

# **PBX expression and regulation in human prostate**

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**Key Words:** prostate cancer; Pre-B-cell leukaemia transcription factor (PBX); androgen

## Abstract

Pre-B-cell leukaemia transcription factors (PBX1, PBX2, PBX3 and PBX4) are part of the TALE (three amino acid loop extension) homeobox gene family. They regulate gene expression programs during development and steroidogenesis. In this study, we evaluated PBX expression in prostate cancer cell lines and tissue sections from radical prostatectomy of prostate cancer patients. We detected PBX1, PBX2, PBX3 and PBX4 at mRNA level in all prostate cell lines tested by using Real-Time RT-PCR. Furthermore, isoforms of PBX1 and PBX3 were observed at the protein level using Western analysis. Androgen down-regulated mRNA levels of PBX1 and PBX4. Hormonal regulation of PBX1 mRNA was also seen in human prostate tissue samples. Although no effects of androgens were observed on PBX3 mRNA level, androgens were shown to decrease the protein level of PBX3 in LNCaP. Immunohistochemical staining of PBX3 showed increased expression of PBX3 in malignant versus benign prostate tissue. Malignant samples were clearly positive for nuclear and cytoplasmic PBX3 staining in luminal cells, whereas benign samples were negative. Distinct nuclear staining in basal cells, however, were observed in benign areas. Up-regulation of PBX3 mRNA was also observed in tumour samples from one of three prostate cancer patients compared to paired malignant biopsy. Our observations showed that members of the PBX family were regulated by androgens through different mechanisms and indicated that PBX3 may play a role in prostate cancer progression.

## Introduction

Transcription factors play a pivotal role in carcinogenesis and have been the focus of many studies. Their function as both activators and repressors of gene regulations during tumorigenesis make them central factors in this process. This also highlights their potential role as candidates for drug targets as well as prognostic and diagnostic markers.

Pre-B-cell leukaemia transcription factor 1 (PBX1) has been identified as a proto-oncogene in pre-B cell acute lymphoblastic leukaemia where it is expressed as a fusion protein with E2A after a chromosomal translocation (Kamps et al.), (Nourse et al.). PBX is part of the TALE (three amino acid loop extension) homeobox gene family and four members of the PBX family is known in human, PBX1, PBX2, PBX3 and PBX4 . Different isoforms generated by alternative splicing of PBX have so far been identified in PBX1 (PBX1a and 1b) and PBX3 (PBX3a, 3b, 3c and 3d) (Monica et al.), (Wagner et al.), (Milech, Kees, and Watt). They show cell-type specific expression pattern and different functional characteristics. PBX interacts with a subset of HOX transcription factors and they are partners of the PNOX (MEIS/PREP) family. More recent studies have shown that they also interact with non-homeodomain transcription factors including nuclear receptors (Laurent et al.).

Besides modifying the transcriptional activity of genes regulated by nuclear receptors (Laurent et al.), PBX promotes the steroid hormone signalling pathway by up-regulating the expression of CYP 17 required for androgen and cortisol biosynthesis. Furthermore, PBX regulates the expression of UGT2B17 which catalyses glucuronidation and thereby removal of steroids from the body (Gregory and Mackenzie). As regulation of steroidogenesis is one of the mechanism involved in development of castration-resistance prostate cancer, we wanted to study expression and regulation of members of the PBX family in prostate cancer.

## **MATERIALS AND METHODS**

### **Cell culture**

The following human prostate cancer cell lines were used: LNCaP, PC3 and DU145 were purchased from ATCC (Rockville, MD, USA). The LNCaP-Rf cell line was kindly provided by Professor Donald Tindall at the Mayo Clinic (Rochester, MN, USA) (Zegarra-Moro et al.). The LNCaP-C4 and -C4-2B cell lines were kindly provided by professor Leland W.K. Chung at Winship Cancer Institute, Emory University (Atlanta, GA, USA) (Thalmann et al.).

LNCaP, PC3 and DU145 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FCS) from InVitrogen (Carlsbad, CA, USA). LNCaP-Rf cells were cultured in RPMI 1640 without phenol red from InVitrogen (Carlsbad, CA, USA) supplemented with 10% charcoal-stripped serum (CSS). CSS was prepared by charcoal treatment of FCS under agitation at 4°C over night, followed by centrifugation and sterile filtration. The LNCaP-C4 and LNCaP-C4-2B cells were cultured in T-medium (D-MEM:F-12 (1:1) + L-Glutamin+15mM HEPES (InVitrogen, Carlsbad, CA) supplemented with 5 µg/ml insulin, 0,0136 ng/ml T3, 5 µg/ml apo-transferin, 0,25 µg/ml biotin, 25 µg/ml adenine, 50 µg/ml streptomycin, 50 µg/ml penicillin and 5% FCS). All cell lines were cultured at 37°C in 5% CO<sub>2</sub>/humidified air. R1881/Methyltrienolone was purchased from Roussel-UCLAF (Romainville, France) whereas other compounds were from Sigma (St.Louis, MO, USA) unless otherwise specified.

### **Gene expression profiling using cDNA microarrays**

Cy3 and Cy5 labeled guanidine isothiocyanate/cesium chloride purified RNA (Chirgwin et al.) from three independent experiments were hybridized to 15K human cDNA microarray (I.M.A.G.E. clones; Norwegian Microarray Consortium). One dye swap experiment was performed with a set of samples. Labeling and hybridization protocols are available at

<http://www.mikromatrise.no/facility/uioid/127>. The images were processed using GenePix Pro4 or 6 and analyzed in BioArray Software Environment (BASE) version 1.2.15 (Saal et al.). The m-array data set and experiment details are available at ArrayExpress ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) accession number E-TABM-78.

### **Semi-Quantitative Real-Time RT-PCR**

Total RNA was isolated using Trizol™ from Invitrogen (Carlsbad, CA, USA). 100 ng of total RNA were included in a one-step RT-PCR reaction using QuantiTect SYBR Green RT-PCR kit from Qiagen (Hilden, Germany) that was performed using MJ Research DNA Engine Opticon Continuous Fluorescence Detection System from MJ Research Inc. (Waltham, MA, USA). RT-PCR cycle conditions were as follow; reverse transcription at 50°C for 30 min and inactivation step at 95°C for 15 min, followed by 40 PCR cycles (15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C), and a final extension at 72°C for 5 min followed by a melting curve analysis. G6PD primers were used for normalization. The  $\Delta C_t$  and  $\Delta\Delta C_t$  formulas were used as described in the protocol from Applied Biosystems (Foster City, CA, USA). The  $\Delta C_t$  formula was only used for data presented in the upper right corner of Figure 4C.. The following primers were used; *G6PD* (NM\_000402): tgcagtgagccagatagc and acagggaggagatgtggttg; *PBX1* (NM\_002585): cggagctggagaaatcag and aatctccttggggagatgg; *PBX2* (NM\_002586): agcaagacatcggggaca and gcagtttagggcgtgttct; *PBX3* (NM\_006195): gcaggaagcaggacatcg and tgacagtcagggcagatgtt; *PBX4* (NM\_025245): gcgaagaagccaaagaagag and ttgccaaccagtttagagacc.

### **Protein extraction and Western analysis**

Cells were harvested in PBS and lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS and Complete protease inhibitor Tablets from Roche

(Mannheim, Germany) ) followed by 30 min rotation at 4°C. The lysate was, centrifuged at 10 000 xg for 30 min and the amount of protein in the supernatant were estimated by Bradford protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). Protein extracts (50 µg/lane) and MagicMark (InVitrogen Carlsbad, CA, USA) were separated on NuPAGE 10% or 4-12% gradient Bis-Tris gels from InVitrogen (Carlsbad, CA, USA) and transferred to PVDF membranes purchased from Millipore Corporation (Billerica, MA, USA) by electro blotting. Membranes were blocked in 5% (w/v) skimmed milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 2 hour at room temperature (RT), and then incubated over night at 4°C with the appropriate primary antibody diluted in TBST. Primary antibodies used were mouse monoclonal anti-PBX 1/2/3/4 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal anti-PBX 3 from Abnova corporation (Heidelberg, Germany), and mouse monoclonal anti- $\alpha$ -Tubulin from Sigma (St.Louis, MO, USA). Membranes were washed 3 x 10 min with TBST and further incubated for 1 hour with anti mouse or anti rabbit IgG horse-radish peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch Ltd (Cambridgeshire, UK). After washing 3 x 10min, membranes were developed using Immobilon™ Western by Millipore (Billerica, MA, USA). Images were generated using a Hamamatsu Digital Camera from Syngene (Cambridge, UK), and quantified using the Multi Gauge software from FujiFilm (Bedford, UK).

