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PPARγ regulates E-cadherin expression and inhibits growth and invasion of prostate cancer.

Jean-Sébastien Annicotte[1] , Irena Iankova[1,*] , Stéphanie Miard[1,*], Vanessa Fritz[2], David Sarruf[1], Anna Abella[1], Marie-Laurence Berthe[3], Danièle Noël[2], Arnaud Pillon[1], François Iborra[3], Pierre Dubus[4], Thierry Maudelonde[3], Stéphane Culine[1], and Lluis Fajas[1, 3+]

[1]Inserm, U540, Equipe Avenir, Montpellier, F-34090, France; [2]Inserm, U475, Montpellier, F-34090, France; [3]Centre Hospitalier Universitaire Arnaud de Villeneuve, Laboratoire de Biologie Cellulaire, Montpellier, F-34090, France; [4]Université Victor Ségalen, EA2406 Histologie et Pathologie Moléculaire, Bordeaux, F-33076 France

+ To whom correspondence should be addressed at :

Equipe Avenir Inserm U540, 60, rue de Navacelles, F-34090 Montpellier, France. E-mail: fajas@montp.inserm.fr. Phone : 0033 467 043 082; Fax : 0033 467 540 598

* These authors contributed equally to this work.

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Abstract

PPARγ might not be permissive to ligand activation in prostate cancer cells. Association of PPARγ with repressing factors or post-translational modifications in PPARγ protein could explain the lack of effect of PPARγ ligands in a recent randomized clinical trial. Using cells and prostate cancer xenograft mice models we demonstrate in this study that a combination treatment using the PPARγ agonist pioglitazone and the HDAC inhibitor valproic acid is more efficient in inhibiting prostate tumor growth than each individual therapy. We show that the combination treatment impairs bone-invasive potential of prostate cancer cells in mice. In addition, we demonstrate that expression of E-cadherin, a protein involved in the control of cell migration and invasion is highly up-regulated in the presence of valproic acid and pioglitazone. We show that E-cadherin expression responds only to the combination treatment, and not to single PPARγ agonists, defining a new class of PPARγ target genes. These results open up new therapeutical perspectives in the treatment of prostate cancer.

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Introduction

Prostate cancer is the most common form of cancer in men, and the second leading cause of cancer deaths. Tumor growth is originally androgen dependent. Androgens exert their effects through activation of the Androgen receptor (AR), a member of the hormone nuclear receptor superfamily. In the mature prostatic gland, AR regulates the expression of genes involved in cell division and proliferation of the epithelial cells (26). AR is also involved in several other aspects of prostate cellular metabolism, including lipid biosynthesis and controls the production of specialized secretory proteins with prostate-restricted expression such as with prostate-specific antigen (26). When prostate cancer is still hormone-dependent, androgen ablation therapy causes regression of the tumor (18), likely through inactivation of the transcription of the AR target genes. However, the durability of this response is inadequate and many men develop recurrent androgen-independent prostate cancer, which has a very poor prognosis (see (11) for review). Other nuclear receptors or locally produced factors that interact with nuclear receptors are likely involved in cell proliferation, differentiation, and apoptosis in the prostate. The peroxisome proliferator-activated receptor gamma (PPARγ) is one of such factors. PPARγ is another member of the hormone nuclear receptor superfamily. As for most of the other members of this family its activity is regulated by ligands. Prostaglandin J2 and the anti-diabetic drugs thiazolidinediones have been determined as natural and synthetic ligands of PPARγ respectively (for review see (9)). PPARγ is highly expressed in the adipose tissue and is required for its development through regulation of the expression of adipocyte-specific genes, such as lipoprotein lipase (LPL), or the fatty acid transport protein aP2. In addition to adipose tissue, PPARγ is expressed in several other tissues, including gut, macrophages, lung, bladder, breast, or prostate, although its function in these tissues remains to be elucidated. Interestingly, PPARγ has been shown to be over-expressed in prostate cancer (15). Whereas the physiological function of PPARγ in normal epithelial cells is largely unknown, PPARγ activation was reported to inhibit the proliferation of prostate carcinoma cells (4, 21, 25, 34), and also other cancer lineages (7). These observations suggest that induction of differentiation by activation of PPARγ may represent a promising novel

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therapeutic approach for cancer, as already demonstrated in xenograft models of prostate (21). In addition, treatment of patients with advanced prostate cancer with the PPARγ agonist troglitazone resulted in the stabilization of prostate-specific antigen levels (25). In contrast, in a large scale placebo-controlled randomized clinical trial, no effects were observed in the PSA doubling time of prostate cancer patients (35). These results suggest that PPARγ is not permissive for activation by ligands in these prostate cancer patients. One interesting hypothesis is that some factors could prevent activation of PPARγ by its ligands in cancer cells. One of such factors is histone deacetylases (HDAC). Deacetylation of histones has been correlated with a transcriptionally silent state of chromatin. Inhibition of HDAC activity by natural or synthetic compounds results in the reversion of the phenotype of tumoral cells into normal cells, or apoptosis of cancer cells (22). Although the precise mechanisms have not been yet elucidated, HDAC inhibition results in the selective induction of endogenous genes that play roles either in differentiation or cell cycle arrest. We demonstrated in previous studies that HDAC3 is complexed with PPARγ in the promoters of PPARγ target genes, and that this association results in the repression of these target genes. HDAC inhibitors, such as valproic acid or sodium butyrate (NaBu) had a synergistic effect with TZDs in the activation of PPAR-target genes (8). Therefore, HDAC inhibition could render PPARγ permissive to activation by its ligands. We show in this study that a combination treatment of HDAC inhibitors and PPARγ agonists results in the arrest of proliferation, increases apoptosis and decreases the invasion potential of prostate cancer cells both *in vitro* and *in vivo*. Furthermore we show that PPARγ agonists increase the expression of E-cadherin mRNA only in the presence of HDAC inhibitors, which define a new class of PPARγ target genes.

Materials and Methods

Materials and oligonucleotides. Pioglitazone was a kind gift of Takeda Pharmaceuticals Industries (Osaka, Japan). Rosiglitazone was purchased from VWR-Calbiochem (Fontenay sous Bois, France). All chemicals, except if stated otherwise, were purchased from Sigma (St Louis, MO, USA). Anti-CDK4 (C-22), anti-PPARγ (H-100 for ChIP, N-20 for immunohistochemistry),

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anti-HDAC-3 (H-99) and anti-PCNA (PC-10) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-acetyl H4 (Lys 12) and anti-Phospho-Rb (ser 807/811) were from Cell Signaling (Beverly, MA, USA), anti-p21 (Ab-1) was from EMD Biosciences (Darmstadt, Germany), anti-p27 was from NeoMarkers (Fremont, CA, USA) and anti-BrdU and anti E-cadherin (NCH-38) antibodies were from Dako (Glostrup, Denmark). The oligonucleotide sequences used for various experiments in this manuscript are available upon request.

Cell culture, transient transfections and siRNA. The LNCaP, DU145, PC3 and the luminescent PC3 (30) prostate cancer cell lines were derived from stocks routinely maintained in the laboratory. Monolayer cell cultures were grown in Ham's F-12 medium supplemented with 10% foetal calf serum (FCS) (Invitrogen, Cergy-Pontoise, France). In all experiments, cells were treated for 48 h with the vehicle DMSO (dilution 1:2000), pioglitazone 5.10^{-6} M, rosiglitazone 5.10^{-6} M, valproic acid (1.5 mM for PC3, 0.75mM for DU145 and 0.375mM for LNCaP) or both pioglitazone and valproic acid. Tansient transfections were performed as described previously (2) and luciferase activity measurements were normalized for ß-galactosidase activity to correct for differences in transfection efficiency. Graph values represent the mean of three independent experiments. For siRNA experiments, smart-pool siRNAs against HDAC3 (Dharmacon, Lafayette, CO, USA) were transfected in PC3 cells using DharmaFECT™ 2 (Dharmacon) following manufacturer's instructions. After 24 h, cells were treated as described above and incubated for 24 h. Effects of the siRNA on HDAC3 mRNA and protein levels are illustrated in figure 7B and C, respectively.

Apoptosis and BrdU assays, flow cytometry analysis and phospho-pRb detection. Proliferating LNCaP, DU145 and PC3 cells were incubated for 48 h with the different treatments as described above. For all immunofluorescence experiments, cells were grown on coverslips. Apoptotic cells were detected using Alexa 568 conjugated-annexin V labeling following manufacturer's instructions (Roche, Basel, Switzerland). For BrdU incorporation, cells were incubated 4h for PC3 and DU145 and 16h for LNCaP in the presence of 100 μM BrdU, harvested and fixed with methanol. An additional treatment of the cells with 1.5 N HCl for 10 min was

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performed. Cells were then incubated with the anti-BrdU antibody (dilution 1:100) for 16 h at 4°C, and BrdU staining was revealed using a Texas-red-conjugated anti-mouse IgG. For phospho-pRb immunofluorescence detection, PC3 cells were harvested after 48 h treatment, fixed in methanol for 10 min at 4°C, and incubated for 16 h at 4°C with the anti-phospho-pRb antibody (dilution 1:50), and phospho-pRb staining was revealed using a Texas-red-conjugated anti-rabbit IgG. At least 500 cells were counted. For FACS analysis, cells were harvested, fixed with EtOH 70%, and DNA was labeled with propidium iodide. Cells were sorted by FACS analysis (Coulter Electronics, Hialeah, FL, USA) and cell cycle profiles were determined using the ModFit software (Becton Dickinson, San Diego, CA, USA).

RNA extraction, RT-PCR and Q-PCR. RNA extraction and reverse transcription were performed as described (3). Q-PCR was carried out using a LightCycler and the DNA double strand specific SYBR Green I dye for detection (Roche). Q-PCR was performed using gene-specific oligonucleotides and results were then normalized to RS9 levels.

Protein extracts and western blot analysis. Protein extracts and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electrotranfer and immunoblotting were performed as described (31).

Kinase assays. CDK4 immunoprecipitation and kinase assays were performed exactly as previously described (1).

***In vivo* murine models of prostate cancer.** Male Rj:NMRI-nu (nu/nu) (Janvier, Le Genest-St-Isle, France) and CD17-SCID/bg (Harlan, Gannat, France) mice were maintained according to European Union guidelines for use of laboratory animals. *In vivo* experiments were performed in compliance with the French guidelines for experimental animal studies (Agreement No. B-34-172-27). For *in vivo* proliferation studies, 3.10^6 luminescent PC3 cells were laterally injected sub-cutaneously (s.c.) in nude mice at 6 weeks of age. 5 days after s.c. injection, cohorts (10 mice/group) were orally administrated the vehicule (0.5% carboxy methyl cellulose (CMC)), pioglitazone (30 mg/kg/d in 0.5% CMC), valproic acid (150 mg/kg/d in 0.5% CMC) or both compounds for a period of 4 weeks. Tumor progression was determined by measuring the volume

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of the tumor with a caliper. Tumor tissues were collected, weighted, fixed in 4% phosphate-buffered formalin and embedded in paraffin for immunohistological analyses. For *in vivo* bone-invasion studies, subconfluent monolayers of luminescent PC3 cells were detached by trypsinization, washed, and resuspended in PBS to the working concentration of 5 x 10^5 cells/10 μl. All tibiae injections were performed on SCID mice (10 mice/group) anesthetized with pentobarbital (50 mg/Kg). The proximal end of the left tibiae bones was exposed surgically in a flex position and 10 μl of PBS containing tumor cells were injected into the bone marrow space with a 26-gauge needle. Mice were treated 7 days after intra-tibiae injection with vehicle (0.5% CMC), pioglitazone (30 mg/kg/d in 0.5% CMC), valproic acid (300 mg/kg/d in 0.5% CMC) or both compounds for a period of 4 weeks and monitored weekly for tumor growth kinetic using bioluminescence imaging with the NightOWL LB981 CCD camera (Berthold Technologies, Bad Wildbad, Germany) and WinLight software (Berthold Technologies). Left and right legs were harvested, and fixed in 4% phosphate-buffered formalin, X-rays of the legs were taken and invasion potential was scored by 4 blind comparisons of X-ray radiographs. Scores ranged from 0 (no invasion) to 4 (high degree of invasion). For both xenograft mouse models, tumor formation was verified one day before starting treatment using bioluminescence imaging. No failure rate for tumor initiation was observed and tumor growth was occurring at the same rate.

