Transcriptional Regulation of Type 11 17β-Hydroxysteroid Dehydrogenase Expression in Prostate Cancer Cells

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Abstract

Type 11 Hydroxysteroid (17-beta) dehydrogenase (HSD17B11) catalyzes the conversion of 5α-androstan-3α,17β-diol into androsterone suggesting that it may play an important role in androgen metabolism. We previously described that overexpression of C/EBPα or C/EBPβ induced HSD17B11 expression in HepG2 cells but this process was not mediated by the CCAAT boxes located within its proximal promoter region. Here, we study HSD17B11 transcriptional regulation in prostate cancer (PC) cells. Transfection experiments showed that the region −107/+18 is sufficient for promoter activity in PC cells. Mutagenesis analysis indicated that Sp1 and C/EBP binding sites found in this region are essential for promoter activity. Additional experiments demonstrated that ectopic expression of Sp1 and C/EBPα upregulated HSD17B11 expression only in PC cell lines. Through DAPA and ChIP assays, specific recruitment of Sp1 and C/EBPα to the HSD17B11 promoter was detected. These results show that HSD17B11 transcription in PC cells is regulated by Sp1 and C/EBPα.

Keywords

1. Introduction

Androgens are essential for the development and regulation of male sexual characteristics (Hayward and Cunha 2000). The biological action of androgenic male sex steroid hormones in prostate tissue is mediated by the androgen receptor (AR). Androgens-activated AR is translocated to the nucleus where it binds to androgen response elements located within promoter regions of androgens target genes (Carson-Jurica et al. 1990; McKenna and O'Malley 2002; Chmelar et al. 2007). This process is well regulated at all stages including at pre-receptor levels which implies the regulation of enzymes that participate in the formation

and degradation of 5α-Dihydrotestosterone (DHT) (Penning et al. 2008). DHT is the most potent androgen and is responsible for the growth, development and maintenance of the normal secretory function of the prostate (Andersson et al. 1991). In adult males testosterone from the Leydig cells of the testis is converted in the prostate into DHT. On the other hand, it has been described that 5α-androstan-3α,17β-diol (3α-Diol) can be converted into DHT with growth consequences for the prostate (Horst et al. 1975). Deregulation of the process of synthesis and degradation of DHT usually results in benign prostatic hyperplasia and/or prostate cancer (PC). PC is the second leading cause of cancer-related in men (Jemal et al. 2009) and approximately 20% of men with this disease develop metastatic cancer requiring systemic therapy that target androgens production or action.

17β-Hydroxysteroid dehydrogenases (HSD17Bs) are the enzymes responsible for reduction or oxidation of sex hormones, fatty acids and bile acids *in vivo* (Moeller and Adamski 2009). All require NAD(P(H)) for their activity. Fourteen HSD17Bs have been identified to date and, with the exception of HSD17B5, an aldo-keto reductase (AKR), all of them are short-chain dehydrogenases/reductases (SDRs) (Day et al. 2008). Type 11 17β-Hydroxysteroid dehydrogenase (HSD17B11) has been shown to have dehydrogenase activity (Li et al. 1998). It converts 3α-Diol into androsterone, which suggests that this enzyme has a role in androgen metabolism (Brereton et al. 2001). HSD17B11, also known as short-chain dehydrogenase/reductase (SDR family) member 8 (DHRS8) and Pan1b, is nearly ubiquitously expressed being highly detected in lung, eyes, liver, pancreas, intestine, kidney, adrenal gland, heart, testis, ovary, placenta and sebaceous gland (Brereton et al. 2001; Chai et al. 2003). Importantly, a recent study demonstrates that HSD17B11 is abundantly expressed in human prostate cancer tissue but not in the normal prostate, suggesting that its expression could be connected with advanced prostate cancer (Nakamura et al. 2009).

HSD17B11 expression has been detected in human steroidogenic cells, including both Leydig and granulosa cells. Importantly, the *HSD17B11* 5'-flanking region contains several steroidogenic factor-1 binding sites, but their functionality is still to be demonstrated (Chai et al. 2003). It has also been shown that peroxisome proliferator-activated receptor α (PPARα) regulates HSD17B11 expression in mouse intestine and liver (Motojima 2004; Yokoi et al. 2007). Although this suggests that HSD17B11 expression is directly regulated by PPARα and its ligand in mouse, promoter sequence up to −1800 bp did not respond to a PPARα ligand in reporter gene assays. Finally, we have described that HSD17B11 is upregulated by C/EBPα and C/EBPβ in the hepatocarcinoma cell line HepG2 but this process was not mediated by the CCAAT boxes located within HSD17B11 proximal promoter (Rotinen et al. 2010).

In order to gain a better understanding of the mechanisms governing the transcriptional regulation of the *HSD17B11* gene and its regulation in prostate cancer, we have cloned and analyzed the 5'-flanking region of the human *HSD17B11* gene between -2016 and +18. Serial deletion of the 5'-flanking region of the *HSD17B11* gene identified the region −107/+18 as the minimal promoter. This region included consensus binding sites for C/EBP, Sp1, GATA and NF-κB transcription factors. Mutagenesis analysis showed that the C/EBP and Sp1 sites were essential for promoter activity. Additional experiments indicated that C/EBPα and Sp1 upregulate, bind and are recruited to the promoter of *HSD17B11* gene. Taken together these results indicate that C/EBPα and Sp1 are involved in the transcriptional regulation of the human *HSD17B11* gene in cultured prostate cancer cells.

2. Materials and methods

2.1. Nucleotide sequence analysis of the *HSD17B11* 5'-flanking region

Putative transcription factor binding sites on the 5'-flanking region of the human *HSD17B11* were identified using the MatInspector program found in the Genomatix Software package (Genomatix, Munich, Germany). *Macaca mulatta*, *Pan troglodytes*, *Mus musculus*, *Rattus norvegicus*, *Equus caballus*, *Canis lupus familiars* and *Bos taurus HSD17B11* orthologs were searched for with the Gene2Promoter (Genomatix Software) program.