### **Prostate tissue specimen**

Matched benign and malignant prostate tissues for mRNA analysis and immunohistochemical staining were derived from radical prostatectomy of 18 and 30 prostate cancer patients, respectively, treated at Oslo Urological University Clinic and obtained from “The Prostate Biobank – a resource for urological research in Norway”(10974). The tissue samples were treated with RNALater at 4°C over night according to the manufacturers protocol (Qiagen,

Hilden, Germany) and stored at  $-80^{\circ}\text{C}$ . The pathology of the samples was verified by an experienced pathologist of urology. Written consent was obtained from all patients and the project has been approved by Regional Committees for Medical and Health Research Ethics.

### **Immunohistochemistry**

Immunohistochemical staining of paraffin embedded tissue sections were performed according to the manufacturer's protocol using Ultravision One detection system from LabVision (Fremont, CA, USA) and IHC Select manual staining system from Chemicon (Temecula, CA, USA). Tissue microarray (TMA) slides were deparaffinised and antigen retrieved in citrate buffer (pH 6) for 30 min at  $100^{\circ}\text{C}$  in the PT module from Lab-Vision (Suffolk, UK). The TMA slides were incubated with anti human PBX3 antibody (dilution 1:50). Non-immune IgG from Vector Laboratories Inc. (Burlingame, CA, USA) was used as negative control. Slides were treated for 70 seconds in hematoxylin. Manual scoring of the staining was done by an experienced pathologist who also verified all pathological gradings. The tissue microarray of specimens from radical prostatectomies contained benign and malignant tissue from 30 patients. Images were captured using Leica DM RA2 microscope and Leica DC 300F camera.

### **Oncomine**

Oncomine is a publicly available database containing microarray results collected from over 350 data sets (Rhodes et al.). The Oncomine database ([www.oncomine.org](http://www.oncomine.org)) was used to search for studies where PBX 1, 2, 3 and 4 expression was detected in prostate cancer. Each PBX variant was queried independently and studies that compared benign and malignant prostate tissue were selected. The normalized expression unit values from each study were used to generate new box plots. All the Oncomine searches were done in August 2009.

## Results

### Expression of PBX mRNAs in prostate cancer cell lines

We have previously reported that approximately 400 genes were differentially regulated in LNCaP cells cultivated for 4 days in 10% charcoal-treated FCS (CSS) compared to LNCaP cells cultivated in 10% foetal calf serum (FCS). One of the genes regulated was PBX1. The result from the micro-array data is shown in Figure 1A, as well as verification of the up-regulation of PBX1 using Real-Time RT-PCR. Four PBX genes (PBX1, PBX2, PBX3 and PBX4) have been identified in the human genome and the regulation of the three other PBX genes were also analysed by Real-Time RT-PCR (Fig1B). Of these genes, PBX4 was the most responsive gene up-regulated at mRNA level in LNCaP cells cultured in CSS for 4 days compared to FCS. PBX2 mRNA level seemed to be slightly up-regulated, whereas the mRNA level of PBX3 was not significantly regulated relative to LNCaP cells cultured in FCS.

As the various PBX variants were shown to be differentially regulated, we analyzed the mRNA expression of the PBX family members in androgen-sensitive and –insensitive prostate cancer cell lines (Fig. 2). All four genes were expressed in the prostate cancer cell lines tested (LNCaP, LNCaP-Rf, LNCaP-C4, LNCaP-C4-2B, PC-3 and DU145). The transcripts were detected at the following Ct values using RNA extracts from LNCaP-cells: PBX1 Ct ~ 31; PBX2 Ct ~ 21; PBX3 Ct ~ 24; PBX4 Ct ~ 25. The expression levels of all PBX transcripts analyzed were higher in LNCaP-Rf cells compared to the other cell lines tested. In case of the other prostate cancer cell lines, the pattern of expression of PBX1 and PBX4 mRNAs seemed to be more similar but different from the pattern of PBX2 and PBX3 transcripts. Whereas the level of PBX2 and PBX3 mRNAs were somewhat higher in DU145 cells than in LNCaP cells, the level of expression of PBX1 and PBX4 mRNAs seemed to be lower in this cell line.

### **Androgen regulation of PBX mRNAs in LNCaP cells**

Charcoal-treatment of serum is used to mimic androgen-depletion or hormonal treatment. Thus the data presented in Figure 1 and 2 indicate that the expression of PBX might be regulated by androgens. In order to test this hypothesis, LNCaP cells were stimulated with 0,1nM R1881 for 4 days (Fig. 3). Androgen stimulation reduced the PBX4 mRNA level relative to LNCaP grown in CSS. Similar the level of PBX1 mRNA was down-regulated although the quantitative effect varied from experiment to experiment. The level of PBX2 and PBX3 mRNAs were not affected by R1881 stimulation of hormone sensitive LNCaP cells.

### **Differential Expression of PBX mRNA in benign versus malignant prostate tissue**

To relate the results from the cell lines to clinically relevant observations, we used the Oncomine database, a publicly available cancer profiling database, to identify studies that contain information about PBX expression levels in prostate cancer specimens. Of the 16 datasets containing information about PBX1 mRNA expression, 14 studies showed reduced levels of PBX1 mRNA in malignant compared to benign prostate tissue ( $p < 0,05$ ), one showed increased levels of PBX1 and one indicated no significant difference (Fig. 4A). Two out of 12 studies showed increased expression of PBX2 and two out of 16 studies showed increased level of PBX3 mRNA in prostate cancer tissue (Data not shown). Only two datasets contained information on PBX4 mRNA expression, and they showed that PBX4 was down regulated in malignant compared to benign tissue.

In order to verify whether PBX is hormonally regulated in humans, we analyzed data from Holzbeierlein et al. (Holzbeierlein et al.) where they compared the gene expression profile in prostatectomy specimens from patients with previously untreated primary prostate cancer with tissue from patients undergone 3 months of androgen ablation therapy. Again,

PBX1 was found to be down-regulated in malignant tissue compared to benign (Fig. 4B). Interestingly, the expression of PBX1 mRNA was up-regulated in patients undergone androgen ablation therapy compared to previously untreated patients. There was no difference in the PBX3 level between the two groups (Data not shown). No data on PBX2 and PBX4 was reported in the study of Holzbeierlein (Holzbeierlein et al.). Interestingly, comparing normal and malignant tissue from the same patient revealed that PBX3 mRNA level was up-regulated in tumor specimens from one-third of the patients whereas it was down-regulated in the other group of patients. Similar results were obtained when we compared the mRNA expression of PBX3 in paired benign and malignant prostate cancer specimens of 18 patient undergone radical prostatectomy at Oslo Urological University Clinic (Fig. 4C). In 7 out of 18 cases, increased level of PBX3 mRNA was observed in tumor. Analyzing the data as groups, benign or malignant, revealed no significant difference in PBX3 mRNA expression (upper right corner of Fig. 4C).

#### **Characterization of PBX protein expression in LNCaP cells.**

To study whether regulation of PBX mRNAs was accompanied by regulation at the protein level, testing of PBX antibodies was performed. Currently, two isoforms of PBX1 (PBX1A and PBX1B) have been described as well as four isoforms of PBX3 (PBX3A, 3B, 3C, 3D). Both PBX1A and PBX3A have a theoretical molecular weight of 47 kDa. The calculated molecular weight for the other variants is: PBX1B (38 kDa), PBX2 (46 kDa), PBX 3B (39 kDa), PBX3C (27 kDa), PBX 3D (18 kDa) and PBX4 (40 kDa). Western analysis of extracts from LNCaP cells using an anti PBX1/2/3/4 antibody recognizing PBX1, PBX2, PBX3 and PBX4, detected multiple bands in the range between 38 KDa and 50 KDa (Fig. 5A, left panel) of which the ~38 kDa, ~40 kDa and ~47 kDa bands were dominant. The size of the upper band in the immunoblot corresponded with the theoretical molecular weights for PBX1A,

PBX2 and PBX3A. In order to identify the various bands, an anti PBX1 antibody as well as anti PBX2, anti PBX3 and anti PBX4 antibodies were tested. The anti PBX1 antibody detected a band corresponding to the theoretical size of PBX1B, but it was barely detectable indicating a low level of PBX1 expression also at protein level in LNCaP cells (data not shown). The anti PBX3 antibody detected three bands that correspond to the theoretical molecular weights of PBX3A, PBX3C and PBX3D (Fig. 5A right panel). The specificity of the antibody was verified by using a peptide representing the epitope of the antibody used. All three bands were blocked when the PBX3 antibody was incubated with the PBX3 competition peptide (data not shown). Using the anti PBX2 and anti PBX4 antibodies on Western, no bands of correct size were observed. By running PCR with primers detecting all PBX3 transcripts, we detected bands corresponding in size to PBX 3A, 3B, 3C and 3D using RNA extracts from LNCaP cells (data not shown).

