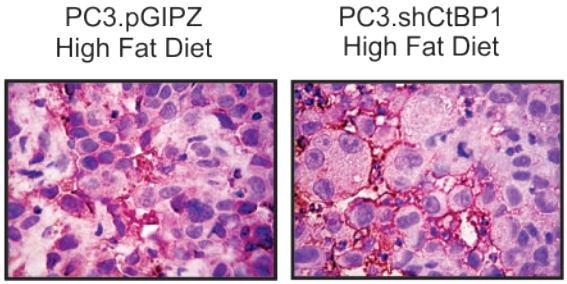
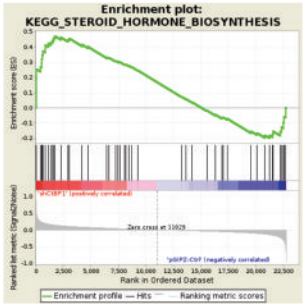
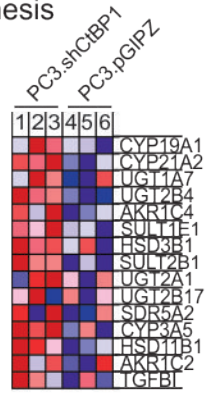


E-Cadherin (CDH1)

D. GSEA

Kegg steroid hormone biosynthesis





E. RT-qPCR

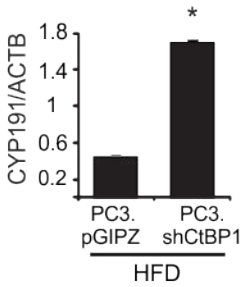
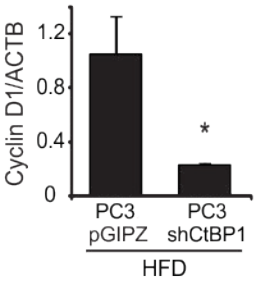
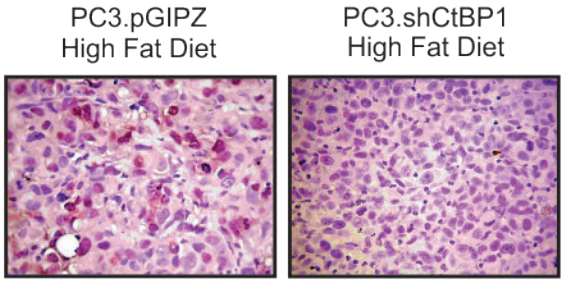


Figure 4

A. RT-qPCR

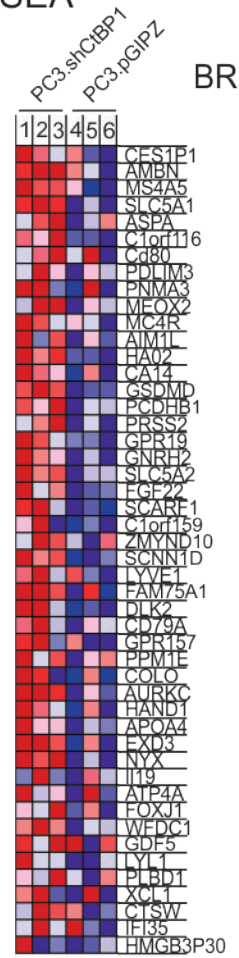


B. IHC

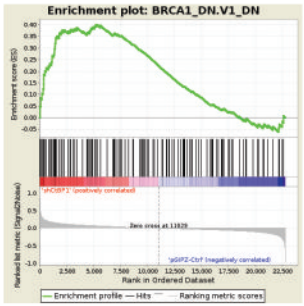


Cyclin D1 (CCND1)