2.2. Generation of human *HSD17B11* 5′-flanking region/luciferase reporter constructs

The 5'-flanking region of the human *HSD17B11* gene was isolated by PCR using the oligonucleotides 5'-AAGGTGGGTGGAACAGGAGATCG-3' (-2016/-1994) and 5'-AAAGAGTAGGGGCGAGAGCAAGG -3' (+18/−5). PCR products were cloned into a pGEM-T Easy plasmid from Promega (Madison, WI). The −2016/+18 bp insert obtained digesting this construct with *Eco*ICRI and *Nco*I was subcloned into the *Sma*I and *Nco*I sites of the pGL3-Basic vector (Promega) to generate the construct pB11-2016. Construct pB11-344 was obtained from pB11-2016 via PCR with the primers 5'-GTGATAAGCAACCTTCAACTATGA-3' and 5'-CTTTATGTTTTTGGCGTCTTCCA-3' and cloning of the PCR product after digestion with *Nco*I into the *Sma*I and *Nco*I sites of the pGL3-Basic vector. Construct pB11-107 was generated by digestion of pB11-2016 with *Nhe*I and circularization of the largest fragment. Constructs pB11-1603 and pB11-1242 were generated from the −2016/+18 pGEM-T Easy, digesting with *Nco*I and either with *Dra*I or *Hinc*II, respectively, and subcloning the resulting fragments into the *Nco*I and *Sma*I sites of the pGL3-Basic vector. The same procedure was used to generate pB11-696, pB11-537 and pB11-150, digesting instead with *Nco*I and either *Bam*HI, *Xho*I or *Hind*III and subcloning the resulting fragments into the *Nco*I and *Bgl*II/*Xho*I/*Hind*III sites of pGL3-Basic vector. For restriction and ligation Promega and Takara Bio Inc. (Otsu, Shiga, Japan) enzymes were used according to manufacturer's instructions. All constructs were confirmed by sequencing. The C/EBPα and C/EBPβ expression vectors were a gift from Dr. Steven L. McKnight (UT Southwestern Medical Center, Dallas, TX). To generate the Sp1 expression vector, Sp1 mRNA was amplified by RT-PCR and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA).

2.3. Site-directed mutagenesis

To generate plasmids bearing mutated consensus-binding sequences for transcription factors, mutagenesis experiments were performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The chimeric luciferase construct containing the −344/+18 region of the *HSD17B11* promoter was used as a template. Oligonucleotides used were designed with the web based Quickchange Primer Design Program (http://www.stratagene.com/qcprimerdesign) and are listed in Table 1A. The mutated luciferase constructs containing the −107/+18 region were obtained by digestion of the pB11-344 mutated constructions with *Nhe*I and circularization of the largest fragments. All mutations were confirmed by sequencing.

2.4. Cell culture, transient transfection and luciferase assay

LNCaP, PC-3, C4-2, HT-29 and HTB-54 cell lines were obtained from American Type Culture Collection (Manassas, VA). EJ138 cell line was obtained from European Collection of Cell Cultures (Porton Down, UK). LNCaP, C4-2, HTB-54 cells were grown in RPMI 1640 (Invitrogen) medium supplemented with 10% FBS and 100 units/mL of penicillin and 100 μg/mL of streptomycin. PC-3 cells were grown in DMEM-F12 (Invitrogen) supplemented with 10% FBS and 100 units/mL of penicillin and 100 μg/mL of streptomycin. HT-29 and EJ138 cells were grown in DMEM-GlutaMAX (Invitrogen)

supplemented with 10% FBS and 100 units/mL of penicillin and 100 μg/mL of streptomycin. Transfections were performed as previously described (Villar et al. 2007). Briefly, cells were seeded in 24-well plates in antibiotic-free medium. The day after, cells were transfected with Lipofectamine LTX Reagent and PLUS Reagent following the Cell-type Specific Transfection Protocols supplied by Invitrogen. After 24 h, cells were harvested and luciferase activities were measured as previously described (Rotinen et al. 2010). Luminescence was measured mixing 20 μL of cell lysate with the Dual-Luciferase Reporter Assay System (Promega) in a Berthold Lumat LB 9501 luminometer (Berthold Technologies, Oak Ridge, TN). Renilla luciferase activity was used to correct the transfection efficiency. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as a standard.

2.5. RT and real time quantitative PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed using AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene) according to manufacturer's protocol. Real-time quantitative PCR was performed using a Chromo-4 Real-Time PCR Detection System (MJ Research, Bio-Rad Laboratories) and Brilliant SYBR green Master Mix (Stratagene). Values were normalized to the expression levels of *GAPDH* and *ACTB* housekeeping genes. Primers used for detecting HSD17B11 mRNA were: sense strand 5'-TGCAATGACGAAGAATAACC-3' and antisense strand 5'-TTGTAAGGCAGCCAGTTC-3'. Primers used for detecting GAPDH were: sense strand 5'-GGAGTCCACTGGCGTCTTC -3' and antisense strand 5'-ATCTTGAGGCTGTTGTCATACTTC -3'. The primer set for human ACTB was purchased from SABiosciences, Qiagen (ACTB PPH00073E-200). Results from four independent experiments each performed in duplicate are expressed as fold change over controls. Statistical analyses were performed using Student's t-test and the differences were considered significant when $p \leq 0.05$.

2.6. Nuclear extraction, DNA affinity precipitation assays (DAPA) and western blot

Nuclear extracts from LNCaP and PC-3 cells were obtained using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL, USA). 5'-biotin end-labeled sense and antisense oligonucleotides corresponding to the normal Sp1, C/EBP and their mutants (Table 1B) of the *HSD17B11* 5'-flanking region were annealed and purified by DNA extraction. Nuclear extracts (500 μg) from LNCaP or PC-3 cells were pre-incubated with 20 μL of streptavidin-agarose for 1h at 4 °C with rotation. The supernatant collected by centrifugation was incubated with 0.5 μg of normal or mutant biotin-labeled probe in binding buffer (20 mM HEPES pH 7.9, 50 mM NaCl, 1 mM DTT, 0.01% NP-40 and 5% Glycerol) overnight at 4 °C with gentle rotation. DNA-protein complexes were washed five times with binding buffer. Pellet was resuspended in 40 μL of 2X protein sample buffer (Bio-Rad Laboratories) and then boiled for 10 min to dissociate complexes. Proteins were resolved by polyacrylamide gel electrophoresis, followed by western blot detection with C/EBPα (N-19) and Sp1 (PEP-2) antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Methods for SDS-PAGE electrophoresis of cellular proteins, and transfer onto nitrocellulose membranes were done as previously described (Alonso et al. 2003).

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIPs were performed using the ChIP-IT Chromatin Immunoprecipitation & Shearing Kit from Active Motif (Rixensart, Belgium) as previously described (Rotinen et al. 2009). Primers used for PCR are listed in Table 1C.

2.8. Statistics

Statistical differences were examined using the SPSS 16.0 for Windows (Chicago, IL, USA). Results are expressed as mean ± SEM for four independent experiments and analyzed by Student's t-test.