Western analysis was performed with extracts from LNCaP cells incubated in FCS or CSS, respectively (Fig. 5A). Up-regulation of the ~47 kDa band was observed using the anti PBX1/2/3/4 antibody. Using the anti PBX3 antibody, a minor stimulatory effect was observed on the ~47kDa band. Densitometric scanning and semi quantitative analysis of the upper band detected by either antibodies indicated that PBX3A was slightly up-regulated at protein level in LNCaP cells cultivated in 10% CSS compared to 10% FCS (Fig. 5B). Alfa-tubulin is shown as loading control (Fig. 5A lower blot).

### **Expression of PBX protein levels in prostate cancer cell lines**

Next, we analyzed the expression of PBX protein levels in the different prostate cancer cell lines. Western analysis was performed using anti PBX1/2/3/4 and anti PBX3 antibodies. Although numerous bands were seen using the anti PBX1/2/3/4 antibody, the two dominating bands corresponds to the bands observed at ~47 kDa and ~40 kDa in Figure 5. As previously

observed, three bands were seen using anti PBX3 antibody of which the ~47 kDa band is shown in Fig. 6A and the densitometric scanning results of the same band presented in Fig. 6B. As observed at mRNA level, increased level of PBX3 was observed in LNCaP-Rf relative to LNCaP cells whereas no significant difference was detected in LNCaP-C4 and -C4-2 cells. PBX3A seemed to be down-regulated in PC-3 and DU145 cells. Alfa-tubulin is shown as loading control.

### **Androgen regulation of PBX3 protein levels in LNCaP**

To further investigate the regulation of PBX expression, we stimulated LNCaP cells with 0, 1 nM R1881 for 1 to 4 days (Fig. 7). Both the anti PBX1/2/3/4 and anti PBX3 antibodies showed decreased levels of the ~47 kDa band after R1881 stimulation (Fig.7A). Down-regulation of PBX3A was detected already after 24 hours of R1881 stimulation, and was further reduced after 4 days of stimulation. Similar regulation of PBX3A was observed when LNCaP cells were stimulated with dihydrotestosterone (data not shown).

### **PBX3 expression and localisation in prostate cancer specimens**

Tissue micro-array slides with benign and malignant tissue cores from patients undergone laparoscopic prostatectomy at Oslo Urological University Clinic (Norway) were analyzed for PBX3 expression. The tissue micro-array contained samples of benign prostate and primary prostate cancer with Gleason score 6-8. Immunohistochemical staining with PBX3 showed nuclear staining of basal cells in benign tissue and no detectable staining in luminal cells (Fig.8A). In malignant tissue, staining was observed both in cytoplasm and nucleus of secretory cells (Fig.8B) showing differential staining intensity in various specimens.(Fig. 8C). No correlation between staining intensity and Gleason score was observed in this study. The results from the immunohistochemical analysis with PBX3 are summarized in Table 1.

Eighteen of the 21 benign samples were negative for nuclear PBX3 staining in secretory cells whereas the remaining three were positive. Of the 30 malignant samples, 29 were positive for both nuclear and cytoplasmic staining with PBX3. In benign samples, two of 21 showed cytoplasmic staining of luminal cells and the remaining 19 were negative. A Pearson's chi-squared test was performed showing a significant difference ( $p < 0.001$ ) between the PBX3 expressions in benign compared to malignant prostate cancer tissue (Table 1).

## **Discussion**

The search for diagnostic and prognostic biomarkers and new drug targets is a multi step and laborious process. Numerous criteria need to be met for biomarkers and drug targets to be considered as potential candidates. Two of these criteria are the functional biological role of the protein in the disease and relevant clinical data linking the candidate to the presence of disease. The results presented in this report can be added to the present data on PBX as potential biomarkers or drug target candidates.

As the various PBX family members have both redundant and specific functions, isoform specific antibodies should be used to increase the knowledge about their role in regulation of cancer progression. Based on the results with PBX1, PBX3 and PBX1/2/3/4 specific antibodies, we were probably able to detect PBX1B and PBX3A, and most likely PBX3C and PBX3D in prostate cancer cell lines (Fig.5A), but these results need to be verified (Rhee et al.). It is unclear why PBX3B was not detected by the ABNOVA PBX3 antibody as we observed this isoform at the transcript level.

As reported in our study, there seem to be an increased expression of PBX3 in malignant versus benign prostate tissue at protein level. Previous work by Crijns and colleagues (Crijns et al.) reported increased PBX immunohistochemical staining in both cytoplasm and nuclei of ovarian tumor cells, when using a PBX1/2/3/4 antibody which does not differentiate between the different PBX members. Immunohistochemical staining of prostate specimens with anti PBX3 antibody showed nuclear staining of basal cells in benign tissue and no detectable staining in luminal cells. While in malignant tissue, staining was detected both in cytoplasm and nucleus in secretory luminal cells.

To strengthen the clinical relevance of PBX, we used the Oncomine database to analyze the PBX mRNAs profile in benign and malignant prostate tissue. We observed a trend towards decreased PBX1 mRNA level in malignant compared to benign tissue. This has also been reported by Crijns and co-workers (Crijns et al.) that used publicly available

Affymetrix datasets to analyze the expression of all four PBX mRNA in a large number of normal and tumor tissue of different origins, including prostate tissue. No significant difference was observed for PBX3 mRNA levels in the analyzed datasets. However, comparing malignant to benign prostate tissue from the same patient we observed either up- or down-regulation of PBX3 mRNA. Further investigation will show whether there is a correlation between clinical/pathologic characteristics and PBX3 expression in prostate cancer.

Promyelocytic leukaemia zinc finger (PLZF), a transcriptional regulator, has been suggested as an early androgen responsive gene with anti-proliferative activity in the prostate (Thirkettle et al.; Jiang and Wang). PLZF has been detected in androgen receptor positive LNCaP cells but not in androgen receptor negative PC3 and DU145 cells. Over-expression of androgen receptor in DU145 cells, however, induced PLZF whereas the level of PBX1 was reduced indicating that PBX1 is androgen regulated as shown in this study (Kikugawa et al.). A putative PLZF-binding site in the PBX1 promoter has been reported (Shiraishi et al.). A preliminary analysis using the TFSEARCH software by our group generated no putative PLZF-binding sites in the PBX3 promoter region. That might explain why we observed no regulation of PBX3 at mRNA level. Nevertheless, androgens regulated PBX3 at level of translation as has been observed in P19 embryonal carcinoma cells following stimulation with all-trans retinoic acid (Knoepfler et al.), (Qin et al.). Whereas PBX1 mRNA levels increased two fold 7 days after all-trans retinoic acid stimulation, the PBX3 mRNA level remained unchanged while the protein level of PBX3 increased.

Interestingly, the expression levels of all PBX family members were highest in LNCaP-Rf cells which are cultivated in growth medium with 10% CSS. The continuous growth in hormone depleted medium could explain the change in PBX expression assuming that PBX induce expression of steroidogenic enzymes as has been observed in granulosa cells

(Ota et al.). Hormonal regulation of PBX was also observed in patients that had undergone 3 months of androgen ablation therapy where an increase in PBX1 mRNA level was detected compared to untreated patients (Fig.4B). Androgens, in contrast to CSS, down-regulated the expression of PBX in prostate cancer cell lines.

In summary, the presented results showed that the PBX family was hormonally regulated in prostate cancer and that further research to determine the functional role of PBX in prostate cancer progression is needed.

## **Acknowledgments**

We thank the Department of Anatomy and Pathology at the Oslo University Hospital, Aker and “The Prostate Biobank – a resource for urological research in Norway” for clinical specimens. Furthermore, we thank Olov Øgren for skilled technical assistance, Morten Wang Fagerland for statistical analysis and Ph.D. Turid Eide for fruitful discussions. This work was supported by Health Region South East, Norwegian Cancer Society, University of Oslo and Oslo University Hospital, Aker.

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**Figure 1: Regulation of PBX mRNA in LNCaP cells.**

The levels of PBX1 mRNA were analyzed in LNCaP cells grown in 10% fetal calf serum (FCS) and 10% charcoal stripped serum (CSS) for 4 days. **A)** PBX1 mRNA expression was analyzed using micro-array analysis (m-array) and Real-Time RT-PCR. **B)** The mRNA levels of the four PBX members, PBX1, PBX2, PBX3 and PBX4, measured by Real-Time RT-PCR. All data are normalized against G6PD and shown relative to FCS and presented as mean  $\pm$  SD (n $\geq$ 3). \* One sample t-test, p<0,05.

**Figure 2: Expression of PBX at mRNA level in prostate cancer cell lines.**

The following cell lines were analyzed by Real-Time RT-PCR; LNCaP, LNCaP-Rf (Rf), LNCaP-C4 (C4), LNCaP-C4-2 (C4-2), PC-3 and DU145. Expression levels are shown relative to LNCaP cells. Data is normalized against G6PD and presented as mean $\pm$  SD (n=3).