Immunohistochemistry (IHC) and histology. IHC was performed as described previously (2). Briefly, after antigen retrieval, 5μm formalin-fixed luminescent PC3 tumor sections were incubated with the anti-PCNA (dilution 1:500), anti-p21 (dilution 1:20) and anti-E-cadherin (dilution 1:25) antibodies and the LandMark™ Prostate Tissue MicroArray (Ambion, Austin, TX, USA) containing 5μm formalin-fixed paraffin-embedded human normal and tumor prostate sections were incubated with the anti-PPARγ (dilution 1:25) or the anti-acetyl H4 antibodies (dilution 1:25). Immunostainings were revealed using peroxydase-conjugated anti-mouse (for PCNA, p21 and E-cadherin, Jackson Immunoresearch, Cambridgeshire, UK), anti-goat (for PPARγ, Jackson Immunoresearch) or anti-rabbit (for acetylated H4, Jackson Immunoresearch) secondary antibodies and the DAB chromogen (DAKO) as a substrate. Sections were

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counterstained with haematoxylin. For E-cadherin, immunofluorescence staining was revealed using a Texas-red-conjugated anti-mouse secondary antibody. Negative controls using mouse, rabbit or goat IgGs were performed and no staining was observed in these conditions. Trained pathologists analyzed the PPARγ, acetyl H4 and E-cadherin stainings. Immunohistochemical quantification was based on two parameters, the intensity of the staining and the percentage of cells positively stained, leading to 4 groups : 0, no staining; 1, weak positive staining; 2, moderate staining and 3, strong staining.

Invasion assay. The Boyden chamber migration assay was performed as described previously (12). Briefly, polycarbonate filters (12 μm pore) were coated with 60 μg of Matrigel (Becton Dickinson). Cells were harvested in medium containing 3% FCS and ligands (vehicle, 5 μM pioglitazone, 1.5 mM valproic acid, or both) and added to the top chamber (1.10^6 cells per chamber). Medium supplemented with 10% FCS and ligands was used in the bottom compartment as a chemo-attractant. To correct for proliferation and/or cell death due to our treatments, cells were cultured in parallel in 12-wells plates in medium containing 3% FCS and ligands (control plate corresponding to total cells). Chambers and plates were incubated for 48 h at 37°C and cells that had traversed the Matrigel and spread on the bottom surface of the filter as well as cells from control plates were then quantified using 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenol tetrazolium bromide and determination of OD_{540}. Experiments were performed in triplicate, and results are expressed as percentage of invading cells relative to total control cells.

Electro mobility shift assays (EMSA). EMSA were performed as described previously (2, 10). Briefly, *in vitro* translated PPARγ and RXRα were incubated for 15 min at 21°C in a total volume of 20 μl binding buffer [(10mM Tris-HCl (pH 7.9), 40 mM KCl, 10% glycerol, 0.05% Nonidet P-40, 1mM DTT and 1 μg poly(dI:dC)] in the presence of 2 ng of a T4-PNK end labeled double-stranded oligonucleotide probe. For gel supershift assay, 2μg of IgG or PPARγ antibody were added to the reaction. DNA-protein complexes were separated by electrophoresis on a 4% polyacrilamide gel in 0.25% TBE at 4°C and 10V/cm.

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Cloning of the E-cadherin and aP2 promoters. The E-cadherin and aP2 promoters were cloned using the BD Advantage GC Genomic polymerase mix (BD Biosciences Clontech, Palo Alto, CA, USA) and genomic DNA as a template. PCR amplifications were performed according to the manufacturer's instructions and cloned in the pGL3-basic vector (Promega Life Science, Madison, WI, USA). A deletion E-cadherin promoter mutant devoid of the PPRE was obtained by PCR using specific primers and cloned as described above. The different pGL3 promoter constructs were sequenced and used in transient transfections.

Co-immunoprecipitation. Immunoprecipitation assays were performed as previously described (8).

Chromatin immunoprecipitation (ChIP) and Re-ChIP. ChIP assays were performed as described previously (3). Re-ChIP assays were performed as described (23). Briefly, proteins from PC3 cells treated for 48 h with different ligands were formaldehyde cross-linked to DNA. After lysis and DNA sonication, proteins were then immunoprecipitated using an anti-PPARγ antibody. After washing, DNA-protein-complexes were subsequently eluted in 10mM DTT for 30 min at 37°C and re-immunoprecipitated using IgG (negative control) or anti-HDAC3 antibody. Cross-linking was then reversed by heating the samples at 65°C for 16 h. DNA was then purified using Qiagen PCR purification kit (Qiagen, Courtabœuf, France), and PCR amplification was performed using promoter-specific oligonuleotide primers.

Statistical analysis. Data are presented as means ± SEM, except for tumor measurements (volume, mass and progression) presented as medians. Group means and medians were compared by factorial analysis of variance (ANOVA). Upon significant interactions, differences between individual group means and medians were analyzed by Fisher's protected least squares difference (PLSD) test. Differences were considered statistically significant at $p < 0.05$.

Results

Synergistic action of PPARγ agonists and HDAC inhibitors in the control of cell proliferation and apoptosis in prostate cancer cells.

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We have previously demonstrated that HDAC inhibitors have a synergistic action with PPARγ agonists in the activation of PPARγ target genes and adipocyte differentiation (8). Since both PPARγ agonists and HDAC inhibitors independently arrest proliferation of prostate cancer cells we wanted to test the synergy of both agents in the control of prostate cancer cell growth. BrdU incorporation studies in the androgen-dependent LNCaP (AR mut, Rb wt, p53 wt) cell line indicated that BrdU-positive cells were significantly decreased upon 48 h of pioglitazone and rosiglitazone treatments compared to control cells (figure 1A, LNCap, 33.8% ± 0.1 for vehicle, 26.8% ± 1.4 for pioglitazone and 27.5% ± 2.5 for rosiglitazone-treated cells). Moreover, a significant decrease in BrdU incorporation was observed when cells were incubated in the presence of the HDAC inhibitor valproic acid (33.8 % ±0.1 for vehicle versus 4.8% ± 0.8 for valproic acid, fig. 1A). Most interestingly, the combination treatment of pioglitazone/valproic acid and rosiglitazone/valproic acid decreased the proliferation index to 1.5% ± 0.1 and 1.6 % ± 0.01, respectively. To further prove that the effect of the combination treatment was independent on the cell line, two androgen-independent prostate cancer cell lines, i.e. DU145 (AR -, Rb -, p53 mt) and PC3 (AR -, Rb wt, p53 -), were subjected to BrdU incorporation (fig. 1B and C). In DU145 cells, PPARγ agonists and valproic acid alone demonstrated no inhibitory effect on proliferation (fig. 1B), whereas in PC3 cells, single valproic acid treatment resulted in a decreased proliferation index (12.6 % ±2.1 for vehicle versus 6.9% ± 1.6 for valproic acid, fig. 1C). Importantly, as observed for LNCaP cells, moderate and strong inhibitory effects on proliferation were obtained when using the combination of PPARγ agonists and valproic acid in DU145 (33.9 % ±4.5 for vehicle versus 24.3% ± 1.8 for pioglitazone/valproic acid and 23.1% ± 2.1 for rosiglitazone/valproic acid) and PC3 cells (12.6 % ±2.1 for vehicle versus 2.1% ± 0.8 for pioglitazone/valproic acid and 1.6% ± 0.7 for rosiglitazone/valproic acid), respectively. Flow cytometry analysis further demonstrated the anti-proliferative effect of our treatments on PC3 cells showing a decrease in the number of cells in the S-phase, concomitant to an increase in the proportion of cells in the G1 phase of the cell cycle compared to vehicle-treated cells (fig. 1D). Similar to BrdU incorporation studies, the combination therapy resulted in the highest

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accumulation of cells in the G1 phase of the cell cycle (fig. 1D). These results suggest an inhibitory effect of the combined treatment of PPARγ agonists and valproic acid on cellular proliferation of several prostate cancer cell lines. Moreover, the effects of the treatment are independent of the AR status of the cells, since LNCaP and PC3 cells responded similarly to the combination therapy. Interestingly, the inhibition of proliferation following treatments was more important in LNCaP and PC3 compared to DU145. Since LNCaP and PC3 are expressing wild type Rb protein and DU145 express mutant Rb protein, this suggests that our treatment efficacy could depend on Rb status and might involve Rb-dependent pathways.

Next we determined the effect of our treatments on apoptosis of PC3 prostate cancer cells. No effect was observed upon pioglitazone treatment, whereas a significant proportion of PC3 cells underwent apoptosis when treated with valproic acid and pioglitazone combined to valproic acid (fig. 1E). Altogether, these results demonstrate that the combination treatment decreases cellular proliferation and increases apoptosis.

Regulation of the expression of cell cycle regulators and pRB phosphorylation by PPARγ agonists and HDAC inhibitors in prostate cancer cells.

Since pioglitazone and valproic acid treatments impact on cellular proliferation of PC3 cells, we next wanted to analyze the expression of cell cycle regulators. Consistent with the observed cell cycle arrest, mRNA and protein expression of the cell cycle inhibitors p19, p21, and p27 were increased in response to pioglitazone, valproic acid, and to a higher extent in response to the combination treatment (fig. 2A, B). Moreover, cyclin D1 (CcnD1) mRNA and protein levels were decreased upon treatment with pioglitazone alone or in combination with valproic acid (fig. 2A, B). Previous reports demonstrated that PPARγ agonists regulate p21 expression in pancreatic and lung cancer cells through interaction with sp1 proteins and binding to sp1 sites on its promoter (13, 16). In addition, it has recently been demonstrated that rosiglitazone post-transcriptionally induces p21 in PC3 cells (28). To clarify whether the increased p21 mRNA and protein expression was mediated by PPARγ transcriptional activity or by indirect mechanisms,

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chromatin immunoprecipitation assays were performed. Using primers amplifying the sp1 sites in the human p21 promoter, previously shown to mediate the effects of PPARγ through sp1 binding (16), we observed that PPARγ was bound to this promoter region in PC3 cells suggesting a transcriptional regulation of the p21 promoter by PPARγ (fig. 2C and supplemental figure S1A). Moreover, we observed an increased acetylation status of histone H4 from vehicle, pioglitazone, valproic acid and pioglitazone plus valproic acid-treated cells, suggesting an increased transcriptional activity of this promoter (fig. 2C and supplemental figure S1A). Since several cell cycle inhibitors are induced upon treatment, we next wanted to study the effect of our treatments on pRB phosphorylation in PC3 cells. pRb phosphorylation levels were dramatically decreased, as assessed by immunofluorescence assays (fig. 2D). Moreover, using an anti-pRb antibody, detecting unphosphorylated and phosphorylated pRb proteins, and an anti-^{P}pRb antibody detecting only the ser807/811 phosphorylated form of pRb, we observed by immunoblotting a decrease in ^{P}pRb in cells treated with pioglitazone, valproic acid or both and an accumulation of unphosphorylated pRb from non treated to pioglitazone plus valproic acid-treated cells, which was consistent with arrested proliferation (fig. 2E). To further assess the participation of the complex cdk4/cyclinD on pRB phosphorylation upon treatments, kinase activity experiments were performed. Immunoprecipitated cdk4 from PC3 cells treated with vehicle was active in non-treated cells whereas it was inactive in cells treated with pioglitazone, valproic acid or both (fig. 2F). Altogether, these results demonstrate that the observed decreased cellular proliferation of PC3 upon treatments could be the result of an increased expression of cell cycle inhibitors, leading to reduced pRB phosphorylation levels.

Inhibition of tumor progression in a mouse model of prostate cancer in response to pioglitazone and valproic acid combination therapy.

To evaluate the *in vivo* effect of a combined therapy of pioglitazone and valproic acid on prostate cancer development, we used an immuno-deficient mouse model in which luminescent PC3 cells were grafted sub-cutaneously, allowing us to follow tumor initiation and progression using

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bioluminescent imaging. We observed no failure in tumor initiation, with 100% grafted cells giving rise to a tumor. No significant differences on tumor volume and mass were observed in mice treated with either pioglitazone or valproic acid, whereas a 40% decrease in tumor volume and mass was observed in mice treated with the combination of pioglitazone and valproic acid, compared to mice treated with vehicle (fig. 3A-B). Consistent with the inhibition of tumor growth, a decrease in cell proliferation in tumors of mice treated with the combination therapy was observed, compared to tumors of mice treated with vehicle, as measured by PCNA staining on histological sections of the tumors (fig. 3C-D). Consistent with the size of tumors (fig. 3A-B), no effect on tumor cell proliferation was observed when each single agent (pioglitazone or valproic acid) was used in the treatment. Further characterization indicated that the expression of p21 was increased in tumors of mice treated with the combination therapy, compared to mice treated with vehicle or single agent therapy (fig. 3E-F), consistent with the observed decrease in cell proliferation. Interestingly, when analyzing other markers of tumors aggressiveness in mice treated with the combination therapy we found increased expression of E-cadherin, which is important in the control of invasion and migration of cancer cells (fig. 3G).

Decreased *in vitro* and *in vivo* invasion potential of prostate cancer cells treated with pioglitazone and valproic acid.

Increased expression of E-cadherin suggested that the combination treatment could have an impact on the invasion and migration potential of prostate cancer cells. We therefore evaluated the effect of the combination of pioglitazone and valproic acid on the invasiveness of LNCaP and PC3 cells using a matrigel assay. Treatments of LNCaP cells with pioglitazone, valporic acid or both had no effect on the invasive potential of these cells (fig. 4A), probably due to the low metastatic potential of this cell line (17, 27) and a weak percentage of cells invading the matrigel membrane in basal conditions (fig. 4A). In PC3 cells which have a high metastatic potential (20, 27) pioglitazone treatment showed no significant effect on invasiveness of the cells compared to the control vehicle-treated cells (fig. 3A; 41.9% ± 2.0 for control, 32.2% ± 8.6 for pioglitazone),

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whereas decreased invasion was observed when PC3 cells were treated with valproic acid (24.0% ± 2.3). Strikingly, a synergistic effect in the inhibition of invasion was observed when a combination of pioglitazone and valproic acid was used (11.4% ± 3.1). These results suggested that the invasion potential of highly metastatic prostate cancer cells, in the presence of the combination treatment, was inhibited.