3. Results

3.1 Predictive nucleotide sequence analysis of the 5'-flanking region of *HSD17B11*

HSD17B11 expression has been shown to be higher in PC relative to normal prostate cells (Nakamura et al. 2009). To better understand the mechanisms that regulate *HSD17B11* gene in PC, up to 2 Kb of the 5'-flanking region of the gene were cloned into a pGL3-Basic vector to form pB11-2016. Sequence analysis showed that it contains several binding sites for transcription factors (Fig. 1A). Transfection of constructs containing serial deletions of the 5'-flanking region showed that this region has more activity in LNCaP than in C4-2 and PC-3 cells (Fig. 1B). Interestingly, construct containing the region −344 to +18 was the most active in all three cell lines. This sequence contains two putative C/EBP binding sites. It also contains putative transcription factor binding motifs for Sp1, KLFs and retinoic acid receptor (RXR) among others. Further deletion of this region showed no significant differences between -344 to -107, which indicates that the region from -107 to +18 is sufficient to maintain promoter activity of *HSD17B11*. This region contains putative binding sites for C/EBP, Sp1, NF-κB and GATA. Data also show that the promoter activity driven by the promoter region -344 to +18 and −107 to +18 are much more active in LNCaP than in C4-2 and PC-3 cells thus suggesting that there is a down regulating site in the region −2016 to −344 in LNCaP.

To explore if these motifs were important for *HSD17B11* promoter activity, we performed site-directed mutagenesis in the pB11-344 and pB11-107 constructs. As shown in Figure 2, mutation of the NF-κB motif reduced significantly the promoter activity of the constructs both in LNCaP and C4-2 cells, but not in PC-3 cells. Conversely, mutation of the GATA motif increased significantly the promoter activity of the constructs only in PC-3 cells. Mutation of the CCAAT box located at -267 had no effect on pB11-344. However mutation of CCAAT box located at -71 reduced the activity of pB11-344 and pB11-107 to 60 and 40% respectively in LNCaP and PC-3 cells, and to less than 20% in C4-2 cells. Likewise, mutation of the Sp1 located at -51 reduced to 40% the activity of pB11-344 and pB11-107 in LNCaP and PC-3 cells, and to 20% in C4-2 cells. Moreover, simultaneous mutation of the Sp1 binding site and the CCAAT box at −71 reduced the promoter activity of the pB11-107mSp1/mCCAAT1 construct to less than 20%. These results clearly show the importance of these motifs for *HSD17B11* promoter activity.

3.2. C/EBPα overexpression stimulates HSD17B11 expression in LNCaP and PC-3 cells

Previously, we described that C/EBPα or C/EBPβ expression induces HSD17B11 expression in the hepatocarcinoma cell line HepG2 (Rotinen et al. 2010). To study if this is a general mechanism that occurs in all tissues, RT-qPCR was performed after C/EBPα or C/EBPβ transfection in PC cell lines (PC-3, LNCaP and C4-2) but also in colon carcinoma HT-29, lung carcinoma HTB-54 or bladder carcinoma EJ138 cell lines. Results showed that C/EBPα increased HSD17B11 expression in each of the PC cell lines tested, while C/EBPβ only did it in PC-3 cells (Fig. 3A). However neither C/EBPα nor C/EBPβ significantly modified HSD17B11 expression in HT-29, HTB-54 or EJ138 cell lines (Fig. 3B) thus confirming that *HSD17B11* regulation is cell type specific.

C/EBPα or C/EBPβ induced HSD17B11 expression in HepG2 cells but this process was not mediated by the CCAAT boxes located within its proximal promoter region (Rotinen et al.

2010). To determine if the HSD17B11 proximal promoter is mediating C/EBPα and/or C/EBPβ-induced HSD17B11 expression in C4-2, LNCaP and PC-3 PC cells, next we performed cotransfection experiments with pB11-107 and C/EBPα or C/EBPβ expression vectors. As shown in Figure 3C, overexpression of C/EBPα stimulated the luciferase activity of normal pB11-107 by 2.65-, 1.7- and 1.5-fold in C4-2, LNCaP and PC-3 cells respectively, but failed to induce the luciferase activity of the mutated construct pB11-107mCCAAT1. However, overexpression of C/EBPβ did not induce significantly *HSD17B11* promoter activity in these cell lines. Taken together, these results indicate that C/EBPα induces *HSD17B11* transcription through its proximal promoter in PC cells.

3.3. C/EBPα interacts with *HSD17B11* promoter

To further explore if the CCAAT box located at -71 is involved in the C/EBPα induced HSD17B11 upregulation, we performed DAPAs in which a biotynilated DNA probe containing *HSD17B11* sequences from −91 to -67 (Table 1B) was incubated with nuclear extracts from LNCaP and PC-3 cells, followed by precipitation with a resin containing streptavidin. Then, DNA/protein complexes were analyzed by western blot (Fig. 3D). Results showed that C/EBPα binds to *HSD17B11* promoter. Importantly, this binding was prevented by a triple mutation within the sequence CCAAT demonstrating that this binding is specific. Finally, we studied if C/EBPα is recruited to the *HSD17B11* promoter in LNCaP and PC-3 cells by ChIP assay. As shown in Figure 3E, C/EBPα was recruited to *HSD17B11* promoter in both cell lines. These results confirm that C/EBPα regulates *HSD17B11* transcription in PC cells.

3.4. Sp1 regulates HSD17B11 expression

As mentioned before, point mutation of the Sp1 site located at -51 reduced promoter activity to 40% in LNCaP and PC-3 cells, and to 20% in C4-2 cells (Fig. 2) thus suggesting that Sp1 could be involved in the transcriptional regulation of the *HSD17B11* gene at basal levels. To confirm this and to determine is Sp1 is involved in the transcriptional regulation of this gene in other tissues, *HSD17B11* mRNA levels were determined after transfection with a Sp1 expression vector in PC cells (LNCaP, PC-3 and C4-2) and also in HT-29, HTB-54 and EJ138 cells. As shown in Figure 4A, Sp1 overexpression significantly induced *HSD17B11* expression in PC cells increasing its levels by more than 50% in all three PC cells. However, Sp1 did not modified HSD17B11 expression in HT-29, HTB-54 or EJ138 cell lines (Fig. 4B) indicating that Sp1-induced HSD17B11 expression is cell type specific. No increase was observed in *ACTB* control.