**Figure 3: Androgen-dependent regulation of PBX mRNA in LNCaP.**

LNCaP cells were pre-treated for 3 days with medium containing 10% CSS. Then the cells were stimulated with 0,1 nM R1881 for 4 days. Total RNA from the samples were used to determine the expression levels of PBX by Real-Time RT-PCR. Data were normalized against G6PD and is presented as mean $\pm$  SD (n $\geq$ 3) relative to CSS.

**Figure 4: Expression of PBX1 mRNA in benign and malign human prostate tissue.**

The Oncomine database was queried for PBX mRNA expression. **A)** The box plot shows the differential expression of PBX1 mRNA between benign (white boxes) and malignant (black boxes) prostate tissue from the 16 studies identified in Oncomine; 1) Lapoint (Lapointe et al.); 2) Vanaja (Vanaja et al.); 3) Liu (Liu et al.); 4) Yu (Yu et al.); 5) Tomlins (Tomlins et al.); 6) Holzbeierlein (Holzbeierlein et al.); 7) Welsh (Welsh et al.); 8) Singh (Singh et al.); 9) Luo

(Luo et al.); 10) Dhanasekaran (Dhanasekaran et al.); 11) Dhanasekaran\_2 (Dhanasekaran et al.); 12) Varambally (Varambally et al.); 13) Nanni (Nanni et al.); 14) Luo\_2 (Luo et al.); 15) LaTulippe (LaTulippe et al.); 16) Magee (Magee et al.). **B**) Holzbeierlein (Holzbeierlein et al.) studied the mRNA expression profile in a group of patients (n=23) with untreated primary prostate cancer and in tissue from patients (n=17) undergone 3 months of androgen ablation therapy with goserelin and flutamide prior to operation. The expression profile of PBX1 mRNA between untreated (black box) compared to hormonal treated (grey box) prostate cancer is shown in the box plot. The white box represents benign samples (n=4). **C**) The level of PBX3 mRNA in prostate cancer specimens from 20 patients treated with radical prostatectomy at Oslo Urological University Clinic, was analyzed using Real-Time RT-PCR. The data was normalized against G6PD and is shown relative to the level of PBX3 expression in normal tissue from the same patient. In the upper right corner of the figure the relative PBX3 expression was calculated using the  $\Delta C_t$  formula. The means values for the benign and malign groups are shown.

**Figure 5: Regulation of PBX at protein level in LNCaP cells.**

The protein level of PBX was analyzed in LNCaP cells grown in 10% fetal calf serum (FCS) or 10% charcoal stripped serum (CSS) for 4 days. **A**) Representative Western blots using PBX1/2/3/4 (PBX) and PBX3 antibodies are shown. Alfa-tubulin antibody was used as loading control. **B**) Densometric scanning of the upper PBX band (~ 47 kDa)(PBX1/2/3/4 antibody) and of PBX3A (~ 47 kDa)(PBX3 antibody) are shown in the histograms. Data are shown relative to FCS and presented as mean  $\pm$  SD (n=3). \* One sample t-test, p<0,05.

**Figure 6: Protein expression of PBX3 in prostate cancer cell lines.**

The expression of PBX3 isoforms were analyzed by Western blot in the following prostate cancer cell lines, LNCaP, LNCaP-Rf (Rf), LNCaP-C4 (C4), LNCaP-C4-2 (C4-2), PC-3 and DU145 cells. **A)** A representative Western blot with the indicated prostate cancer cell lines probed with PBX1/2/3/4 (PBX), PBX3 (PBX3A band shown) and alfa-tubulin antibodies. **B)** Densitometric scanning of the 47kDa band detected by the PBX3 antibody (PBX3A) relative to LNCaP. Data is presented as mean $\pm$  SD (n=3).

**Figure 7: Androgen regulation of PBX3 in LNCaP cells.**

LNCaP cells were pre-treated for 3 days with medium containing 10% CSS followed by stimulation with 0,1 nM R1881 (androgen) for 1 - 4 days. **A)** Representative Western blots probed with either PBX1/2/3/4, PBX3 (PBX3A presented) or alfa-tubulin antibodies are shown. **B)** Densitometric scanning results of the PBX3A band relative to CSS are shown in the histogram. Data is presented as mean $\pm$  SD (n=3).

**Figure 8: Expression of PBX3 in human prostate cancer specimens.**

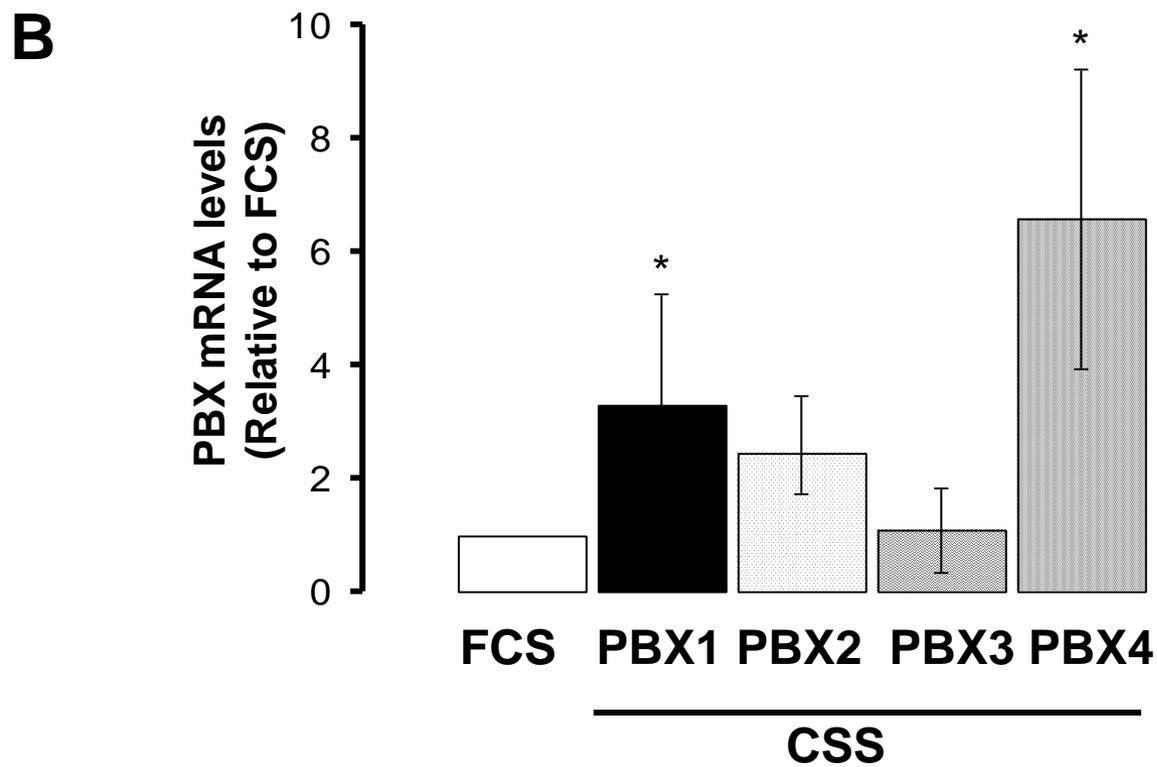
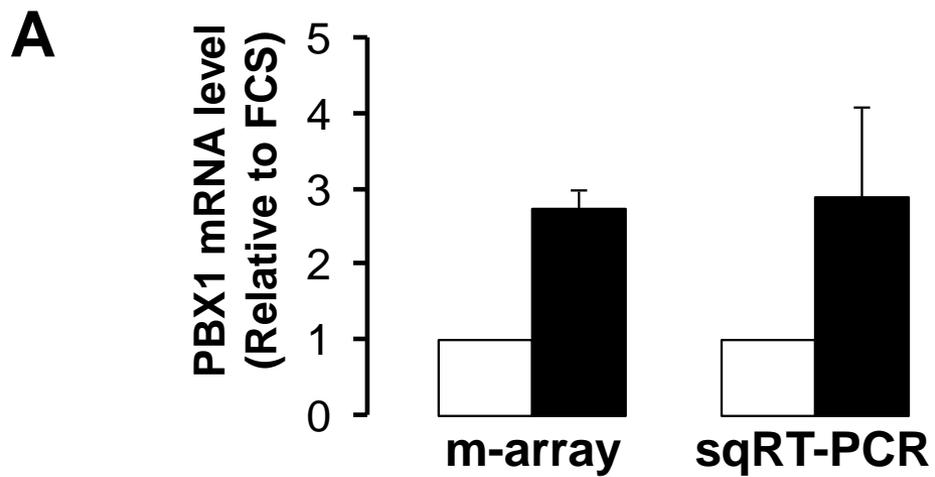
Representative images obtained after immunohistochemical staining of a tissue micro-array containing benign prostate hyperplasia (**A**), primary prostate cancer Gleason grade 3 (**B**) and Gleason grade 4 (**C**) using the anti PBX3 antibody.