These data prompted us to study the effect of pioglitazone and valproic acid on the inhibition of invasion *in vivo*. Prostate cancer cells preferentially invade bone. We therefore used a bone invasion model by intra-tibially injecting luminescent PC3 cells in immunodeficient mice. Mice were treated thereafter for 30 days with the combination therapy pioglitazone and valproic acid. *In vivo* imaging techniques using a CCD camera facilitated the follow up of tumor initiation and growth in these animals by quantification of the luciferase signal after intraperitoneal luciferine injection. As described for sub-cutaneous xenograft, no failure in tumor initiation was observed. To characterize the *in vivo* bone invasion potential of our xenografted-PC3 cells, X-ray analysis of the legs were performed and bone destruction was scored from 0 (no destruction, thus no invasion) to 4 (high degree of bone destruction, demonstrating a high invasion potential, fig. 4B). X-ray analysis of treated mice showed preservation of bone structure and density, whereas massive bone destruction was observed in non-treated mice (fig. 4C). Moreover, histological analysis of the tibiae demonstrated that tumor cells engrafted in mice treated with vehicle destroyed the tibial bone and spread both in the join and in the skeletal muscle, whereas PC3 cells from pioglitazone plus valproic acid-treated mice remained inside the central bone cavity (fig 4D), reinforcing the scoring data presented in figure 4C. These results demonstrate that the combination of pioglitazone and valproic acid is effective in the inhibition of invasion of prostate cancer cells in bone.

Increased expression of E-cadherin mRNA in prostate cancer cells in response to pioglitazone and valproic acid treatment.

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Inhibition of invasion of prostate cancer cells was likely the result, at least in part, of increased expression of E-cadherin. Since PPARγ and HDACs are key regulators of gene transcription we tested the hypothesis that E-cadherin expression could be regulated at the transcriptional level by pioglitazone and valproic acid. In LNCaP, we failed to induce E-cadherin mRNA expression upon pioglitazone, valproic acid or both molecules, suggesting that in this cell line PPARγ might have no transcriptional effect on genes involved in migration processes (fig. 5A, LNCaP). This idea is reinforced by the invasion results showing that migration of LNCaP cells is not modified upon PPARγ agonists nor HDACi (fig. 4A). In the androgen-independent and highly metastatic PC3 cell line, no significant induction of E-cadherin mRNA levels were observed after pioglitazone treatment of PC3 cells. In contrast, valproic acid significantly induced E-cadherin mRNA up to 10-fold compared to vehicle-treated cells (fig. 5A-B). Interestingly the combination of pioglitazone and valproic acid further increased E-cadherin mRNA expression (70-fold induction, fig. 5A, Q-PCR and B, semi-quantitative RT-PCR). Consistent with the mRNA data, the association of pioglitazone and valproic acid resulted in an increase in E-cadherin protein levels compared to vehicle-treated PC3 cells (fig. 5C).

Computational analysis of the E-cadherin promoter identified a PPARγ Response Element (PPRE), located at nucleotides –2476 to –2464 from the transcription initiation start site, highly conserved when compared to the PPRE found in PPARγ target genes such as the HMG-CoA synthetase, the aP2 and the LPL promoters (fig. 5D). EMSA analysis using the PPRE found in the E-cadherin gene as a probe indicated that the *in vitro* translated PPARγ-RXRα heterodimer specifically bound to this element as demonstrated by the use of a competitor probe containing a consensus PPRE, and the use of a PPARγ antibody, which supershifted the retarded PPARγ-containing band (fig. 5E). No binding was observed when the reticulocyte lysate or the *in vitro* translated PPARγ or RXRα were used alone (fig. 5E, lanes 1, 2 and 3). These results suggested that the PPARγ/RXRa heterodimer could regulate the expression of E-cadherin through direct binding to its promoter.

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To determine whether PPARγ/RXRα could activate the human E-cadherin promoter *in vitro*, COS cells were then co-transfected with a PPARγ expression vector and with the full-length E-cadherin promoter containing the PPRE driving the expression of the luciferase gene or a deletion mutant devoid of this PPRE. No effect of pioglitazone on E-cadherin promoter activity was observed in the presence of PPARγ expression vectors, whereas valproic acid induced up to 3-fold E-cadherin promoter activity. Consistent with increased E-cadherin mRNA expression (fig. 5A-B), the combination of pioglitazone and valproic acid had synergistic effects and induced up to 5-fold the activity of the full length E-cadherin promoter in COS (fig. 5F). This synergistic effect was abrogated when the PPRE of the E-cadherin promoter was deleted (fig. 5F), suggesting that PPARγ was mediating the synergistic effects. The same results were obtained when PC3 cells were transiently transfected (data not shown). Interestingly, the E-cadherin gene contains a functional PPRE that is responsive to PPARγ, but only in the presence of HDAC inhibitors.

E-cadherin is a new class of PPARγ target genes responding only to the combination treatment.

To further elucidate the molecular mechanisms underlying this particular effect of PPARγ on the expression of E-cadherin, we first tested the presence of the HDAC3 repressor protein in the PPARγ complex in PC3 cells by co-immunoprecipitation studies. We first verified that our treatments had no impact on PPARγ and HDAC3 protein levels, as demonstrated by immunoblotting (fig. 6A). When protein extracts from PC3 cells were immunoprecipitated using an anti-HDAC3 antibody endogenous PPARγ protein was associated to HDAC3 in the control, pioglitazone and valproic acid-treated cells, and was minimally detected in cells co-treated with pioglitazone and valproic acid (fig. 6B). To further prove that HDAC3 is associated with PPARγ and represses its transcriptional activity, chromatin immunoprecipitation studies of the E-cadherin promoter were performed. A 414 bp fragment of the human E-cadherin promoter containing the binding site of PPARγ was amplified by PCR when anti-PPARγ was used to

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immunoprecipitate chromatin from vehicle, pioglitazone, valproic acid and pioglitazone plus valproic acid-treated cells (fig. 6C, PPRE and supplemental figure S1B). Interestingly, a PCR amplification product was observed when anti-HDAC3 was used to immunoprecipitate chromatin from either vehicle, pioglitazone, or valproic acid-treated cells, whereas no amplification was observed when immunoprecipitated chromatin from cells treated with the combination of pioglitazone and valproic acid was used as a template nor when non-specific IgGs were used to immunoprecipitate the chromatin (fig. 6C, PPRE and supplemental figure S1B). Moreover, when using an anti-acetylated histone H4 antibody, the E-cadherin promoter could be amplified in valproic acid and pioglitazone plus valproic acid treated cells indicating that, in these conditions, the E-cadherin promoter was activated (fig. 6C, PPRE and supplemental figure S1B). Binding of PPARγ and HDAC3 was specific to the PPARγ binding site of the E-cadherin promoter, since no amplification of a promoter region located outside of the PPRE was observed (fig. 6C, non PPRE). However, when chromatin was immunoprecipitated using an anti-acetylated histone H4, we observed amplification of the region devoid of the PPRE after treatments of the cells with valproic acid and pioglitazone plus valproic acid, and to a much lesser extent with pioglitazone, suggesting that this region is also transcriptionally active (fig. 6C, non PPRE). To further prove the direct association of HDAC3 with PPARγ on the E-cadherin promoter, we performed Re-ChIP experiments. After a first chromatin immunoprecipitation using an anti-PPARγ antibody, we performed a second immunoprecipitation using an anti-HDAC3 antibody or non-specific IgGs. As observed for ChIP experiments, the same fragment of the human E-cadherin promoter was amplified by PCR when anti-HDAC3 was used to immunoprecipitate chromatin from vehicle, pioglitazone or valproic acid treated cells (fig. 6D and supplemental figure S1C), demonstrating that HDAC3 forms a complex with PPARγ in PC3 cells on the E-cadherin promoter even in the presence of valproic acid. No association of PPARγ and HDAC3 to the E-cadherin promoter was observed when chromatin from cells treated with a combination of pioglitazone and valproic acid was used (fig. 6D and supplemental figure S1C).

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Finally, we asked whether other known PPARγ target genes, such as aP2 responded similar to E-cadherin to the treatments. In contrast to what observed for the E-cadherin gene, aP2 mRNA expression was induced more that 100-fold in PC3 cells treated with pioglitazone compared to cells treated with vehicle (fig. 6E). Surprisingly, only minor effects on aP2 mRNA expression were observed upon treatment with valproic acid (fig. 6E). Furthermore, combination treatment of pioglitazone and valproic acid induced aP2 mRNA expression at similar levels as observed for pioglitazone treatment alone (fig. 6E). These results suggested that PPARγ differentially regulated transcription in the context of the E-cadherin or the aP2 genes. Transient transfection assays in COS and PC3 (data not shown) cells using the aP2 luciferase-based promoter construct were consistent with this hypothesis. As observed for the aP2 mRNA expression, pioglitazone induced the aP2 promoter activity, whereas no effect on luciferase activity was observed upon valproic acid treatment (fig. 6F). Moreover, no additive effect of the association of pioglitazone and valproic acid on luciferase activity was observed for this promoter (fig. 6F). To elucidate the molecular mechanism underlying the observed effects, Re-ChIP experiments were performed on the aP2 gene as described above. A 567 bp fragment of the human aP2 promoter containing the PPRE was amplified by PCR when anti-HDAC3 was used to re-immunoprecipitate PPARγ-immunoprecipitated chromatin from vehicle-treated PC3 cells (fig. 6G and supplemental figure S1D). In contrast to E-cadherin gene promoter, HDAC3 was not present on the aP2 promoter of PC3 cells treated with pioglitazone, valproic acid or both suggesting that HDAC3 is not associated with PPARγ in these conditions on the aP2 promoter (fig. 6G and supplemental figure S1D). Altogether, our data suggest that the E-cadherin and aP2 genes are differentially transcriptionally regulated by PPARγ. Regulation of E-cadherin expression by PPARγ requires inhibition of HDACs regardless of the presence of PPARγ ligands, whereas in the context of the aP2 promoter, PPARγ ligands are sufficient to induce expression.

HDAC3 mediates repressive effects on PPARγ -mediated E-cadherin promoter activity.

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We observed by ChIP experiments that HDAC3 is recruited on the E-cadherin promoter upon pioglitazone or valproic acid treatment. To further prove that HDAC3 mediates repressive effects on the E-cadherin promoter, we first evaluated the effect of the transient over-expression of HDAC3 on PPARγ-mediated E-cadherin promoter activity. COS cells were transiently co-transfected with the PPARγ expression vector, the full-length E-cadherin promoter driving the expression of the luciferase gene and increasing amount of the HDAC3 expression vector (fig. 7A). The combination of pioglitazone and valproic acid induced the E-cadherin promoter activity in the absence of HDAC3 (fig. 5E and 7A). Interestingly, when increasing amount of HDAC3 were co-transfected with PPARγ, a strong decrease in the E-cadherin promoter activity was obtained, suggesting a repressive role for HDAC3 on the E-cadherin gene (fig. 7A). To specifically evaluate the consequence of loss of HDAC3 expression, siRNA experiments were performed. Transfection of validated HDAC3 siRNA in PC3 resulted in a 80% reduction in endogenous HDAC3 mRNA and protein levels, as demonstrated by QPCR and immunoblotting (fig. 7B and C, respectively). siRNA-mediated HDAC3 knock-down increased endogenous E-cadherin mRNA expression in PC3 treated with pioglitazone (2-fold induction, fig. 7D), whereas no effect of pioglitazone was observed with the control siRNA (fig. 7D). These results suggest that HDAC3 represses PPARγ transcriptional activity on the E-cadherin gene in PC3 cells upon pioglitazone treatment.

E-cadherin expression is decreased whereas PPARγ expression and deacetylated histone H4 are increased in human prostate cancer.