To confirm if the Sp1 binding site located within the promoter is involved in the Sp1-induced *HSD17B11* expression we cotransfected PC cells with pB11-107 or pB11-107mSp1 and a Sp1 expression vector. Sp1-induced HSD17B11 luciferase activity was observed in all three PC cell lines. Interestingly, Sp1 induced promoter activity only in the wild type construct pB11-107 but failed to do it in the mutated construct pB11-107mSp1 (Fig. 4C) showing that the Sp1 binding site is required for this induction.

3.5. Sp1 interacts with *HSD17B11* promoter

To further investigate if the Sp1 binding site is involved in *HSD17B11* transcriptional regulation we performed DAPA experiments. With this purpose, a DNA probe containing either the *HSD17B11* Sp1 binding site or its mutated form (Table 1B) was incubated with nuclear extracts of PC-3 and LNCaP cells. Figure 4D shows that Sp1 binds to *HSD17B11* normal promoter sequence *in vitro* and, more importantly, that mutation of this GC box prevented Sp1 binding. Finally, Sp1 binding to *HSD17B11* promoter *in vivo* was confirmed in a ChIP assay performed with an antibody against Sp1 (Fig. 4E). All together these results

clearly demonstrate that Sp1 is involved in the transcriptional regulation of HSD17B11 in PC cells.

4. Discussion

Here, we describe that transcription of the human *HSD17B11* gene is regulated by Sp1 and C/EBPα in PC cells. Human *HSD17B11* gene was cloned in an attempt to expand the 11β-hydroxysteroid dehydrogenase (HSD11B) family isolating *HSD11B1* homologous cDNAs (Li et al. 1998). These enzymes play pivotal roles in modulating tissue levels of glucocorticoids and are thus excellent mediators of paracrine and autocrine actions (Chapman et al. 2009). Early experiments testing HSD17B11 enzymatic activity showed that this protein, which is 22.9% identical to HSD11B1 (Brereton et al. 2001), has 17β-hydroxysteroid dehydrogenase activity. The highest amount of activity was seen with 3α-Diol where ~ 30% of the substrate was converted to androsterone (Li et al. 1998; Brereton et al. 2001).

Androgen metabolism is well regulated in prostate. Regulation of ligand access to the AR is one of the most important aspects of this process (Penning 2010). In normal circumstances, prostate cells capture circulating testosterone produced in the Leydig cells and reduce it to DHT by 5α-reductase (Russell and Wilson 1994). DHT is responsible for the growth, development, and maintenance of the normal secretory function of the prostate. Upon DHT binding, the activated AR dissociates from its cytoplasmic chaperone complex and undergoes a conformational change inducing nuclear translocation (Marcelli et al. 2006), dimerization (Centenera et al. 2008) and binding to specific DNA sequences known as androgen response elements usually located upstream of the target gene sequence (Claessens et al. 2001). AR will then recruit different transcription factors including both, general transcription factors and coregulatory proteins (Chmelar et al. 2007) which lead to the transcriptional regulation of AR-target genes. Although it is known that androgen metabolism is altered in PC the mechanisms remain unclear (Penning 2010).

3α-Diol is considered a weak androgen with no ability to transactivate AR due to its low affinity for the receptor. However, studies on rats, dogs, marsupials and humans have demonstrated that 3α-Diol can be converted back into DHT to stimulate growth of prostate (Horst et al. 1975; Jacobi et al. 1978; Shaw et al. 2000; Wilson et al. 2003). AKR1C2, an enzyme that converts DHT in 3α-Diol in human prostate, is overexpressed in epithelial cells from PC. Since AKR1C2 activity deprives AR from its ligand, it has been suggested that this enzyme might contribute to progression towards androgen independence in PC (Rizner et al. 2003). Since HSD17B11 converts 3α-Diol into androsterone thus reducing its intracellular levels, it is possible that this enzyme also contributes to progression to androgen independence in PC. Interestingly, HSD17B11 expression has been shown to be upregulated in human PC cells (Nakamura et al. 2009). Here we show that mutation of the binding sites for C/EBPα and Sp1 decreases HSD17B11 promoter activity to less than 20% in PC cells (Fig. 2). Since C/EBPα and Sp1 overexpression induced HSD17B11 expression in the all three PC cell lines tested (PC-3, LNCaP and C4-2 cells) but not in the colon carcinoma HT-29, lung carcinoma HTB-54 or bladder carcinoma EJ138 cell lines (Fig. 3A, 3B, 4A and 4B), our results clearly indicate that this regulation is cell type specific. We also show that mutation of the overlapping GATA and NF-κB binding sites affects HSD17B11 promoter activity in a cell type specific manner (Fig. 2). Additional work is required to unravel the role of these motifs in HSD17B11 transcription. Interestingly, sequence alignment of the 5'-flanking region of the *HSD17B11* of human (hsa), Macaca mulatta (mcc), Pan troglodytes (ptr), Mus musculus (mmu), Rattus norvegicus (rno), Equus caballus (ecb), Canis lupus familiares (cfa) and Bos taurus (bta) showed that all these elements are almost identical among these species (Fig. 5). Moreover, the sequence from -145 to +18 of

the human *HSD17B11* gene seems to be almost identical to the compared orthologs, which indicates that HSD17B11 could be regulated in the same way in all species.

In conclusion, here we show that the basal level of expression of the human *HSD17B11* gene is regulated by Sp1 and C/EBPα in cultured PC cells. This regulation requires binding and recruitment of these transcription factors to *HSD17B11* 5'-flanking sequence. Since HSD17B11 is overexpressed in PC these results provide a new insight that will help to understand the mechanisms that deregulate the expression of this gene in PC.