**TABLE 1: Scoring of immunohistochemical staining of PBX3 in secretory cells of human prostate specimens.**

Nuclear immunoreactivity	Negative	Positive	Total
Benign	18	3	21
Malign	1	29	30
Cytoplasmic immunoreactivity	Negative	Positive	Total
Benign	19	2	21
Malign	1	29	30

Pearson's chi-squared test was performed showing a significant difference between benign and malign nuclear staining and between benign and malign cytoplasmic staining ( $p < 0.001$ ).

Fig. 1



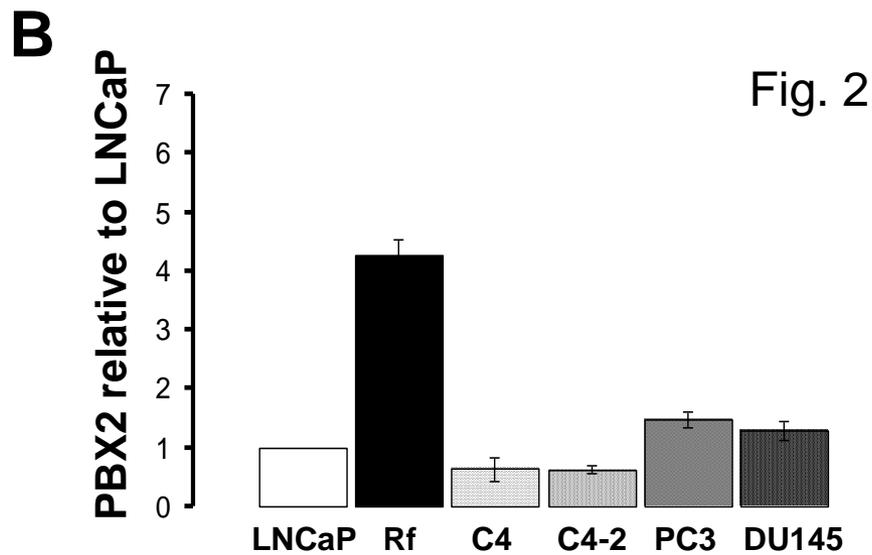
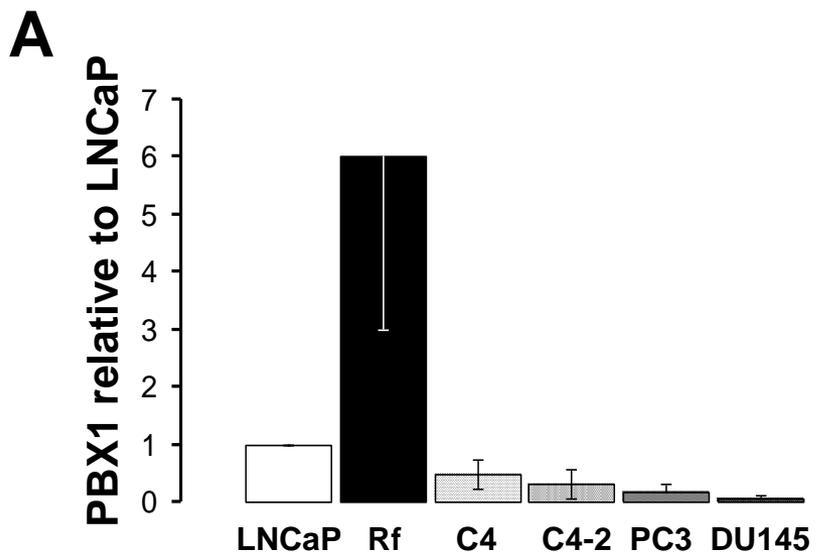