One important requirement in order to insure a successful therapy using a combination of PPARγ agonists and HDAC inhibitors is that PPARγ is expressed in prostate cancer, and that histones are deacetylated. Consistent with previous studies (32) we found by IHC studies that PPARγ was mainly not expressed in normal prostate (fig. 8A). PPARγ expression was absent in 42.9% and 65.5% of normal prostate and benign prostate hyperplasia,respectively and 42.9% of normal prostate expressed low levels of PPARγ (fig. 8A and table 1). However a strong expression was

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found in prostate cancer with 100% of prostate cancers expressing PPARγ at different levels (fig. 8A and table 1). Furthermore, a gradual increase in PPARγ staining was observed from differentiated (gleason < 7, 62.5% of cancer expressed PPARγ) to undifferentiated adenocarcinomas (gleason ≥ 7, more than 80% of cancer are positive for PPARγ protein) (table 1). In contrast to PPARγ expression, acetylation status of histone H4 were found to be inversely correlated with the aggressiveness of prostate cancer. In the normal prostate, histone H4 was often acetylated (fig. 8B and table 2). 81% of normal prostate biopsies were positively stained (table 2, score 1+2) whereas acetylated histone H4 was mostly not detected in aggressive prostate cancer (table 2, score 0+1, 87.5%, 60% and 78.6% of prostate cancer tissues with gleason < 7, = 7 and > 7 have negative or weak acetylated H4, respectively), indicating high histone deacetylase activity in prostate cancer. Furthermore, we correlated the expression of PPARγ with acetylated histone H4 in each individual tumor with different Gleason scores (table 3). Interestingly we found that tissues that were positively stained for both PPARγ and acetylated H4 were tumor prostate with Gleason ≤ 7 (table 3). In these tissues, we also observed positive staining for PPARγ and negative staining for acetylated H4 (25% of cancer with Gleason < 7 and 50% with Gleason = 7 are PPARγ + and AcH4 -, respectively ; table 3). Aggressive prostate cancers were mostly positive for PPARγ and negative for acetylated H4 (64.3 % of cancer with Gleason > 7 are PPARγ + and AcH4 -, table 3). Most of the normal prostate tissues were negatively and positively stained for PPARγ and acetylated H4, respectively (47.6 % of normal prostate are PPARγ - and AcH4 +, table 3). These data demonstrate that PPARγ expression and acetylation status of histone H4 are often inversely correlated in aggressive prostate cancer. Importantly, these results support the use of HDAC inhibitors and PPARγ agonists in the treatment of prostate cancer. Finally, consistent with previous results (19, 29), we showed that E-cadherin expression is lost in most of prostate adenocarcinomas samples (fig. 8C), suggesting that the association of PPARγ agonists and HDAC inhibitors might be of interest to reinduce E-cadherin expression and subsequently inhibits invasion.

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Discussion

PPARγ is over-expressed in prostate cancer (15). Whereas the physiological function of PPARγ in normal epithelial cells is largely unknown, PPARγ activation inhibits the proliferation of malignant cells from prostate carcinoma (4, 21, 25, 34), among others. These observations suggest that induction of differentiation by activation of PPARγ may represent a promising novel therapeutic approach for cancer, as already demonstrated for liposarcoma (6) and in xenograft models of prostate (21). In addition, treatment of patients with advanced prostate cancer with the PPARγ agonist troglitazone, resulted in a high incidence stabilization of prostate-specific antigen levels (25). These studies were however limited to a reduced number of patients. A larger prospective, randomized, placebo controlled clinical trial analyzed the effects of rosiglitazone on the PSA doubling time in patients with biochemical disease progression after radical prostatectomy and/or radiation therapy. In this study, no effects of rosiglitazone were observed in disease progression in these patients (35). Despite technical caveats in the interpretation of PSA doubling time measurements, this study showed that PPARγ ligands are not efficient in this subset of patients. One interesting hypothesis is that PPARγ could be insensitive to ligand activation in prostate cancer because its activity is repressed by the action of upstream events. This was demonstrated in a study showing that sustained activation of PPARγ by the new PPARγ activator R-etodolac required the presence of HER2 inhibitors, suggesting that the HER2 pathway, likely through MAPK phosphorylation of PPARγ, abrogated the effects of PPARγ activity through degradation of this nuclear receptor (14). In this scenario, PPARγ ligands cannot activate PPARγ as a result of its degradation. We show in our study that, similar to what is observed for the HER2-PPARγ axis, inhibition of HDAC activity is required to achieve maximal PPARγ activation in prostate cancer cells. We have previously shown that PPARγ is part of a HDAC3-containing repressor complex in the presence of PPARγ ligands, and we characterized a PPARγ-HDAC3 direct interaction (8). We believe that in prostate cancer cells HDACs are fully active (fig. 8), and therefore PPARγ activity is repressed in these cells even in the presence of

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ligands. HDAC inhibition has been shown to result in decreased proliferation of several cancer cells (22). We found that histone H4 acetylation levels were decreased in prostate cancer tumors, although the precise correlation between histone acetylation level and tumor stage is more complex (33).

We found that PPARγ might control tumor growth at two different levels. First, this nuclear receptor might exert anti-proliferative effects through regulation of the expression of cell cycle regulators. This is consistent with previous studies showing decreased expression of cyclin D1 upon PPARγ agonists treatment in cancer cell lines (38). Second, we found that the combination treatment abrogated the invasive potential of prostate cancer cells. It is known that E-cadherin is one of the major factors that inhibit metastasis and invasion of prostate cancer cells through maintenance of the adherens junctions important for epithelial cell-cell adhesion, and inhibition of epithelial-to-mesenchymal transition (EMT), which is a required event in cancer progression. Downregulation of E-cadherin expression contributes to certain aspects of oncogenesis (5), and it has been observed in 50% of prostate cancers (24, 36, 37). We consistently found increased expression of E-cadherin in PC3 cells treated with the combination therapy. Furthermore, we show that E-cadherin is a bona fide PPARγ target gene. In contrast to classical PPARγ target genes, regulation of E-cadherin expression in response to PPARγ ligands both at the promoter and RNA levels requires, however, the presence of HDAC inhibitors to fully achieve maximal stimulation. This is consistent with our hypothesis that PPARγ is not permissive for activation by ligands when complexed with HDACs. This is demonstrated by our ChIP experiments, which show that despite PPARγ being bound to the promoter of the E-cadherin gene in the presence of ligand, the promoter is not active, as shown by transient expression experiments and E-cadherin mRNA quantification. The lack of activity is most likely the result of the presence of HDAC3 in this PPARγ complex on the PPAR binding site of the E-cadherin promoter, a phenomenon that we cannot explain and are currently investigating. However, in the presence of HDAC inhibitors and PPARγ ligands HDAC3 is absent from the PPARγ complex in the E-cadherin gene promoter, and consequently the promoter is active, as suggested by the activity of the E-cadherin promoter.

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The finding that E-cadherin expression responds to PPARγ agonists only in the presence of HDAC inhibitors defines a new class of PPARγ target genes.

In support of this we show that classical PPARγ target genes, such as aP2 responded to PPARγ with a 6-fold activation in the absence of HDAC inhibitors (fig. 6). This suggests that the sensitivity of PPARγ repression to HDACs is different depending on the context of the promoter of the PPARγ-target gene. We can conclude from our results that a combination therapy using PPARγ agonists and HDAC inhibitors might be considered for the treatment of prostate cancer.

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References

1. **Abella, A., P. Dubus, M. Malumbres, S. G. Rane, H. Kiyokawa, A. Sicard, F. Vignon, D. Langin, M. Barbacid, and L. Fajas.** 2005. Cdk4 promotes adipogenesis through PPARgamma activation. Cell Metab **2:**239-49.

2. **Annicotte, J. S., C. Chavey, N. Servant, J. Teyssier, A. Bardin, A. Licznar, E. Badia, P. Pujol, F. Vignon, T. Maudelonde, G. Lazennec, V. Cavailles, and L. Fajas.** 2005. The nuclear receptor liver receptor homolog-1 is an estrogen receptor target gene. Oncogene **24:**8167-75.

3. **Annicotte, J. S., E. Fayard, G. H. Swift, L. Selander, H. Edlund, T. Tanaka, T. Kodama, K. Schoonjans, and J. Auwerx.** 2003. Pancreatic-duodenal homeobox 1

Hal author manuscript inserm-00096196, version 1

regulates expression of liver receptor homolog 1 during pancreas development. Mol Cell Biol **23:**6713-24.

4. **Butler, R., S. H. Mitchell, D. J. Tindall, and C. Y. Young.** 2000. Nonapoptotic cell death associated with S-phase arrest of prostate cancer cells via the peroxisome proliferator-activated receptor gamma ligand, 15-deoxy-delta12,14-prostaglandin J2. Cell Growth Differ **11:**49-61.

5. **Cano, A., M. A. Perez-Moreno, I. Rodrigo, A. Locascio, M. J. Blanco, M. G. del Barrio, F. Portillo, and M. A. Nieto.** 2000. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol **2:**76-83.

6. **Demetri, G. D., C. D. Fletcher, E. Mueller, P. Sarraf, R. Naujoks, N. Campbell, B. M. Spiegelman, and S. Singer.** 1999. Induction of solid tumor differentiation by the peroxisome proliferator-activated receptor-gamma ligand troglitazone in patients with liposarcoma. Proc Natl Acad Sci U S A **96:**3951-6.

7. **Fajas, L., M. B. Debril, and J. Auwerx.** 2001. Peroxisome proliferator-activated receptor-gamma: from adipogenesis to carcinogenesis. J Mol Endocrinol **27:**1-9.

8. **Fajas, L., V. Egler, R. Reiter, J. Hansen, K. Kristiansen, S. Miard, and J. Auwerx.** 2002. The retinoblastoma-histone deacetylase 3 complex inhibits the peroxisome proliferator-activated receptor gamma and adipocyte differentiation. Developmental Cell **3:**903-910.

9. **Fajas, L., J. C. Fruchart, and J. Auwerx.** 1998. Transcriptional control of adipogenesis. Current Opinions in Cell Biology **10:**165-173.

10. **Fajas, L., R. L. Landsberg, Y. Huss-Garcia, C. Sardet, J. A. Lees, and J. Auwerx.** 2002. E2Fs regulate adipogenesis. Developmental Cell **3:**39-49.

11. **Feldman, B. J., and D. Feldman.** 2001. The development of androgen-independent prostate cancer. Nat Rev Cancer **1:**34-45.

Hal author manuscript inserm-00096196, version 1

12. **Glondu, M., E. Liaudet-Coopman, D. Derocq, N. Platet, H. Rochefort, and M. Garcia.** 2002. Down-regulation of cathepsin-D expression by antisense gene transfer inhibits tumor growth and experimental lung metastasis of human breast cancer cells. Oncogene **21:**5127-34.

13. **Han, S., N. Sidell, P. B. Fisher, and J. Roman.** 2004. Up-regulation of p21 gene expression by peroxisome proliferator-activated receptor gamma in human lung carcinoma cells. Clin Cancer Res **10:**1911-9.

14. **Hedvat, M., A. Jain, D. A. Carson, L. M. Leoni, G. Huang, S. Holden, D. Lu, M. Corr, W. Fox, and D. B. Agus.** 2004. Inhibition of HER-kinase activation prevents ERK-mediated degradation of PPARgamma. Cancer Cell **5:**565-74.

15. **Hisatake, J. I., T. Ikezoe, M. Carey, S. Holden, S. Tomoyasu, and H. P. Koeffler.** 2000. Down-Regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor gamma in human prostate cancer [In Process Citation]. Cancer Res **60:**5494-8.

16. **Hong, J., I. Samudio, S. Liu, M. Abdelrahim, and S. Safe.** 2004. Peroxisome proliferator-activated receptor gamma-dependent activation of p21 in Panc-28 pancreatic cancer cells involves Sp1 and Sp4 proteins. Endocrinology **145:**5774-85.

17. **Hoosein, N. M., D. D. Boyd, W. J. Hollas, A. Mazar, J. Henkin, and L. W. Chung.** 1991. Involvement of urokinase and its receptor in the invasiveness of human prostatic carcinoma cell lines. Cancer Commun **3:**255-64.

18. **Huggins, C.** 1967. Endocrine-induced regression of cancers. Science **156:**1050-4.

19. **Jaggi, M., S. L. Johansson, J. J. Baker, L. M. Smith, A. Galich, and K. C. Balaji.** 2005. Aberrant expression of E-cadherin and beta-catenin in human prostate cancer. Urol Oncol **23:**402-6.

20. **Keer, H. N., F. D. Gaylis, J. M. Kozlowski, H. C. Kwaan, K. D. Bauer, A. A. Sinha, and M. J. Wilson.** 1991. Heterogeneity in plasminogen activator (PA) levels in human

Hal author manuscript inserm-00096196, version 1

prostate cancer cell lines: increased PA activity correlates with biologically aggressive behavior. Prostate **18:**201-14.

21. **Kubota, T., K. Koshizuka, I. A. Williamson, H. Asou, J. W. Said, S. Holden, I. Miyoshi, and H. P. Koeffler.** 1998. Ligand for peroxisome proliferator activated receptor γ (troglitazone) has potent anti-tumor effects against human prostate cancer both in vitro and in vivo. Cancer Research **58:**3344-3352.

22. **Marks, P., R. A. Rifkind, V. M. Richon, R. Breslow, T. Miller, and W. K. Kelly.** 2001. Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer **1:**194-202.

23. **Metivier, R., G. Penot, M. R. Hubner, G. Reid, H. Brand, M. Kos, and F. Gannon.** 2003. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell **115:**751-63.

24. **Morton, R. A., C. M. Ewing, A. Nagafuchi, S. Tsukita, and W. B. Isaacs.** 1993. Reduction of E-cadherin levels and deletion of the alpha-catenin gene in human prostate cancer cells. Cancer Res **53:**3585-90.

25. **Mueller, E., M. Smith, P. Sarraf, T. Kroll, A. Aiyer, D. S. Kaufman, W. Oh, G. Demetri, W. D. Figg, X. P. Zhou, C. Eng, B. M. Spiegelman, and P. W. Kantoff.** 2000. Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. Proc Natl Acad Sci U S A **97:**10990-5.