Acknowledgments

Abbreviations

HSD	hydroxysteroid dehydrogenase
C/EBP	CCAAT enhancer binding protein

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A

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-2016  AAGGTGGGTGGAACAGGAGATCGAGATTGAGACCATCCTAGCCAACATGGGGAAACCCTGTCTACTAAAA
-1946  ATACAAAAATTAGCTGGGTGTGGTGGTGCGTGCCTGTAGTCCCAGCTACTTGGGAGGCTGAGGCAGGAGA
-1876  ATCGCTTGAACCTGGGAGGTGGAGGTTGAGGTTGCTGTGAGCCAAGATTTCGCCACTGCACTCCAGCCTG
                             RXR/NF-1
-1806  GAGACAGAGGGAGACTCCTTTAAAAAAAAAAAAAAATAGCATAGCTAGAGCCTCAATATATGAAACTCAAC
-1736  ATAGTTGAAACACTTCTTATAGTAGATCACCATGTCAGATGTAATTTATTCCTGTAGGTGTTGGACCAAA
                             YY-1
-1666  AAATTGACAAACACTGAACATATGTGTAGCCCCAGTTACACTTTTACTCACTGTGTAGATTTTAAAGTTG
              SRF                        AP-1
-1596  ATCTAACAGATTTATTCATTTACAGTATAGGTATAAATATATCCTGACTCTACTTTTCTCTATTTCCTAG
                                          AP-1
-1526  GAGGCTTTTGAAAATCAAATGAAATAATATATGTGGAAGAACACTTTGTAAACTTTCTATACAAGTGTTT
                                          PRE
-1456  TGTAAATTTGGATTTGGAGGCTATCCAAAAAAAGTTGTAGATATTCTGAATTCAAAGAATTCTATAATG
       C/EBP        NF-1
-1386  GGGTTCACTTTTGTGAGGCTGCCACTATCCCTTCATCCCCTATAACAACGTATGGCTGCTTAAGTAAGAG
-1316  GGACAATCGAGTTTGAACTATTTGTGAACTTTGATTTTTTTGTGTGATCTTTCTGGCTCTTAAATATGAA
                  RXR
-1246  AGTTGACTATTGTCACACAACAAAGTCCCTTCCCAAGCACTGGAGTGTCTTGCGCAAAACCCAAATCCAA
                                              C/EBP
-1176  TGTCTGTCAAAAATGAGTTGGAAAAAAATGCATGCAATACCATTATTAAGCTCTTACTGGACACAAGCCC
-1106  TAGTTAGGTCTTTTCTTCCAGTAACCAATCTCTAGAATTTTTTCAAGTCACTAGTTAGCATATACTGAAT
                           CCAATbox
-1036  ACCCATTTATATAGGCTACAAAACCAAATGTTACATAGGTAATCTTTAGGCTAAATGATTTGCTATTTAT
                                                   CCAATbox
 -966  TCACTTGAATAAAGATAAAATTCCTAAAGTACAACATATATTTTTTATTTTCAAACTCTAATAGGTCAAG
 -896  TGAAAAATGATTATTCAAATATACTCTTCTTTCTTGTGGAAACAATCCTTTAATACGATAACAATTTTTT
                C/EBP
 -826  CTGGCGCTTGATTGAAGCTGTCCTTGAACAGACAAAATTTCCCCTGTCTTCCAGAAATCGTGCTTAGTTC
                     SF-1
 -756  AAACTAAAATGTGATGTAGCGCAATTTCTCAGCTGGAAGCAGAACGATTGGTCTTTTCAGGATCCTTTAA
                               CCAATbox
 -686  GATAGGAATCTACATTTGCATCCTCTATCTAGTTTTTGTTTTTTAGGACAAAGTCTGGCCTTTTCGTCCA
 -616  GGCTGGAGTGCAGTGGCGCGATCTTCACTCACTGTAGCCTCCACCTCCTGGACTCAAGCAATTCCCGTGC
                            AP-1
 -546  CTCAGTCTCTCGAGTAGCTGGAACTACAGGCACACGCCACTACGCCTGGCTAATTAAAAAAAAAAAAAAA
 -476  GAGACCGGGGGTCTCCCTATGTTGTTCAGGCTGATCTCGAACTCCTGGGGTCAAGCGATCCTCCCGCCAG
              NF-KB               GATA-1/RXR               GATA-2
 -406  GGCTTCTCAAAGTGCCAGGATCACAGGCGAGAGCCGCCGCGCCCAGCGGCACCCCCTATCTAGTGATAAG
               RXR
 -336  CAACCTTCAACTATGAAAGAAGCATCTGGCCTGTGGACACTGTCGGTTATCCTTGGAACTTATCCAATTT
                                 AP-2            RXR         C/EBP
 -266  AGGAAAACAAGTGAGCAACTAACCCTCCAAGGCCGGCTCACGACACCGGGAGAAAGTGGTGTAAAAGACA
                              SF-1
 -196  ATCGCGGCCACACACCGCGTGGGGGCTGCGCAAAGCAGAACTAGGAAGCTTTGCTCCGGTCTGGACTTCG
 -126  TCCCTCCCAGATCTCTGAGCTAGCAAGCACTGCAGCCCGAGCCAATTGCGAAAGACCAACAAAGCCCAGC
       Sp1/RXR                                     C/EBP              Sp1
  -56  CCAGCGGAAGGAACGGTTTCGGAGTTGTTTTTCTTTGATACGGGAGTTCCTCCTTGCTCTCGCCCCTACT
                                      GATA-1/NF-KB                  KLFS
  +18  CTTT
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B

Figure 1. Transcriptional Activity of the *HSD17B11* 5'-flanking region in PC cell lines
A) Flanking sequence of the human *HSD17B11* gene indicating putative binding sites for transcription factors (underlined). The transcriptional start site is indicated with bold faced