Fig. 2

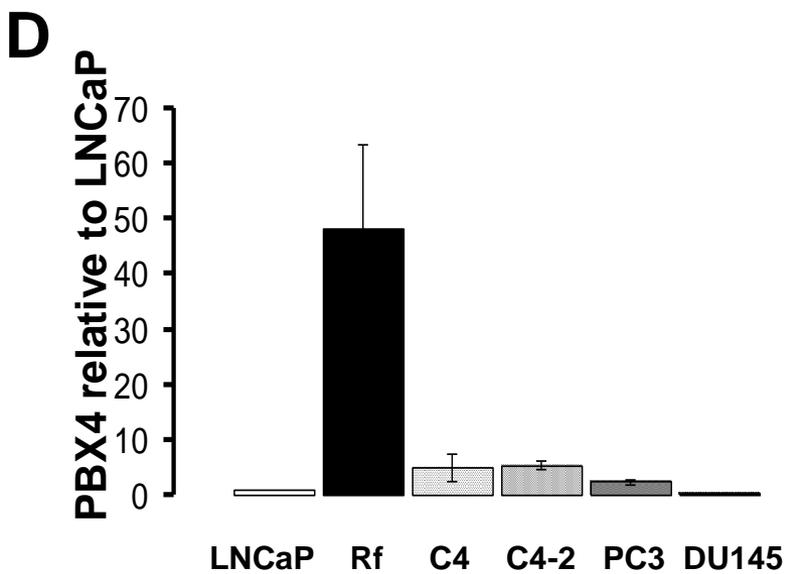
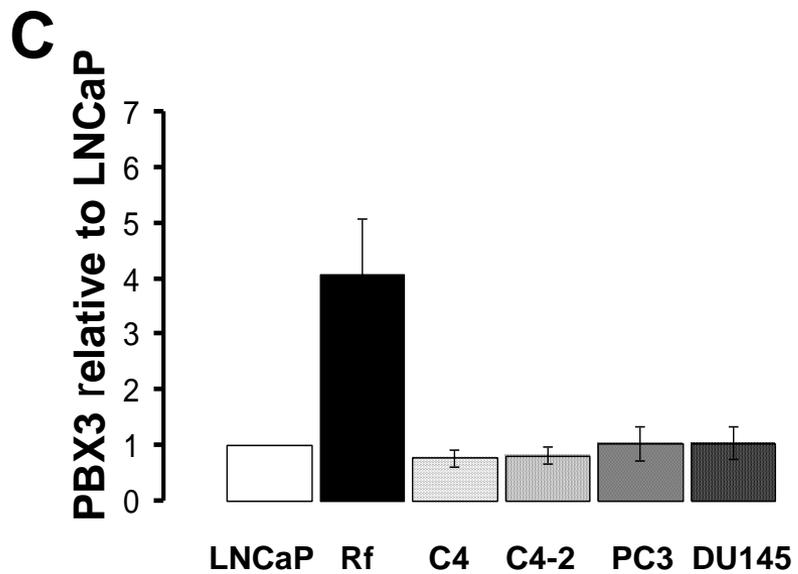
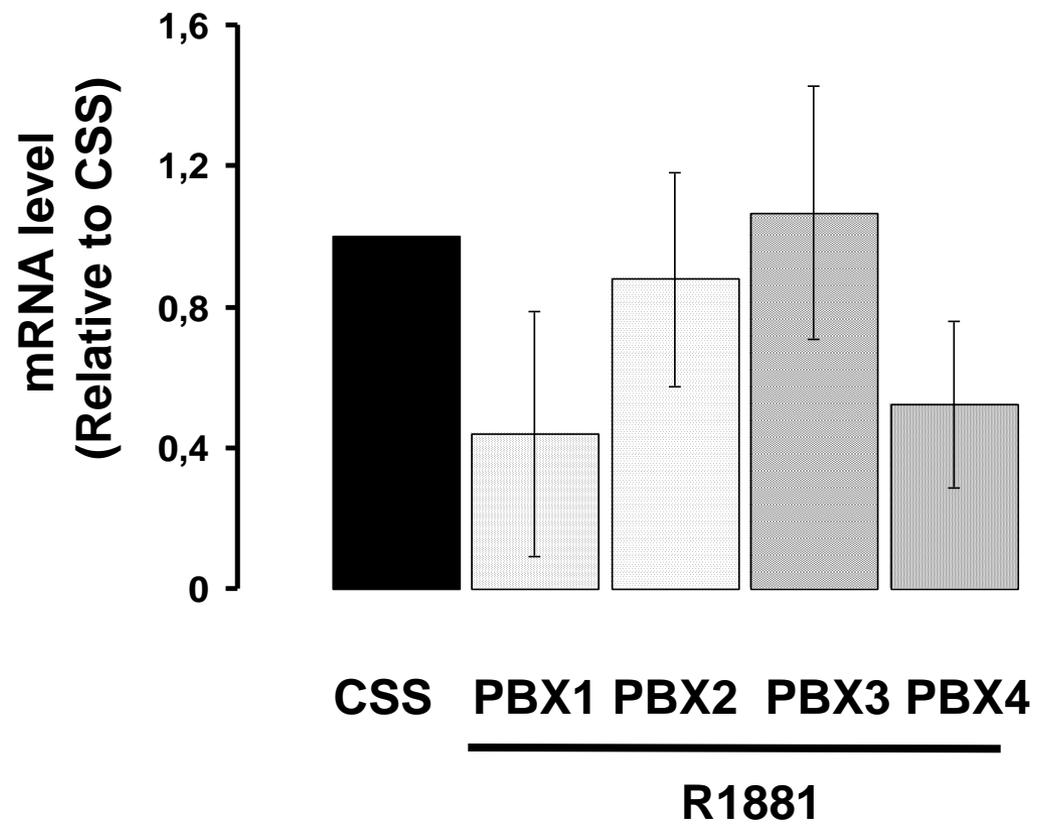
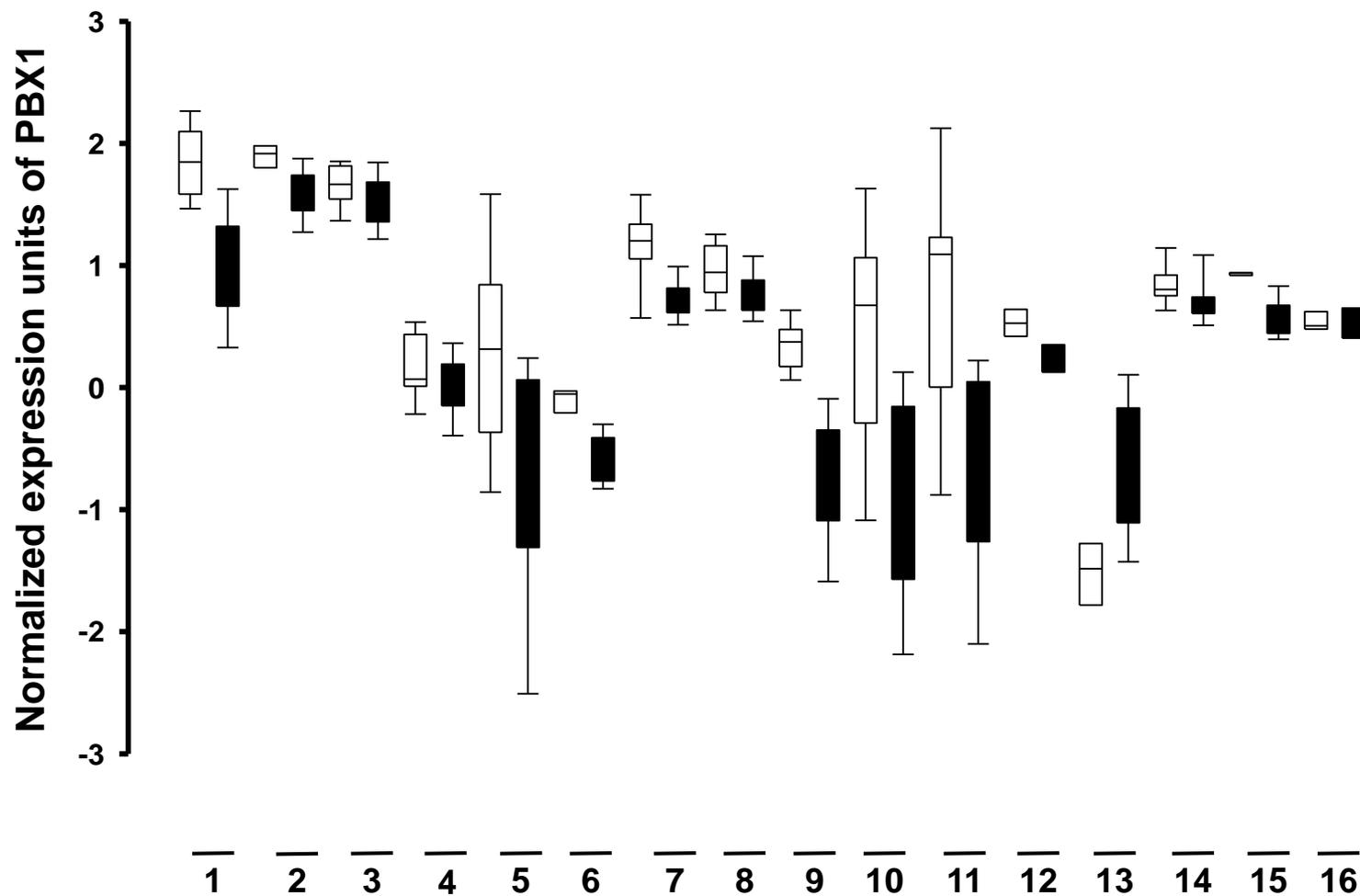


Fig. 3



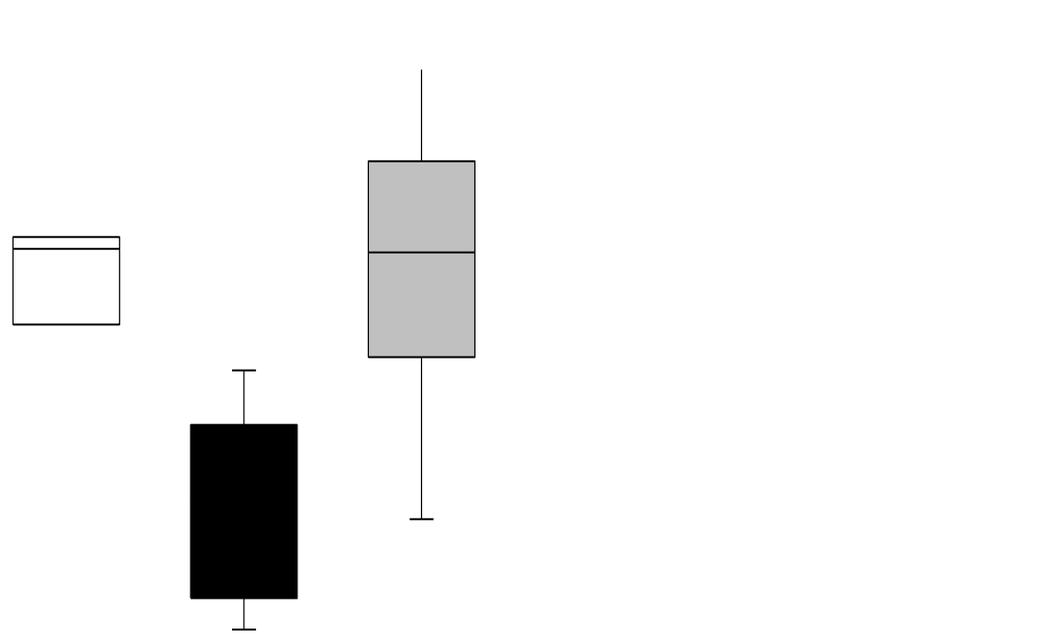
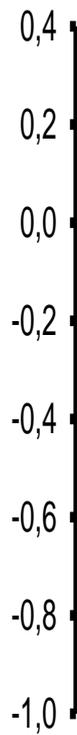
**A**