26. **Nelson, P. S., N. Clegg, H. Arnold, C. Ferguson, M. Bonham, J. White, L. Hood, and B. Lin.** 2002. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A **99:**11890-5.

27. **Pulukuri, S. M., C. S. Gondi, S. S. Lakka, A. Jutla, N. Estes, M. Gujrati, and J. S. Rao.** 2005. RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo. J Biol Chem **280:**36529-40.

Hal author manuscript inserm-00096196, version 1

28. **Radhakrishnan, S. K., and A. L. Gartel.** 2005. The PPAR-gamma agonist pioglitazone post-transcriptionally induces p21 in PC3 prostate cancer but not in other cell lines. Cell Cycle **4:**582-4.

29. **Rubin, M. A., N. R. Mucci, J. Figurski, A. Fecko, K. J. Pienta, and M. L. Day.** 2001. E-cadherin expression in prostate cancer: a broad survey using high-density tissue microarray technology. Hum Pathol **32:**690-7.

30. **Rubio, N., M. M. Villacampa, N. El Hilali, and J. Blanco.** 2000. Metastatic burden in nude mice organs measured using prostate tumor PC-3 cells expressing the luciferase gene as a quantifiable tumor cell marker. Prostate **44:**133-43.

31. **Sarruf, D. A., I. Iankova, A. Abella, S. Assou, S. Miard, and L. Fajas.** 2005. Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor gamma. Mol Cell Biol **25:**9985-95.

32. **Segawa, Y., R. Yoshimura, T. Hase, T. Nakatani, S. Wada, Y. Kawahito, T. Kishimoto, and H. Sano.** 2002. Expression of peroxisome proliferator-activated receptor (PPAR) in human prostate cancer. Prostate **51:**108-116.

33. **Seligson, D. B., S. Horvath, T. Shi, H. Yu, S. Tze, M. Grunstein, and S. K. Kurdistani.** 2005. Global histone modification patterns predict risk of prostate cancer recurrence. Nature **435:**1262-6.

34. **Shappell, S. B., R. A. Gupta, S. Manning, R. Whitehead, W. E. Boeglin, C. Schneider, T. Case, J. Price, G. S. Jack, T. M. Wheeler, R. J. Matusik, A. R. Brash, and R. N. Dubois.** 2001. 15S-Hydroxyeicosatetraenoic acid activates peroxisome proliferator- activated receptor gamma and inhibits proliferation in PC3 prostate carcinoma cells. Cancer Res **61:**497-503.

35. **Smith, M. R., J. Manola, D. S. Kaufman, D. George, W. K. Oh, E. Mueller, S. Slovin, B. Spiegelman, E. Small, and P. W. Kantoff.** 2004. Rosiglitazone versus placebo for men with prostate carcinoma and a rising serum prostate-specific antigen level after radical prostatectomy and/or radiation therapy. Cancer **101:**1569-74.

Hal author manuscript inserm-00096196, version 1

36. **Umbas, R., W. B. Isaacs, P. P. Bringuier, H. E. Schaafsma, H. F. Karthaus, G. O. Oosterhof, F. M. Debruyne, and J. A. Schalken.** 1994. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res **54:**3929-33.

37. **Umbas, R., J. A. Schalken, T. W. Aalders, B. S. Carter, H. F. Karthaus, H. E. Schaafsma, F. M. Debruyne, and W. B. Isaacs.** 1992. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res **52:**5104-9.

38. **Wang, C., M. Fu, M. D'Amico, C. Albanese, J. N. Zhou, M. Brownlee, M. P. Lisanti, V. K. Chatterjee, M. A. Lazar, and R. G. Pestell.** 2001. Inhibition of cellular proliferation through IkappaB kinase-independent and peroxisome proliferator-activated receptor gamma-dependent repression of cyclin D1. Mol Cell Biol **21:**3057-70.

Figure legends

Figure 1. Proliferation of prostate cancer cells in response to PPARγ agonists and HDAC inhibitor.

A, **B, C,** Quantification of BrdU incorporating LNCaP (**A**), DU145 (**B**) and PC3 (**C**) cells treated with vehicle, pioglitazone, rosiglitazone, valproic acid, or a combination of both PPARγ agonists and HDAC inhibitor. At least 500 cells were counted under the microscope. Asterisks indicate statistically significant results here and in subsequent figures (ANOVA; ns, not significant; *= $0.01 \leq p < 0.05$; **= $0.001 \leq p < 0.01$; ***= $p < 0.001$).

D, Flow cytometry analysis of PC3 cells in response to pioglitazone, valproic acid or both. Fraction of cells in the G0/G1, S, or G2/M phases of the cell cycle is indicated.

E, Quantification of apoptosis of PC3 cells in response to pioglitazone, valproic acid or both.

Figure 2. Analysis of cell cycle regulators in response to PPARγ agonist and HDAC inhibitor.

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A, Quantification of mRNA expression by Q-PCR of the indicated genes in PC3 cells in response to pioglitazone, valproic acid or both. Results were normalized for the expression of RS9 mRNA.

B, Immunoblotting of the indicated proteins in PC3 cells treated as indicated in **A**. The corresponding fold induction compared to non-treated cells is indicated below the image.

C, Chromatin immunoprecipitation assays (ChIP) showing binding of PPARγ and HDAC3 to the human p21 promoter in a region containing sp1 sites and the presence of acetylated histone H4 in this region. PC3 cells were treated as in **A**.

D, Quantification of pRb phosphorylation levels in PC3 cells following treatment as described in **A**. At least 500 cells were counted under fluorescence microscope for detection of phospho pRb after using an anti-phospho RB antibody.

E, Western blot analysis of PC3 whole cell extracts treated as described in **A**. The proteins detected with specific antibodies and the fold induction are indicated.

F, CDK4 activity in PC3 cells. SDS-PAGE autoradiography showing phosphorylated purified pRb by immunoprecipitated CDK4 from vehicle, pioglitazone, valproic acid and pioglitazone plus valproic acid-treated PC3 cells.

Figure 3. *In vivo* analysis of tumor development in nude mice in response to pioglitazone and valproic acid after PC3 cell-graft.

A, **B**, Volume (**A**) and weight (**B**) of luminescent PC3 tumors in nude mice treated for 4 weeks with vehicle, pioglitazone (Pio), valproic acid (Val) or both (Pio+Val) as described in the Materials and Methods section. The number of mice used and the median value in each group is indicated.

C, Micrography representative of PCNA staining (red arrow) by IHC of tumor sections in mice treated with vehicle, pioglitazone (Pio), valproic acid (Val) or both (Pio+Val).

D, Quantification of PCNA staining represented in **C**. Four fields per section were analyzed for PCNA staining indicative of cell proliferation. Sections of tumors of all mice were analyzed. At least 500 cells were counted per tumor.

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E, Micrography representative of p21 staining (red arrow) by IHC of tumor sections in mice treated with vehicle, pioglitazone (Pio), valproic acid (Val) or both (Pio+Val).

F, Quantification of p21 staining represented in **E** was obtained as described in **D**.

G, Micrography representative of E-cadherin staining by immunofluorescence (white arrow) of sections of tumors in mice treated with vehicle, pioglitazone (Pio), valproic acid (Val) or both (Pio + Val).

Figure 4. Analysis of invasive potential of prostate cancer cells both *in vitro* and *in vivo* in response to valproic acid and pioglitazone treatments.

A, Invasive capacity of LNCaP and PC3 cells in Matrigel-coated membrane in response to pioglitazone, valproic acid, or both as indicated. % invasion represents the proportion of plated cells that migrated through the membrane.

B, Representative X-ray analysis and scores of the PC3 engrafted-tibiae of SCID mice after 21 days of treatment with vehicle, pioglitazone, valproic acid or a combination of pioglitazone and valproic acid. Xenografted tibiae were scored from 0 to 4 depending on the invasion degree : no invasion, score = 0; weak and localized sign of invasion (star), score = 1; regular features of invasion, score = 2 (arrowhead); strong marks of bone destruction, score = 3; complete bone destruction (inside the white dotted line), score = 4. Location of femur and tibiae bone structure is indicated.

C, Qualitative *in vivo* invasion analysis of X-ray. X-ray radiographs were blindly scored for bone invasion potential and results are presented as relative percentage of scores > 3.

D, Haematoxylin/eosin staining of intra-tibial tumors demonstrating invasion of tumor cells from mice treated with vehicle in the join (arrowhead) and in the skeletal muscle (arrows), whereas PC3 tumors from pioglitazone plus valproic acid-treated mice remained in the bone cavity (asterisk).

Figure 5. E-cadherin expression, *in vitro* binding by PPARγ/RXRα and transactivation assays in response to pioglitazone and/or valproic acid treatments.

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A, Quantification of mRNA expression by QPCR of the E-cadherin gene in LNCaP and PC3 cells in response to pioglitazone, valproic acid, or both. Results were normalized for the expression of RS9 mRNA.

B, Semi-quantitative RT-PCR imaging showing expression of the E-cadherin mRNA in PC3 cells in response to pioglitazone, valproic acid, or both.

C, Western blot analysis of PC3 whole cell extracts treated as described in **A**. The proteins detected with specific antibodies and the fold induction are indicated.

D, Computational analysis of the regulatory region of the human E-cadherin gene demonstrating the presence of a potential PPAR response element (PPRE). Comparison of this PPRE with the PPRE of classical PPARγ target genes is illustrated.

E, *In vitro* binding of the PPARγ/RXRα heterodimer to the E-cadherin promoter. EMSA analysis of the radiolabeled PPRE of the E-cadherin promoter incubated with unprogrammed reticulocyte lysate (lane 1), *in vitro* translated RXRα (lane 2), PPARγ (lane 3), or both (lane 4 to 11). Double-stranded cold oligonucleotides, representing either the E-cadherin PPRE ($PPRE_{E\text{-}cad}$), the consensus PPRE ($PPRE_{cons}$) or the mutated E-cadherin PPRE ($PPRE_{mut}$), were included in the competition assays (lanes 5 to 8). Incubation of an anti-PPARγ antibody resulted in a supershifted band (lane 10, black arrowhead) whereas no modification in PPARγ/RXRα binding was observed with IgG (lane 9). No binding was observed when a radiolabeled mutated E-cadherin PPRE ($PPRE_{mut}$) was used as a probe (lane 11). ns, non specific binding; fp, free probe.

F, Pioglitazone and valproic acid treatments modulate the E-cadherin promoter activity. Relative luciferase activity as determined after co-transfection of COS cells with the PPARγ expression vector and the empty, the E-cadherin promoter or the E-cadherin promoter deletion mutant reporter constructs. Cells were treated as indicated.

Figure 6. Differential HDAC3 recruitment and *in vivo* binding of PPARγ to the E-cadherin and aP2 promoters in response to pioglitazone and/or valproic acid treatments.

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A, Western blot showing PPARγ and HDAC3 expression in PC3 cells treated with pioglitazone, valproic acid or both. Fold induction is indicated.

B, Immunoprecipitation assays showing interaction between PPARγ and HDAC3. Extracts from PC3 cells treated with vehicle, pioglitazone, valproic acid or both were immunoprecipitated with IgG or anti-HDAC3 or directly analyzed for the presence of PPARγ (Input). Western blot analysis revealed the presence of PPARγ in HDAC3 immunoprecipitates.

C, ChIP demonstrating binding of PPARγ and HDAC3 to the E-cadherin promoter. Cross-linked chromatin from PC3 cells treated with vehicle, pioglitazone, valproic acid or both was incubated with antibodies against PPARγ, HDAC3, acetylated H4 or IgG. Immunoprecipitates were analyzed by PCR using specific primers for the PPRE present in the E-cadherin promoter (PPRE) or primers amplifying a region outside the PPRE (non PPRE). As a control, a sample representing 10% of the total chromatin was included in the PCR (Input).

D, Re-ChIP assays demonstrating interaction between HDAC3 and PPARγ on the E-cadherin promoter. Chromatin prepared from PC3 cells treated with vehicle, pioglitazone, valproic acid or both was subjected to the ChIP procedure with the antibody against PPARγ and re-immunoprecipitated using IgG or anti-HDAC3 antibody. Immunoprecipitates were analyzed as described in **C**.

E, Quantification of mRNA expression by Q-PCR of the aP2 gene in PC3 cells in response to pioglitazone, valproic acid, or both. Results were normalized for the expression of RS9 mRNA.

F, Activity generated by the aP2-Luc reporter cotransfected with the PPARγ expression vector. Experiments were performed either without stimulation (vehicle) or in the presence of pioglitazone, valproic acid or both.

G, Re-ChIP assays demonstrating interaction between HDAC3 and PPARγ on the aP2 promoter. Chromatin was prepared and subjected to the Re-ChIP procedure as described in **D**. Immunoprecipitates were analyzed using primers specific for the aP2 promoter.

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Figure 7. Effects of HDAC3 over-expression on the E-cadherin promoter and HDAC3 knockdown on E-cadherin mRNA in response to pioglitazone.