letters. **B)** Luciferase reporter constructs carrying serial deletions of the *HSD17B11* 5'-flanking region (left panel) were transiently transfected into LNCaP (white bars), PC-3 (black bars) or C4-2 (stripped bars) cells. pGL3-Basic and pGL3-Promoter vectors were used as transfection controls. Luciferase activity was normalized to the internal transfection efficiency control (pRL-SV40) and is expressed relative to the activity of the pGL3-Promoter vector. Each value represents the mean ± SEM of four independent experiments, each performed in triplicate. Significant differences to the largest construct (pB11-2016) are denoted by asterisks (*p≤0.05, **p≤0.01).
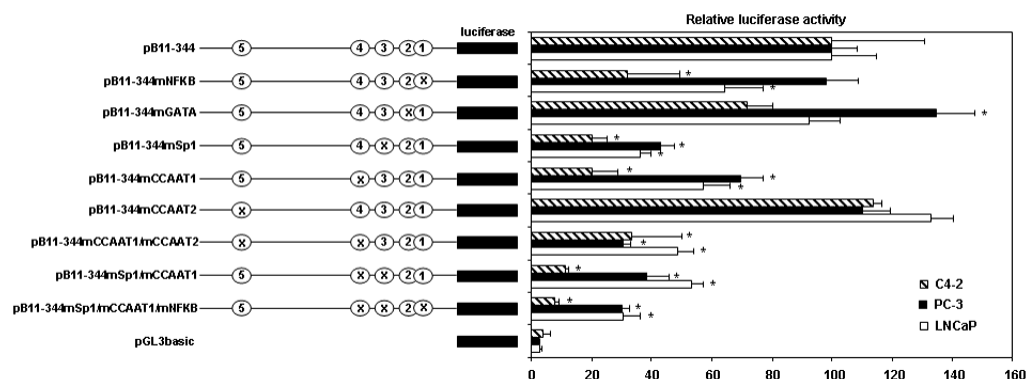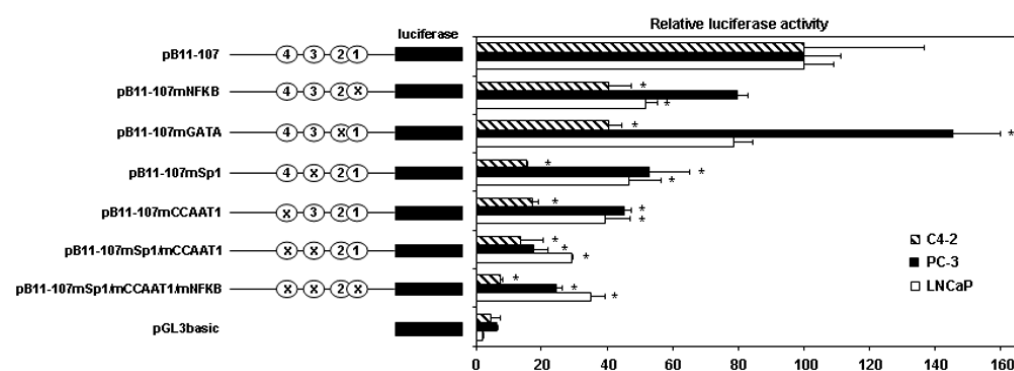
Figure 2. The CCAAT box located at -71 and the Sp1 binding motif located at -51 are important for *HSD17B11* promoter activity
Luciferase activity of normal and mutated pB11-344 (**A**) and pB11-107 (**B**) was determined in LNCaP, PC-3 and C4-2 cells. Transcription factor binding sites are shown as ovals and referred as 1 (NF-κB located at -18/-6), 2 (GATA located at -24/-12), 3 (Sp1 located at -51/-65), 4 (CCAAT box located at -71/-84) and 5 (CCAAT box located at -267/280); mutations are indicated with a cross. Luciferase activity is referred to the normal pB11-344 (A) or pB11-107 (**B**). Each value represents the mean ± SEM of four separate experiments, each performed in triplicate. Significant differences (p≤0.05) to the reference constructs are denoted by asterisks (*).
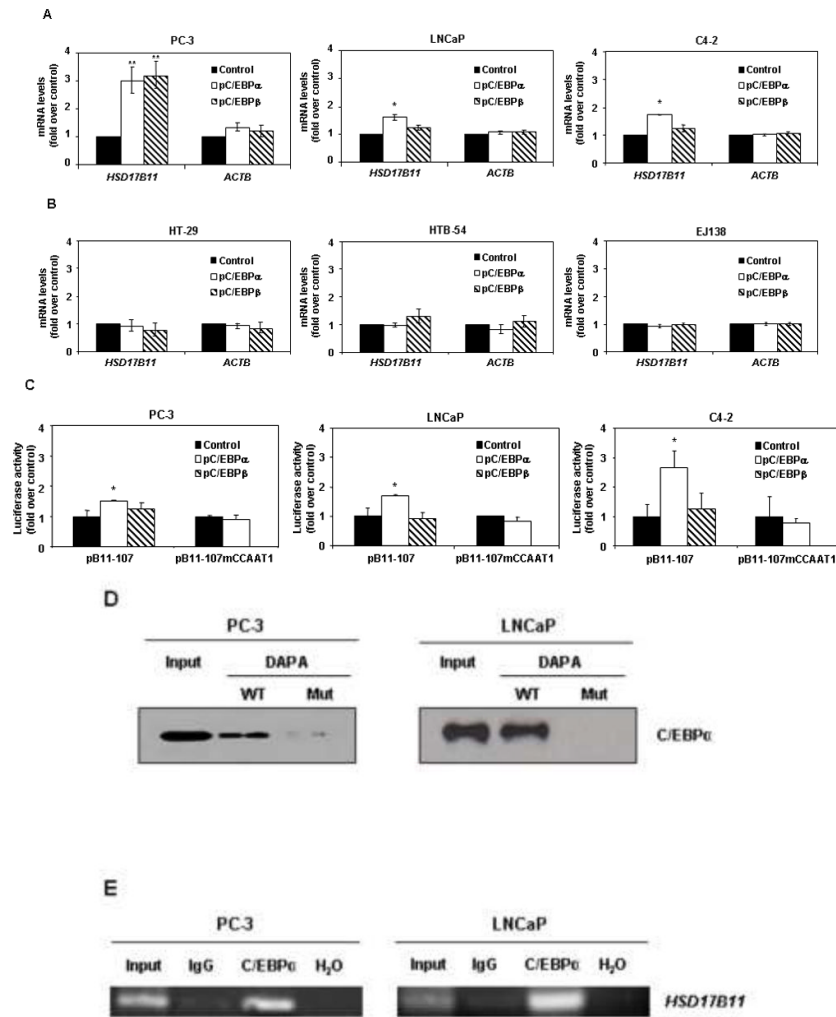
Figure 3. C/EBPα upregulates *HSD17B11* transcription in PC cells through the CCAAT box located at -71