Fig. 4



**B**

Normalized expression units of PBX1



C

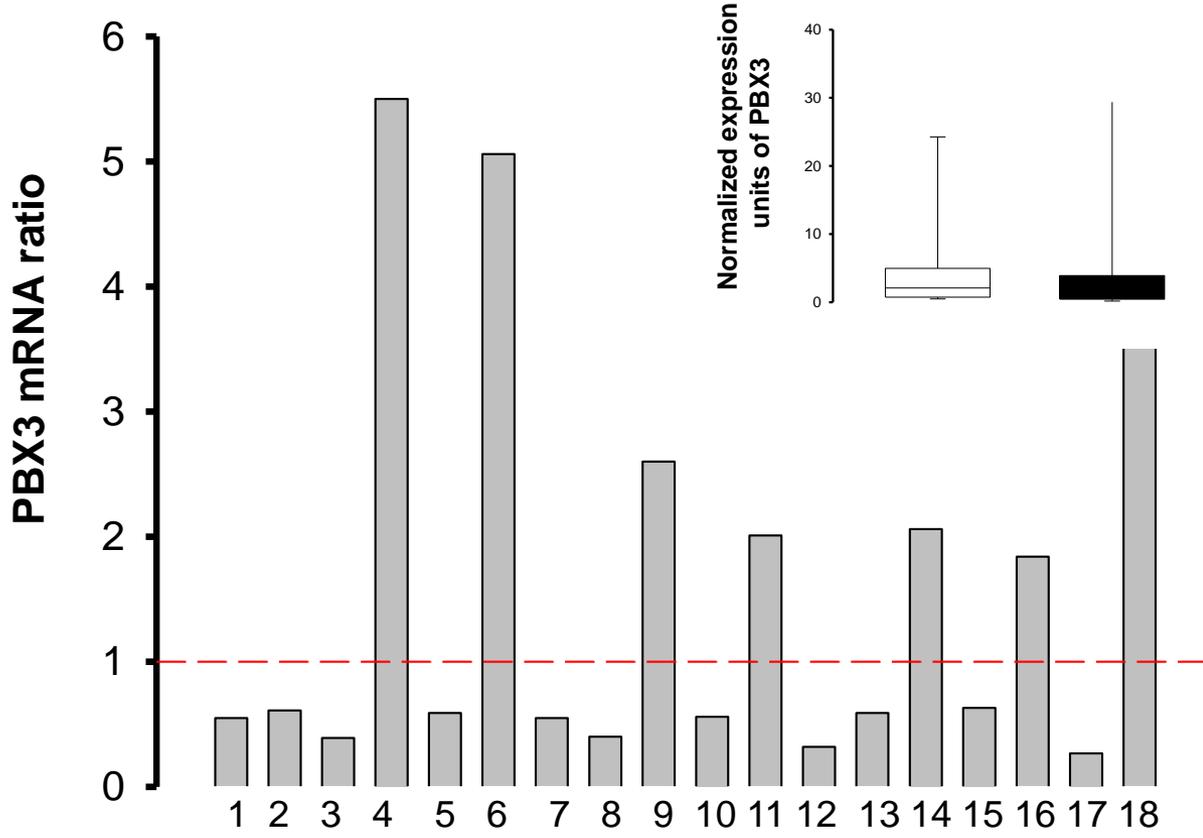
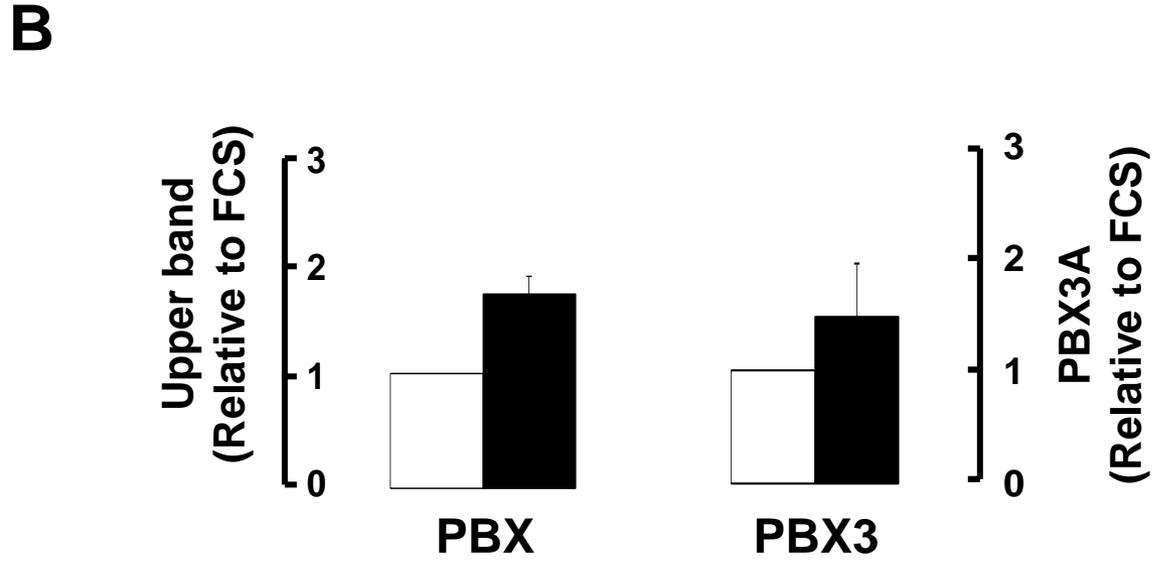
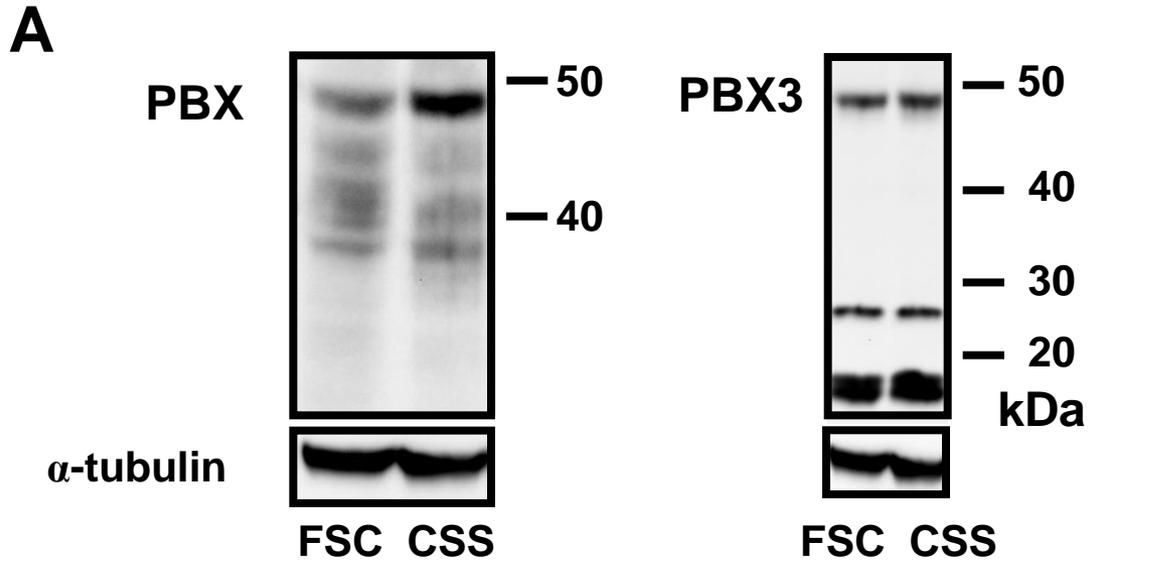