A, Activity generated by the E-cadherin-Luc reporter cotransfected with the PPARγ expression vector and increasing amount of the HDAC3 expression vector. Experiments were performed either without stimulation (vehicle) or in the presence of pioglitazone, valproic acid or both.

B, **C**, Q-PCR (**B**) and western blot (**C**) analysis showing knockdown expression of HDAC3 expression in PC3 cells transfected with a control or HDAC3 siRNA.

D, Quantitative real-time PCR showing E-cadherin gene expression in control versus HDAC3 knockdown in PC3 cells treated as indicated.

Figure 8. Analysis of PPARγ, E-cadherin expression and histone H4 acetylation in human normal and neoplastic prostate.

A, Micrography representative of human PPARγ staining (red arrow) by IHC of sections of normal prostate, prostatic intra-epithelial neoplasia (PIN) and prostatic adenocarcinoma. A weak to no staining (black arrow) was observed in normal prostatic gland.

B, Micrography representative of acetylated histone H4 staining (red arrow) by IHC of sections of normal prostatic gland and prostatic adenocarcinoma. No immunostaining (black arrow) was observed in prostatic adenocarcinoma.

C, Micrography representative of human E-cadherin staining by IHC of Tissue MicroArray (TMA) sections of normal prostate and prostatic adenocarcinoma obtained after radical prostatectomy. A strong staining was observed in normal prostate (red arrow), whereas no staining (black arrow) was observed in adenocarcinoma.

Table 1, PPARγ expression in normal, benign prostate hypertrophy (BPH) and prostate cancer.

Table 2, Acetylation status of histone H4 in normal, benign prostate hypertrophy (BPH) and prostate cancer.

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Table 3, Correlation between PPARγ expression and acetylated H4 in normal, benign prostate hypertrophy (BPH) and prostate cancer.

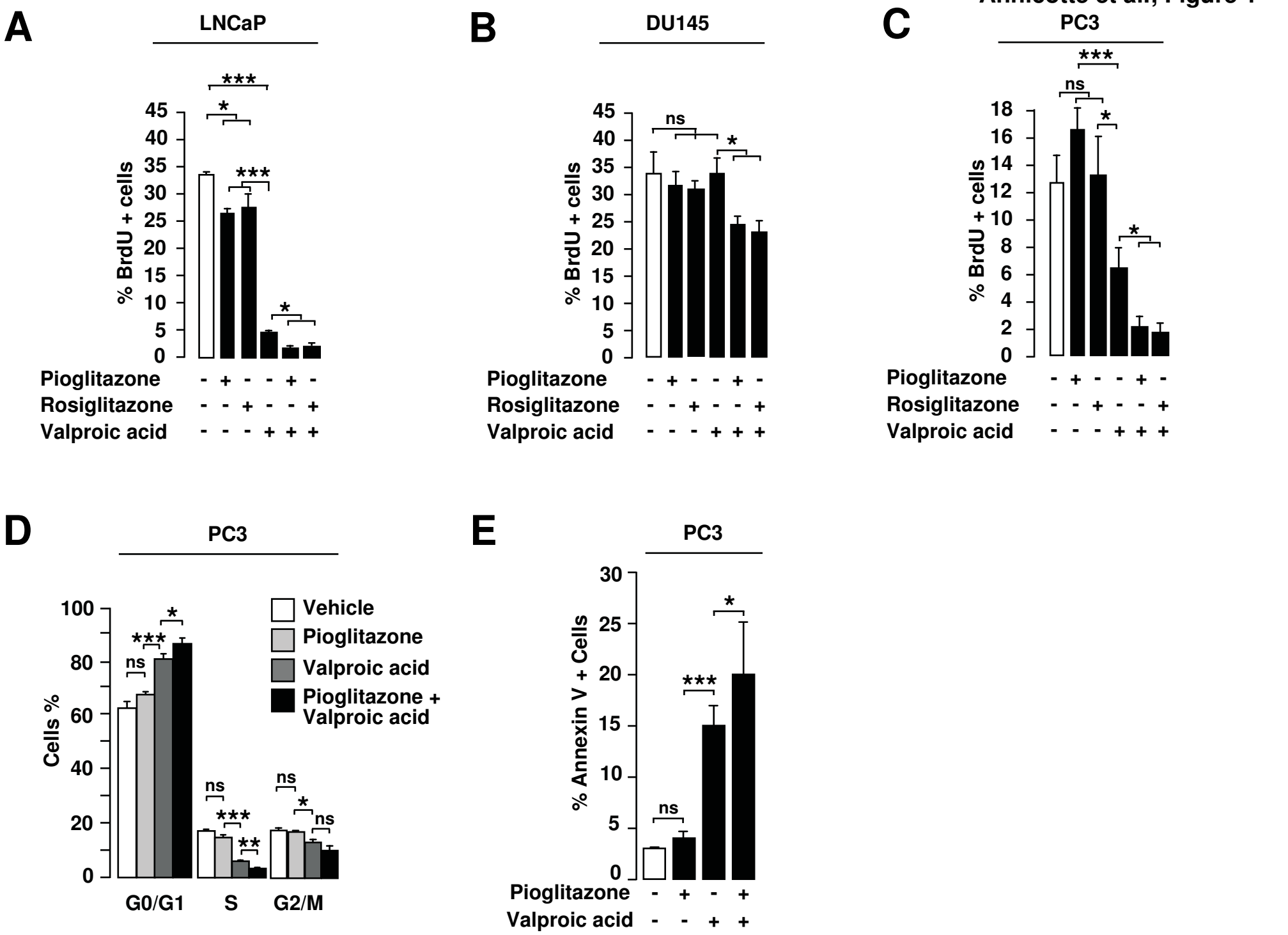
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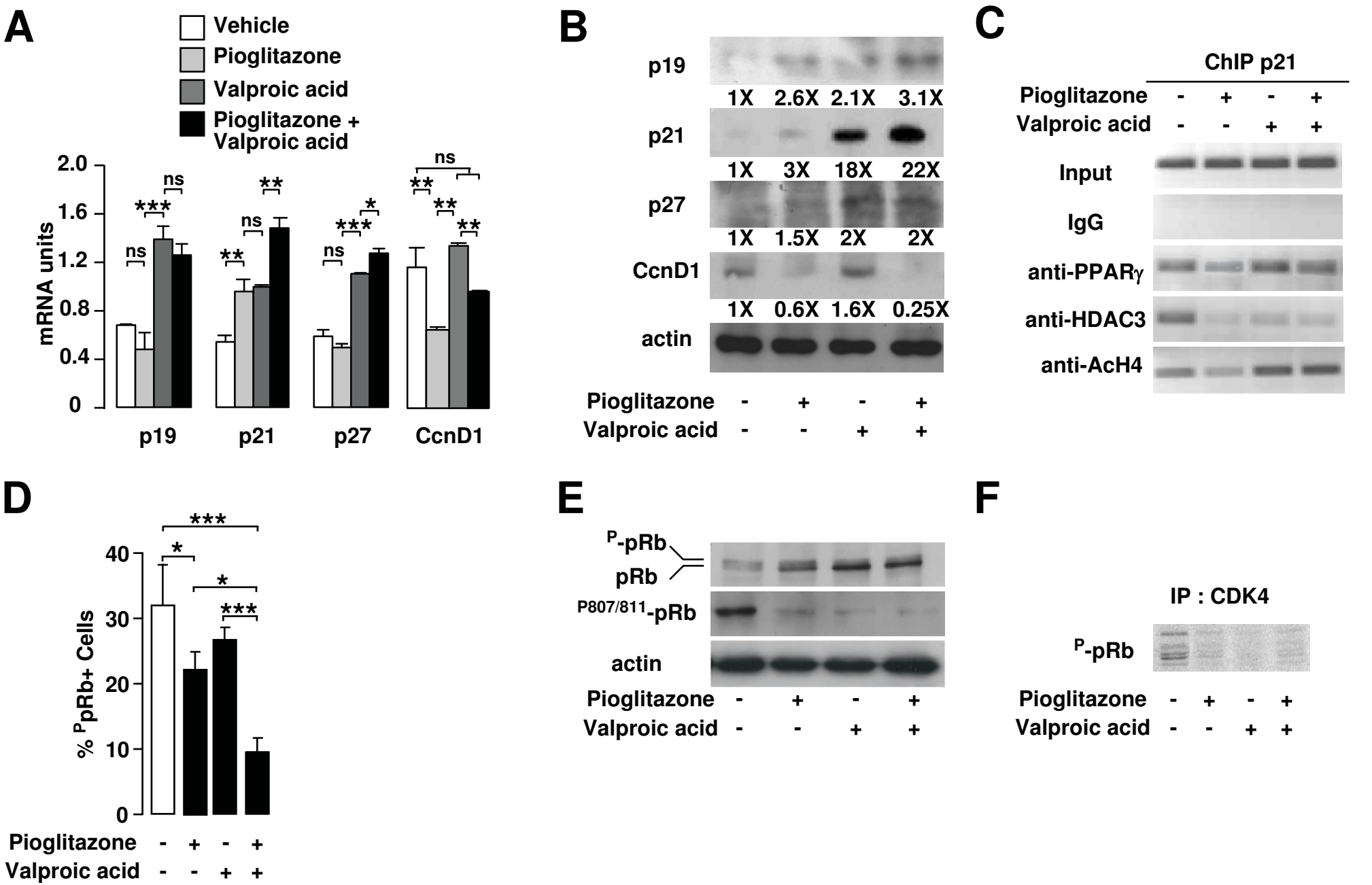
Figure S1. Quantification of ChIP analysis

A, **B**, Quantification of PPARγ and HDAC3 occupancy on and H4 acetylation status of the p21 (**A**) and E-cadherin (**B**) promoter in PC3 cells treated by vehicle, pioglitazone, valproic acid and both after ChIP analysis.

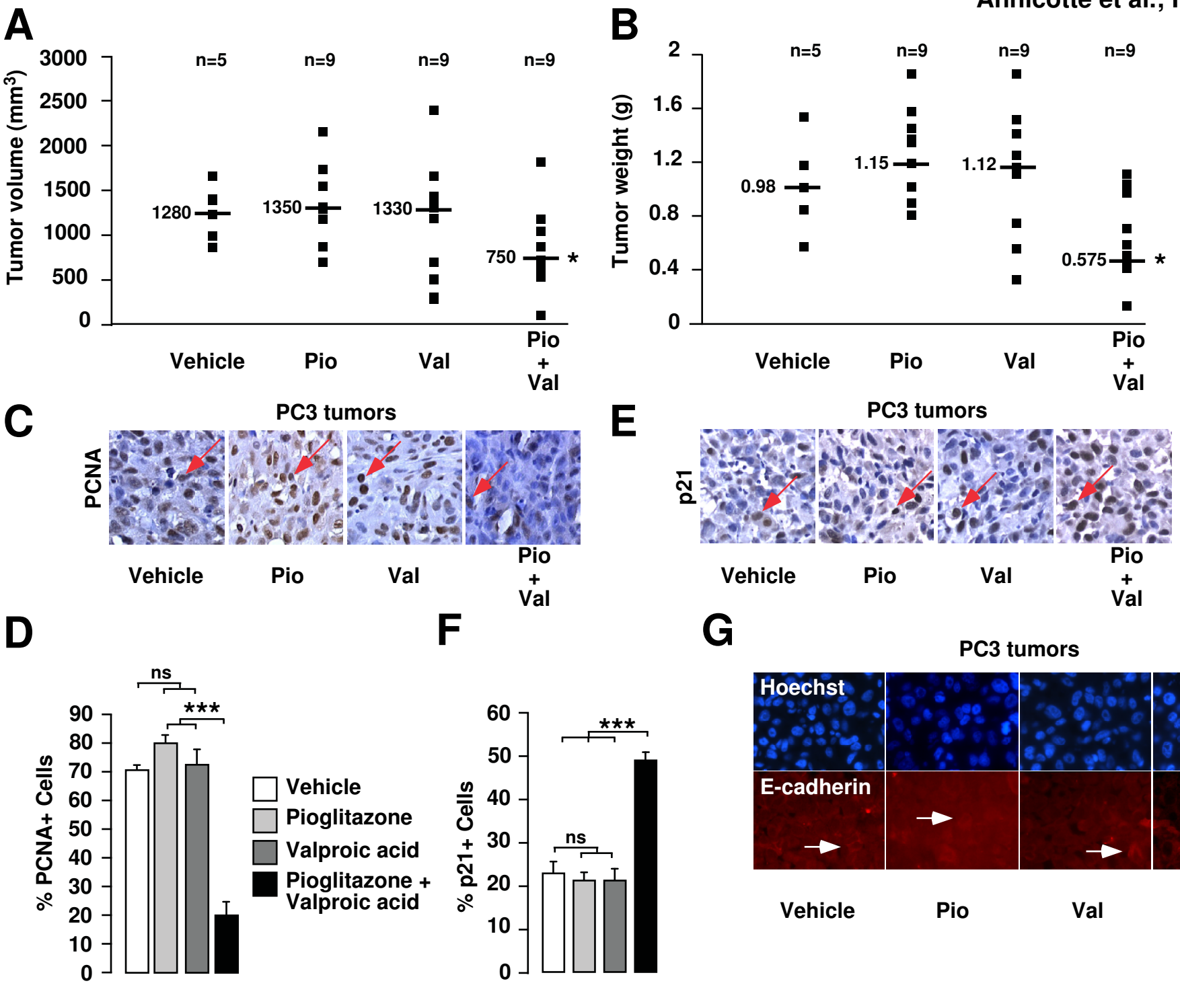
845 **C**, **D**, Quantification of HDAC3 occupancy on the E-cadherin (**C**) and aP2 (**D**) promoter in PC3 cells treated by vehicle, pioglitazone, valproic acid and both Re-ChIP analysis.