A) HSD17B11expression levels were determined by RT-qPCR in PC-3, LNCaP and C4-2 cells transfected with either C/EBPα or C/EBPβ. Results are expressed as fold over control (empty vector transfected cells). Significant differences to the reference construct are denoted by asterisks (*p≤0.05, **p≤0.01). **B)** HSD17B11 expression levels were determined by RT-qPCR in HT-29, HTB-54 and EJ138 cells transfected with either C/EBPα or C/EBPβ. Results are expressed as fold over control (empty vector transfected cells). **C)** Transfection assays showing that expression of C/EBPα but not C/EBPβ, transactivates *HSD17B11* promoter activity and that mutation of the CCAAT box located at -71 impairs this induction. Promoter activity is expressed as fold over control (cells transfected with empty vector). Each value represents the mean ± SEM of four independent experiments, each performed in triplicate. Significant differences (p≤0.05) to the reference construct are denoted by asterisks (*). **D)** DNA Affinity Precipitation Assay (DAPA) experiments showing that C/EBPα binds to the normal *HSD17B11* probe (Table 1B) but not to the mutated one. **E)** ChIP assays showing that C/EBPα is recruited to the HSD17B11 promoter in PC cells at basal conditions. Results of amplification of soluble chromatin before immunoprecipitation are shown as control (input).
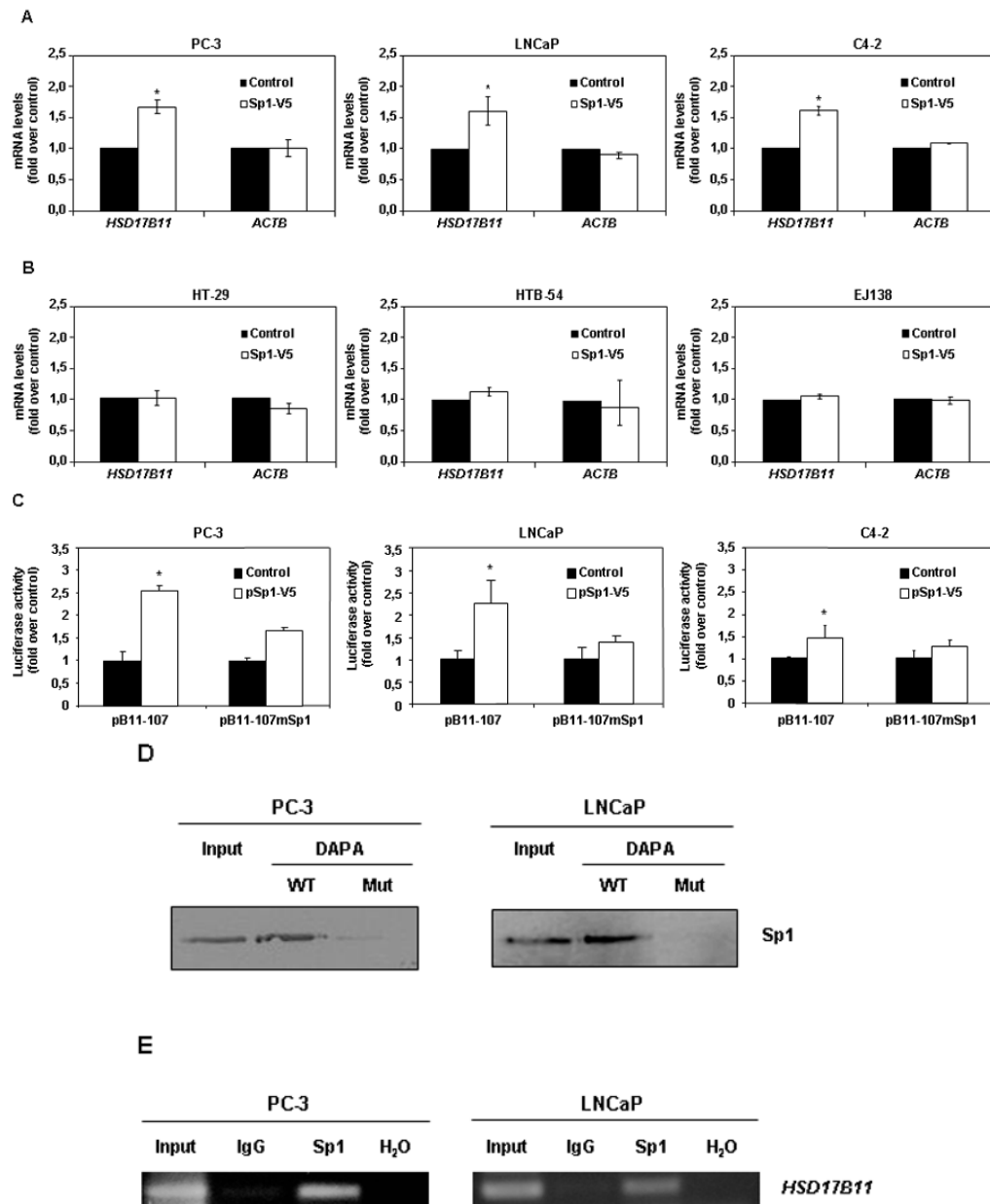
Figure 4. Sp1 transactivates HSD17B11 in PC cells
A) PC-3, LNCaP and C4-2 cells were transfected with either a Sp1 expression vector or an empty vector. RT-qPCR shows that Sp1 expression induces HSD17B11 transcription. *GAPDH* and *ACTB* housekeeping genes were both used as controls. **B)** HSD17B11 expression levels were determined by RT-qPCR in Sp1 transfected HT-29, HTB-54 and EJ138 cells. Results are expressed as fold over control (empty vector transfected cells). **C)** Transfection experiments showing that Sp1 expression induced *HSD17B11* promoter activity. Results are expressed as fold over control (empty vector). Each value represents the mean ± SEM of four independent experiments, each performed in triplicate. Significant differences (p≤0.05) to the reference construct are denoted by asterisks (*). **D)** DNA Affinity Precipitation Assay (DAPA) experiments showing that Sp1 binds to the normal *HSD17B11* probe (Table 1B) but not to the mutated one. **E)** ChIP assays showing that Sp1 is

recruited to the *HSD17B11* promoter *in vivo* in PC cells at basal conditions. Results of amplification of soluble chromatin before immunoprecipitation are shown as control (input).

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                                        Sp1/RXR
hsa   -145   TGCTCCGGTCTGGACTTCGTCCCTCCCAGATCTCTGAGCTAGCAAGCA-----------C
mcc   -130   TGCTCGGGTCTGGACTTCGTCCCTCCCAGATCTCTGAGCGAGCAAGCA-----------C
ptr   -130   TGCTCCGGTCTGGACTTCGTCCCTCCCAGATCTCTGAGCTAGCAAGCA-----------C
mmu   -185   GGCTTGCCTCTGAACTTCGTCCCTCCCAGATTTCCAAGCCAGCGAGCAGTTGCTCTG-AC
rno   -184   GGCTTGCCTCTGAACTTCGTCCCTCCCAGATTGCCAAGCCAGCGAGCctTTGCTCTG-AC
ecb   -178   TGCTCTGCCCCGGACTTTGTCCCTCCCAGATTTCTGAGCTGGCGAGCAGTGGCTCCGCGC
cfa   -143   TGCCCTCCCCTGGATTTTGTCCCTCCCAGATTTCTGAGCTGGCGAGCAGCCGCTCCGCAC
bta   -121   CGCTCTTGGCTGGGTTTTGTCCCTCCCAGATTTCTGAGCTGGCGAGCAGTCTCTCCGCAC

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              ..        .  .   .. ............... . ... .. ...            .