Fig. 5



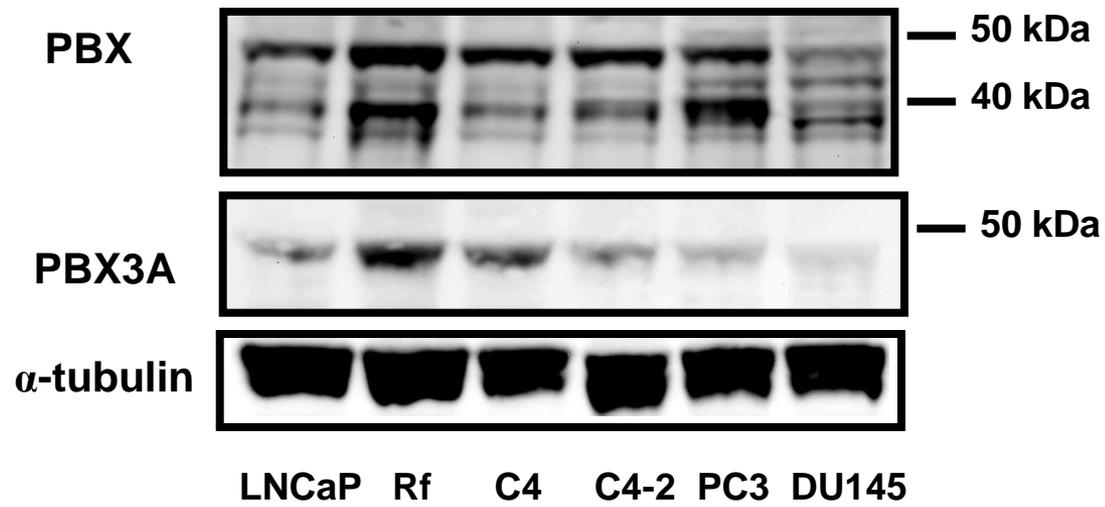
**A**

Fig. 6

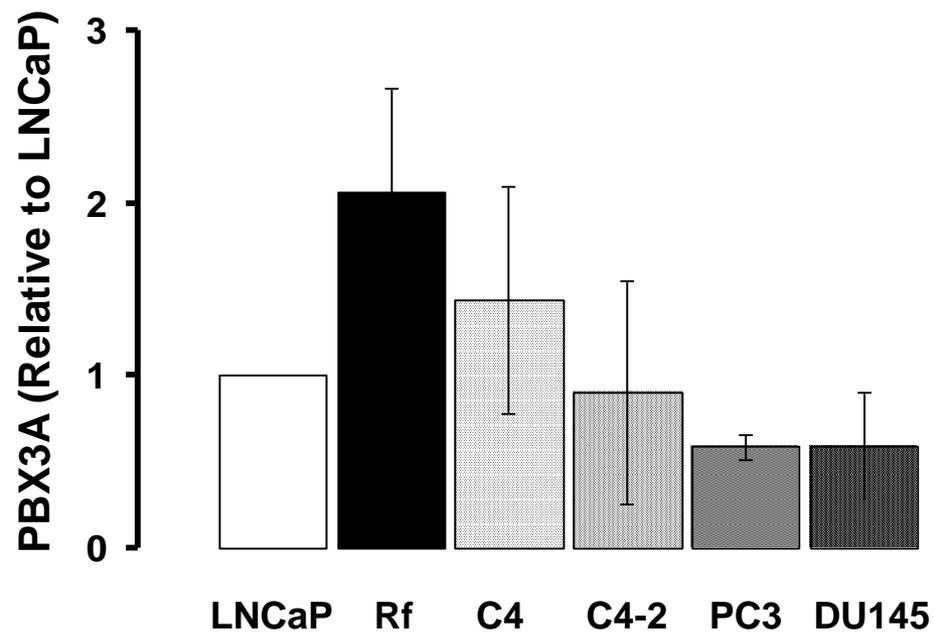
**B**

Fig. 7

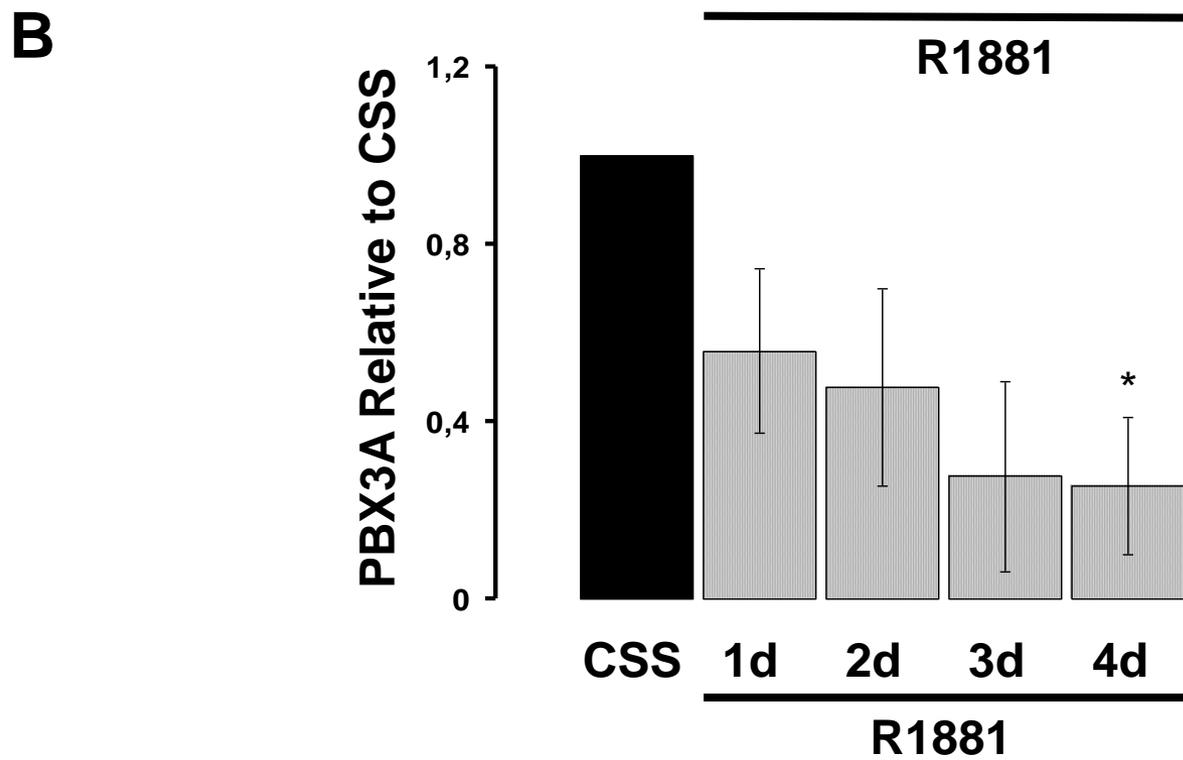
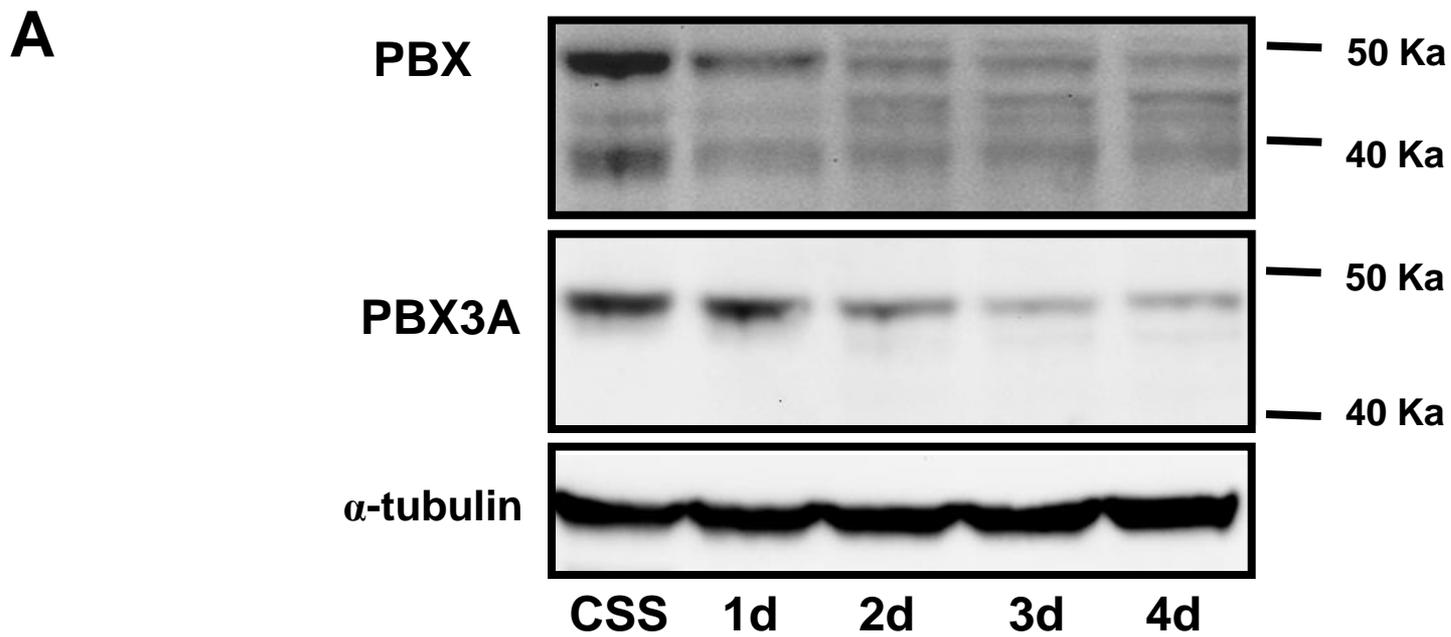
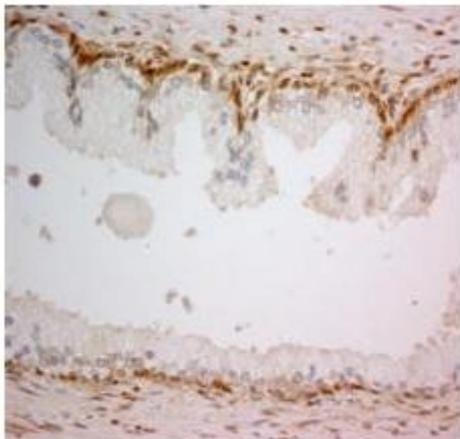
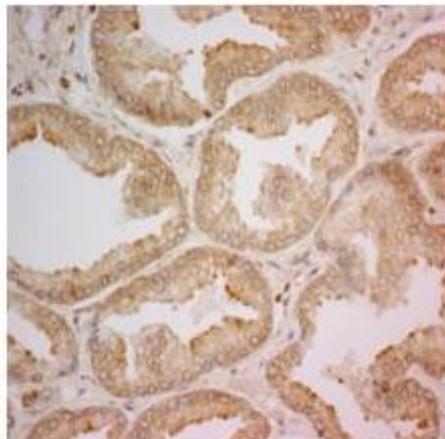


Fig. 8

**A**



**B**



**C**