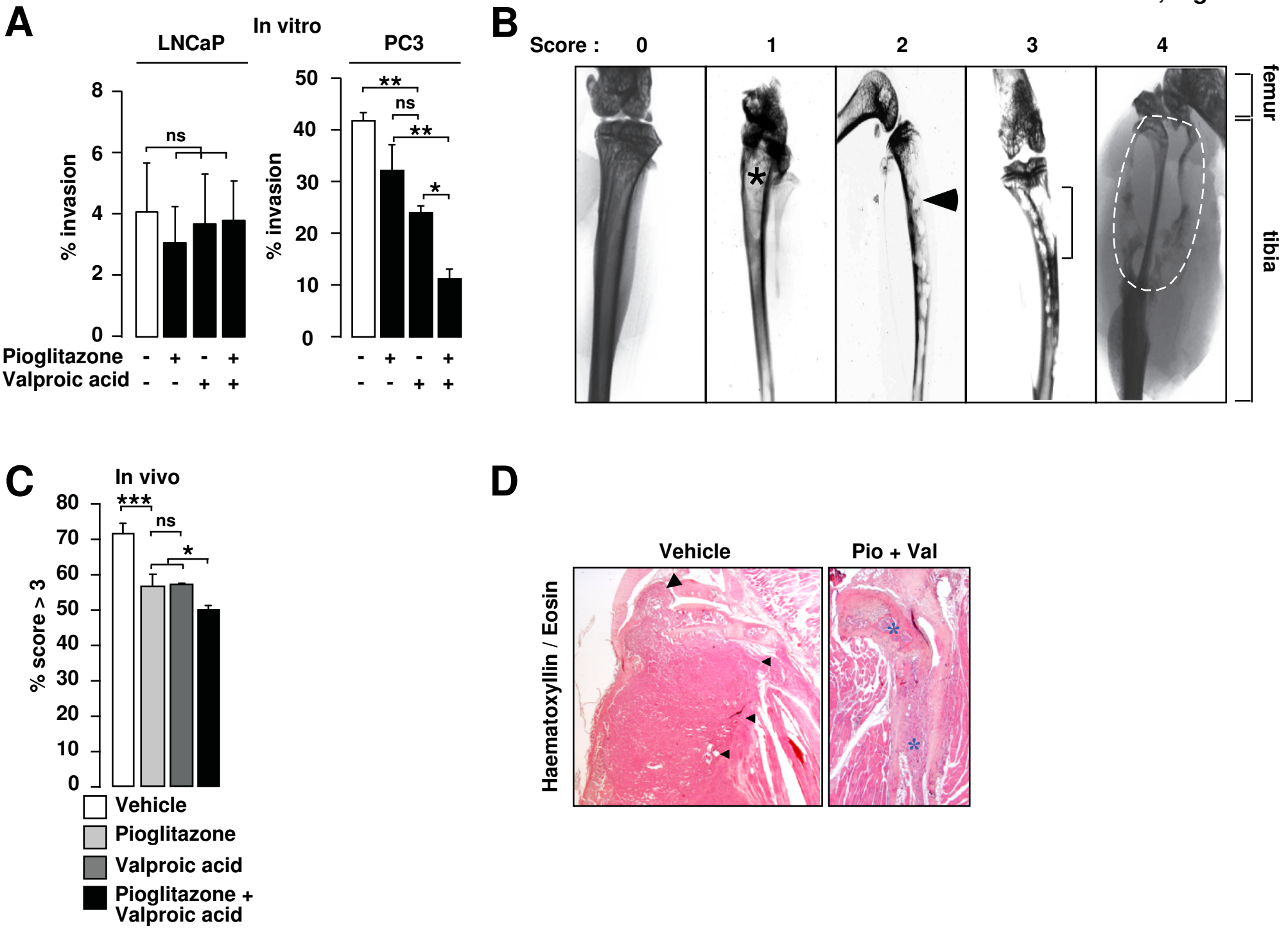
Annicotte et al.; Figure 1

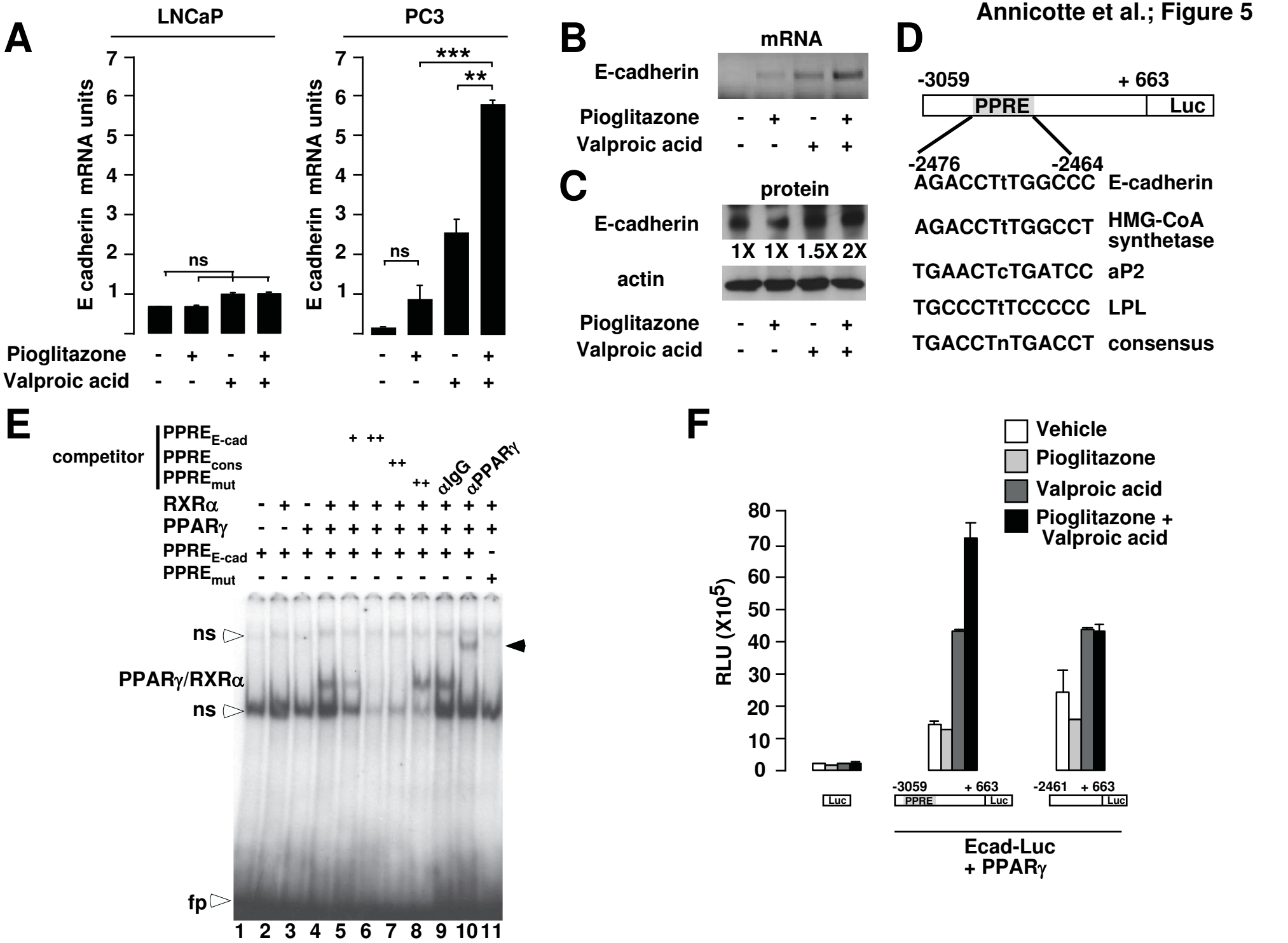


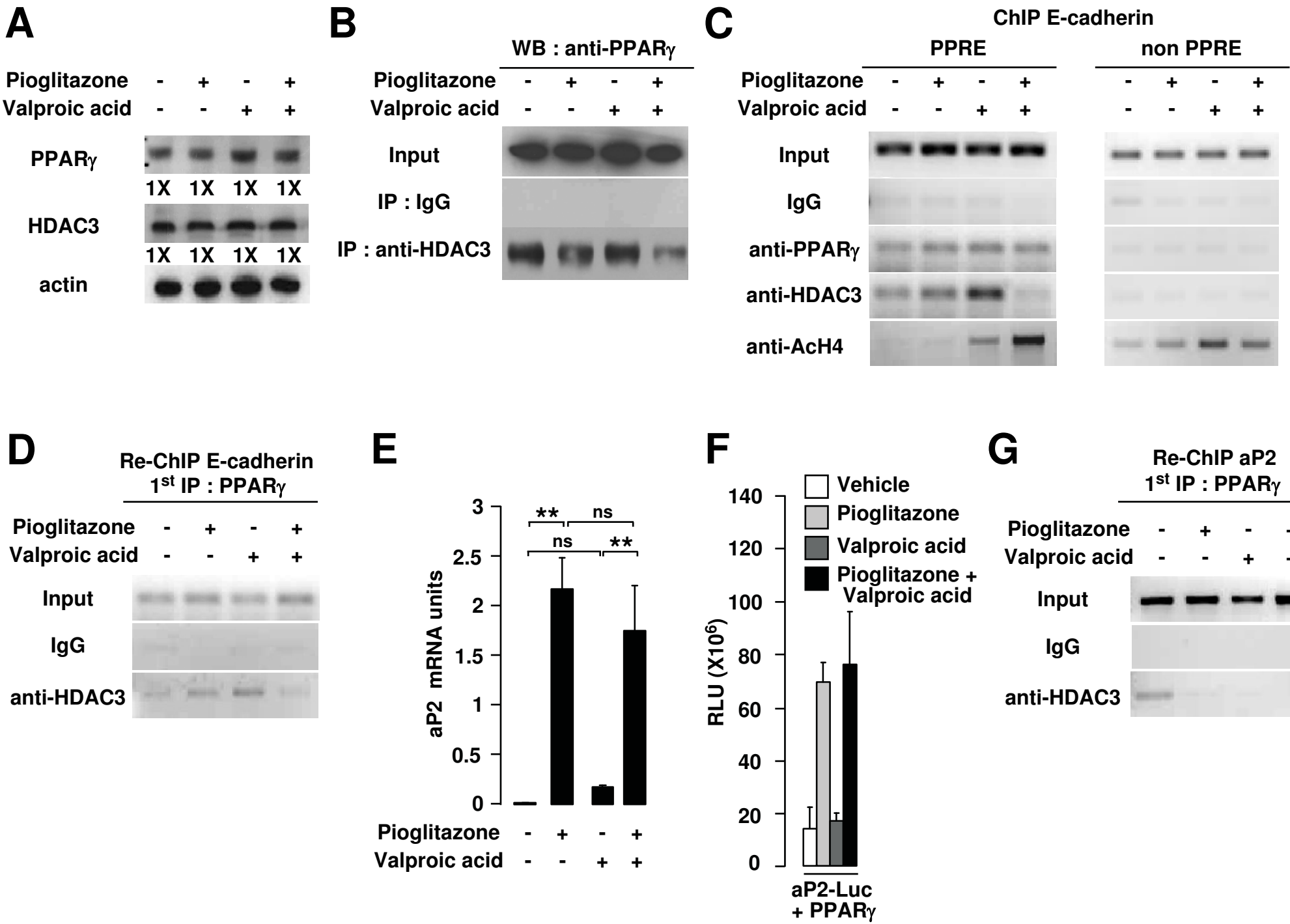

A
Vehicle
Pioglitazone
Valproic acid
Pioglitazone + Valproic acid
mRNA units
2.0
1.6
1.2
0.8
0.4
0
ns
***
**
*
p19
p21
p27
CcnD1
B
p19
1X 2.6X 2.1X 3.1X
p21
1X 3X 18X 22X
p27
1X 1.5X 2X 2X
CcnD1
1X 0.6X 1.6X 0.25X
actin
Pioglitazone - + - +
Valproic acid - - + +
C
ChIP p21
Pioglitazone - + - +
Valproic acid - - + +
Input
IgG
anti-PPARγ
anti-HDAC3
anti-AcH4
D
% PpRb+ Cells
40
30
20
10
0
***
*
Pioglitazone - + - +
Valproic acid - - + +
E
P-pRb
pRb
P807/811-pRb
actin
Pioglitazone - + - +
Valproic acid - - + +
F
IP : CDK4
P-pRb
Pioglitazone - + - +
Valproic acid - - + +