                        CCAAT                              Sp1
hsa   -96    TGCAGCCCGAGCCAATTGCGAAAGACC-AACAAAGCCCAGCCCAGCGGAAGGAACGGTTT
mcc   -81    TGCAGCCCGAGCCAATTGCGAAAGACC-AACAAAGCCCAGCCCAGCGGAAGGAACGGTTT
ptr   -81    TGCAGTCCGAGCCAATTGCGAAAGACC-AACAAAGCCCAGCCCAGCGGAAGGAACGGTTT
mmu   -126   TGCAGTCCGAGCCAATGGCAAAAACCCTCCTGAAGCCCGGCCCCGCGGAAGGAATGGTTT
rno   -125   TGCAATCCGAGCCAATGGCAAAAACCCTTCTGAAGCCCGGCCCCGCGGAAGGAATGGTTT
ecb   -118   TGCAGCCCCAGCCAATTGCAGACGACCTAACCga----GGCCAGCCGGAAGGTGCGGTTT
cfa   -83    TGCAGCCCGAACCAATTGTGAAAGACCTAACCAAGCCCAGCCCAGCGGAAGGAACGGTTT
bta   -61    TGCAGCCTGAGCCAATTGCCAAAGACCTAGCCAAGCCCAGCCCAGCGGAAGGAGCGGTTT

             ..... ............... ....... . .  ..........  .................
             ....  .  .  ..... .   .    ..                  ...  ........   .....


                            GATA-1    NF-κβ                      KLFs
hsa   -37    CGGAGTTGTTTT----TCTTTGATACGGGAGTTCCTCCTTGCTCTCGCCCCTACTCTT-T
mcc   -22    CGGAGTTGTTTT----TCTTTGATAGGGGAGCTCTTCCTTGCTCTCGCCCCTACCCTT-T
ptr   -22    CGGAGTTGTTTT----TCTTTGATACGGGAGTTCCTCCTTGCTCTCGCCCCTACTCTT-T
mmu   -66    CCGAGTTGTTTGTTTCTCTTTAATAGGGGAACTCTGCCTTGCTTAAGCTTTTACTCT---
rno   -65    TTGAGTTGTTTGTTTCTCTTTAATAGGGGAACTCTGCCTTGCCCACGCTTTTACTTT---
ecb   -62    CGAGTTG--TTT----GTTCTTATAAAGGAGCCCTTCCTCGTTCTTGCTTTTTATTCCCT
cfa   -23    CAGAGTTGTTGGTTTCTCTTTGATAAAGAAGCCCTTGCTTGCTCTTGCTTTTTATTCCAT
bta   -1     CGGAGTTGTTTGTTTCTCTCCGATAAAGGAGCCCTTCCTTGTTCTCGCTTTTgtgctcgc

             . .........       ..... ...  ..... ............ ..  .   .
             .  .          .   ... . .  .   .. .        ..    .
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Figure 5. Sequence alignment of *HSD17B11* orthologs
Sequence alignment of the 5'-flanking region of *HSD17B11* of humans (hsa), *Macaca mulatta* (mcc), *Pan troglodytes* (ptr), *Mus musculus* (mmu), *Rattus norvegicus* (rno), *Equus caballus* (ecb), *Canis lupus familiares* (cfa), and *Bos taurus* (bta). Putative transcription factor binding motifs are boxed. The (:) symbol below the alignment denotes complete similarity among the sequences; while (·) indicates that at least 6 of the 8 nucleotides match.

Table 1

Sequences of sense and antisense oligonucleotide primers used for (A) site-directed mutagenesis, (B) DNA affinity precipitation assays and (C) amplification of immunoprecipitated chromatin.

A. Sequences of oligonucleotides used as primers for Site-Directed Mutagenesis experiments.	
Oligonucleotides	**Sequence (5' to 3')**
HSD17B11-mC1 (f) HSD17B11-mC1 (r)	GCAAGCACTGCAGCCCGAGC<u>ACTG</u>AGCGAAAGACCAACAAAGCCC GGGCTTTGTTGGTCTTTCGC<u>TCAGT</u>GCTCGGGCTGCAGTGCTTGC
HSD17B11-mC2 (f) HSD17B11-mC2 (r)	GCCTGTGGACACTGTCGGTTATCCT<u>AC</u>GAACTTATCC<u>GG</u>TTTAGG CCTAAA<u>CC</u>GGATAAGTTC<u>GT</u>AGGATAACCGACAGTGTCCACAGGC
HSD17B11-mSp1 (f) HSD17B11-mSp1 (r)	CCAATTGCGAAAGACCAACAAAG<u>AGT</u>AGCCCAGCGGAAGG CCTTCCGCTGGGCT<u>ACT</u>CTTTGTTGGTCTTTCGCAATTGG
HSD17B11-mNFKB (f) HSD17B11-mNFKB (r)	TCGGAGTTGTTTTTCTTTGATACG<u>AAAGG</u>CCCTCCTTGCTCTCGCCCCTA TAGGGGCGAGAGCAAGGAGG<u>GCC</u>T<u>TT</u>CGTATCAAAGAAAAACAACTCCGA
HSD17B11-mGATA (f) HSD17B11-mGATA (r)	GGAACGGTTTCGGAGTTGTTTTTCTTT<u>ATT</u>GCGGGAGTTCCTCCT AGGAGGAACTCCCGC<u>AAT</u>AAAGAAAAACAACTCCGAAACCGTTCC

B. Sequences of oligonucleotides used as probes for DAPA experiments.	
Oligonucleotides	**Sequence (5' to 3')**
HSD17B11-CCAAT (f) HSD17B11-CCAAT (r)	**BioG/** CCCGAGCCAATTGCGAAAGACCAAC GTTGGTCTTTCGCAATTGGCTCGGG
HSD17B11-CCAATmut (f) HSD17B11-CCAATmut (r)	**BioG/** CCCGAGC<u>ACTG</u>AGCGAAAGACCAAC GTTGGTCTTTCGC<u>TCAGT</u>GCTCGGG
HSD17B11-Sp1 (f) HSD17B11-Sp1 (r)	**BioG/**AACAAAGCCCAGCCCAGCGGAAGGA TCCTTCCGCTGGGCTGGGCTTTGTT
HSD17B11-Sp1mut (f) HSD17B11-Sp1mut (r)	**BioG/**AACAAA<u>GAGT</u>AGCCCAGCGGAAGGA TCCTTCCGCTGGGCT<u>ACT</u>CTTTGTT

C. Sequences of oligonucleotides used as primers for amplification in ChIP experiments.	
Oligonucleotides	**Sequence (5' to 3')**
promHSD17B11 (f) promHSD17B11 (r)	GCAAAGCAGAACTAGGAAGC GCGAGAGCAAGGAGGAAC