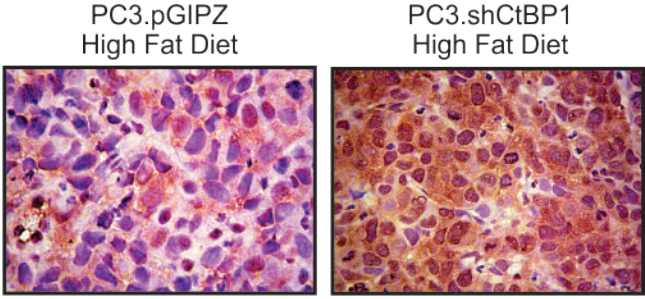
C. GSEA



BRCA1 DN V1 DN



D. IHC



BRCA1

Figure 5

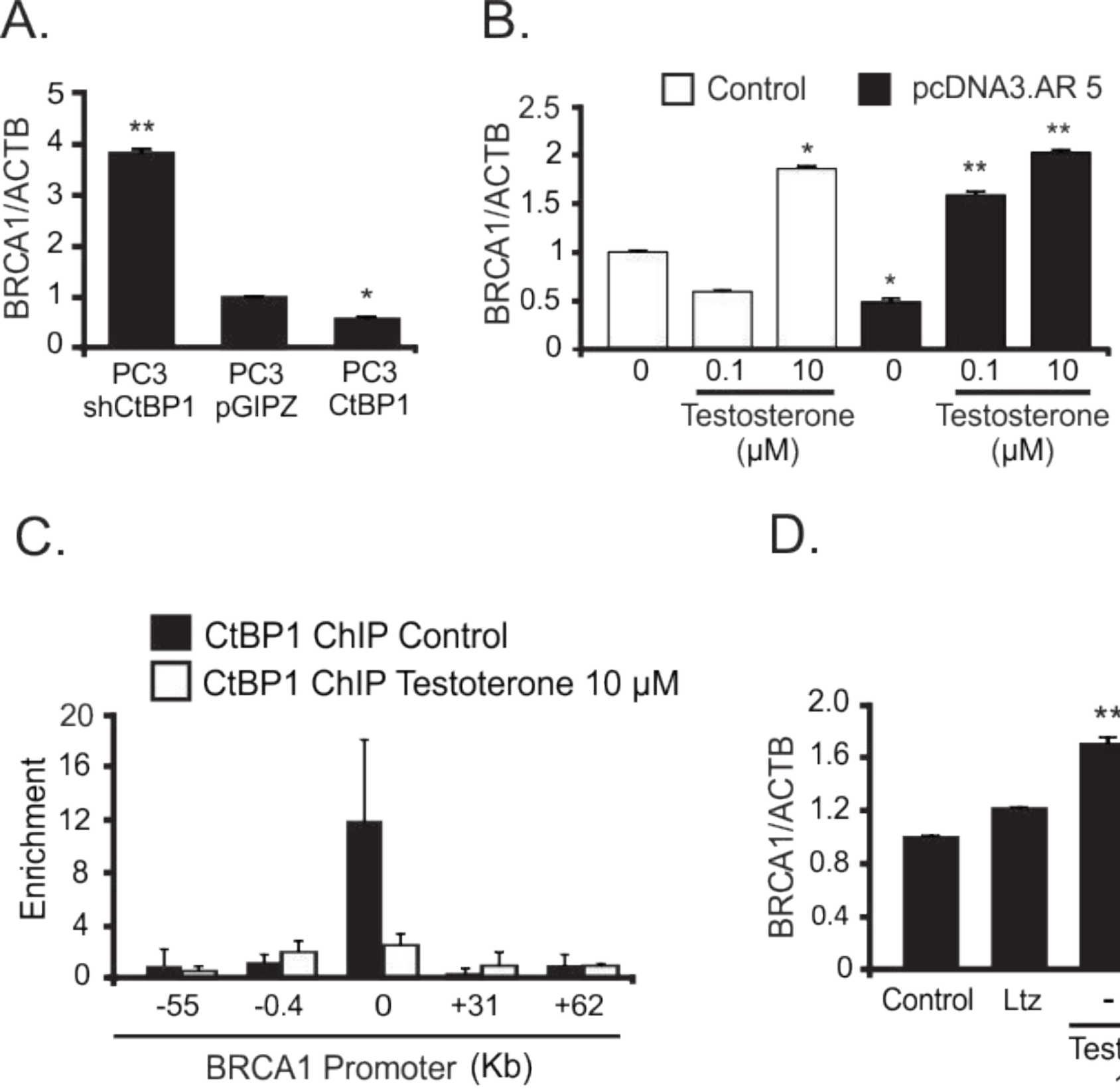


Figure 6