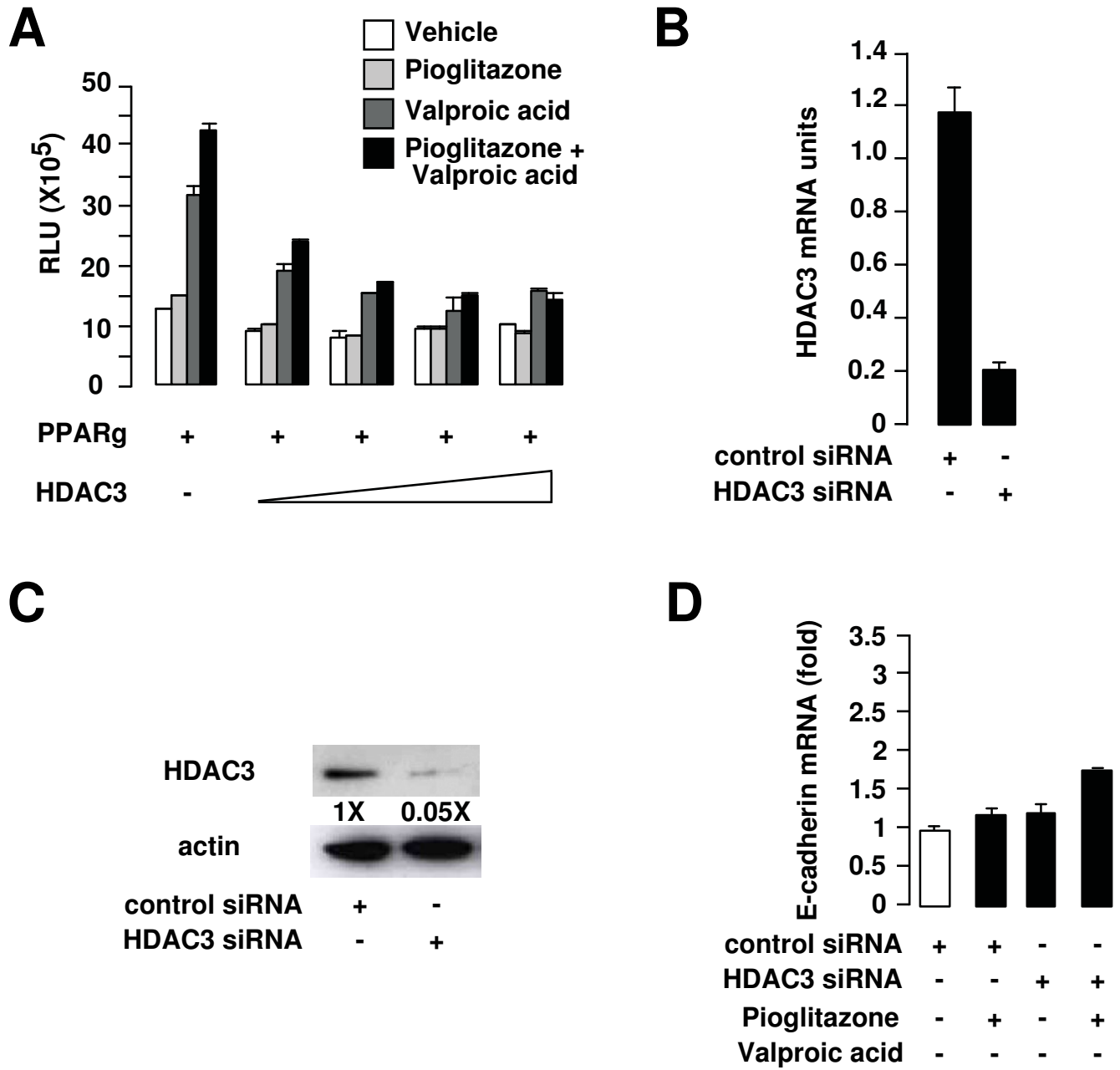
Annicotte et al.; Figure 3

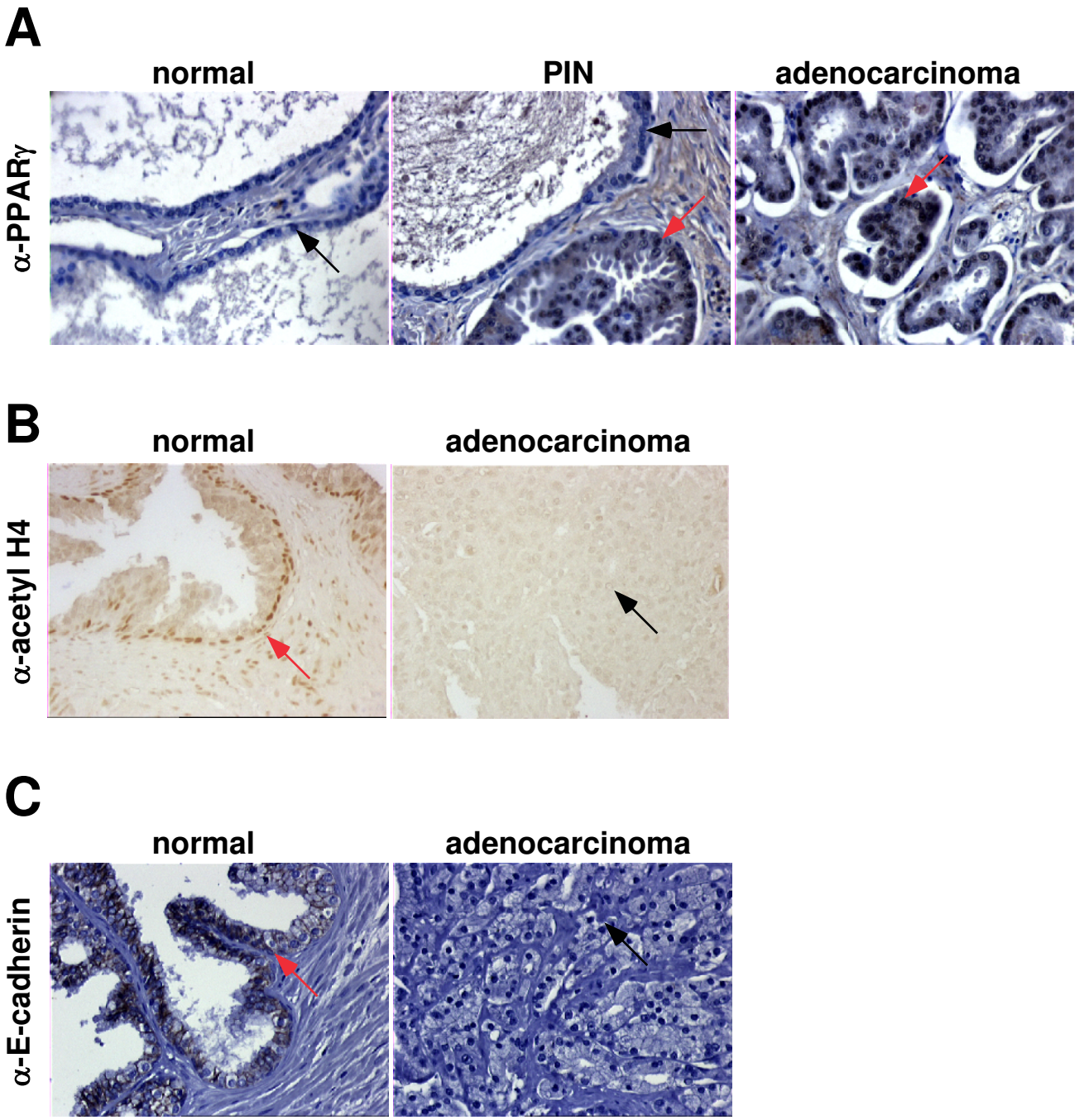



A
In vitro
LNCaP
PC3
% invasion
ns
**
ns
**
*
Pioglitazone
Valproic acid
B
Score :
0
1
2
3
4
femur
tibia
C
In vivo
% score > 3
***
ns
*
Vehicle
Pioglitazone
Valproic acid
Pioglitazone + Valproic acid
D
Vehicle
Pio + Val
Haematoxylin / Eosin


A
LNCaP
PC3
E cadherin mRNA units
ns
***
**
Pioglitazone
Valproic acid
B
mRNA
E-cadherin
Pioglitazone
Valproic acid
C
protein
E-cadherin
1X 1X 1.5X 2X
actin
Pioglitazone
Valproic acid
D
-3059
+ 663
PPRE
Luc
-2476
-2464
AGACCTtTGGCCC E-cadherin
AGACCTtTGGCCT HMG-CoA synthetase
TGAACTcTGATCC aP2
TGCCCTtTCCCCC LPL
TGACCTnTGACCT consensus
E
competitor
PPRE E-cad
PPRE cons
PPRE mut
αIgG
αPPARγ
RXRα
PPARγ
PPRE E-cad
PPRE mut
ns
PPARγ/RXRα
ns
fp
1 2 3 4 5 6 7 8 9 10 11
F
Vehicle
Pioglitazone
Valproic acid
Pioglitazone + Valproic acid
RLU (X10^5)
-3059
+ 663
-2461
+ 663
PPRE
Luc
Ecad-Luc
+ PPARγ


A
Pioglitazone - + - +
Valproic acid - - + +
PPARγ
1X 1X 1X 1X
HDAC3
1X 1X 1X 1X
actin
B
WB : anti-PPARγ
Pioglitazone - + - +
Valproic acid - - + +
Input
IP : IgG
IP : anti-HDAC3
C
ChIP E-cadherin
PPRE
non PPRE
Pioglitazone - + - + - + - +
Valproic acid - - + + - - + +
Input
IgG
anti-PPARγ
anti-HDAC3
anti-AcH4
D
Re-ChIP E-cadherin
1st IP : PPARγ
Pioglitazone - + - +
Valproic acid - - + +
Input
IgG
anti-HDAC3
E
aP2 mRNA units
3
2.5
2
1.5
1
0.5
0
** ns
ns **
Pioglitazone - + - +
Valproic acid - - + +
F
RLU (X10^6)
140
120
100
80
60
40
20
0
Vehicle
Pioglitazone
Valproic acid
Pioglitazone + Valproic acid
aP2-Luc + PPARγ
G
Re-ChIP aP2
1st IP : PPARγ
Pioglitazone - + - +
Valproic acid - - + +
Input
IgG
anti-HDAC3


A
Vehicle
Pioglitazone
Valproic acid
Pioglitazone + Valproic acid
RLU (X10^5)
50
40
30
20
10
0
PPARg + + + + +
HDAC3 -
B
HDAC3 mRNA units
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
control siRNA + -
HDAC3 siRNA - +
C
HDAC3
1X 0.05X
actin
control siRNA + -
HDAC3 siRNA - +
D
E-cadherin mRNA (fold)
3.5
3
2.5
2
1.5
1
0.5
0
control siRNA + + - -
HDAC3 siRNA - - + +
Pioglitazone - + - +
Valproic acid - - - -


A
normal
PIN
adenocarcinoma
α-PPARγ
B
normal
adenocarcinoma
α-acetyl H4
C
normal
adenocarcinoma
α-E-cadherin

Annicotte et al.; Table 1

Tissue Types (total samples)	PPARγ expression Scores (%) 0	1	2	3
normal (21)	9 (42.9)	9 (42.9)	3 (14.2)	0 (0)
BPH (8)	5 (65.5)	2 (25)	1 (12.5)	0 (0)
Gleason < 7 (8)	0 (0)	3 (37.5)	5 (62.5)	0 (0)
Gleason = 7 (10)	0 (0)	1 (10)	1 (10)	8 (80)
Gleason > 7 (14)	0 (0)	2 (14.3)	4 (28.6)	8 (57.1)

Annicotte et al.; Table 2

	H4 acetylation			
Tissue Types (total samples)	**Scores (%)**			
	0	**1**	**2**	**3**
normal (21)	**4 (19)**	**7 (33.3)**	**10 (47.7)**	**0 (0)**
BPH (8)	**3 (37.5)**	**2 (25)**	**3 (37.5)**	**0 (0)**
Gleason < 7 (8)	**1 (12.5)**	**6 (75)**	**1 (12.5)**	**0 (0)**
Gleason = 7 (10)	**3 (30)**	**3 (30)**	**4 (40)**	**0 (0)**
Gleason > 7 (14)	**6 (42.9)**	**5 (35.7)**	**3 (21.4)**	**0 (0)**

Annicotte et al.; Table 3

Tissue Types (total samples)	PPARg - AcH4 -	PPARg - AcH4 +	PPARg + AcH4 -	PPARg + AcH4 +
normal (21)	7 (33.3)	10 (47.6)	1 (4.8)	3 (14.3)
BPH (8)	3 (37.5)	2 (25)	1 (12.5)	2 (25)
Gleason < 7 (8)	2 (25)	0 (0)	2 (25)	4 (50)
Gleason = 7 (10)	0 (0)	0 (0)	5 (50)	5 (50)
Gleason > 7 (14)	0 (0)	1 (7.1)	9 (64.3)	4 (28.6)

Annicotte et al.; supplemental figure 1

