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## Reducing prohibitin increases histone acetylation, and promotes androgen independence in prostate tumours by increasing androgen receptor activation by adrenal androgens

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### Abstract

Prostate cancers, initially responsive to anti-androgen therapies, often advance to a hormone-refractory “castrate resistant” stage (CRPC). However the androgen receptor (AR) pathway remains active and key for cell growth and gene expression within tumours, even in the apparent absence of hormone. Proposed mechanisms to explain progression, including AR amplification/ mutation, are insufficient to completely explain CRPC and possible roles of AR cofactors such as prohibitin are poorly understood. We investigated whether prohibitin loss could sensitise prostate cancer cells and tumours to adrenal gland-derived androgens which persist even after androgen ablation, hence contribute to development of CRPC. Using a pair of prostate cancer cell lines, inducibly expressing ectopic cDNA or RNAi for PHB, responses to different androgens and hormone concentrations were studied both *in vitro* and *in vivo*. PHB was found at the promoters of several genes, both AR and non AR-regulated, and knockdown increased histone acetylation at these promoters. Further, PHB knockdown increased rate of AR ligand-induced chromatin binding, and binding rate and occupancy of AR upon the *PSA* promoter. This resulted in increased cell growth and AR activity in response to all androgens, including promoting a response to the weaker adrenal androgens previously absent at physiological concentrations. *In vivo* this had functional consequences such that PHB knockdown resulted in androstenedione being sufficient to promote tumour growth, under conditions mimicking those in patients undergoing androgen ablation therapy.

We conclude that reduction in prohibitin levels is sufficient to lower the threshold of AR activity *in vitro* and *in vivo*; this may be via a general increase in histone acetylation that could potentially affect signalling by other transcription factors. Prohibitin loss may provide a mechanism for progression to CRPC by sensitizing prostate cancer cells to “castrate” conditions i.e. low levels of testicular androgens in the continued presence of weak adrenal and dietary androgens.

### Keywords

prohibitin; androgen receptor; prostate; androgens; castrate-resistant prostate cancer; corepressor

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## Introduction

Prostate cancer is the most commonly diagnosed cancer in males (1), and tumour growth is initially androgen-dependent. Cellular and physiological responses to androgens are mediated by the androgen receptor (AR), which is key to prostate cancer progression and treatment (2, 3). There are three sources of androgenic steroids: the testes, which synthesise testosterone from adrenal precursors; the adrenal glands, which secrete the weakly androgenic precursors androstenedione (ASD) and dehydroepiandrosterone (DHEA); and diet. 5 $\alpha$ -reductase enzymes in androgen-dependent tissues such as the prostate convert testosterone into the more potent dihydrotestosterone (DHT), which binds AR with higher affinity. Ligand-activated AR binds to androgen response elements (AREs) located in the regulatory regions of target genes, thus influencing the rate of gene transactivation by recruiting gene activation complexes to the promoters of these genes and dismissing repressive proteins, leading to androgen-dependent cell growth and function (4-9).

Currently the mainstays of prostate cancer treatment are chemical castration using LHRH analogues to ablate testicular androgens, and anti-androgens which block androgen signalling at the level of the AR. Initially successful, such hormonal therapies inevitably fail and patients relapse with “castrate-resistant” prostate cancer (CRPC). Resistance results from clonal selection of cells that circumvent androgen requirement by mechanisms including AR gene mutation or receptor amplification (4-7, 10-12). Additionally, reduced levels of repressor proteins may increase the activity of the AR due to a lack of repressive action.

One such co-repressor is prohibitin (PHB) (13), previously found to be down-regulated by androgen treatment (14). PHB has multiple roles in the cell, including as a chaperone complex in the inner mitochondrial membrane (15), an attenuator of Raf-Mek and Akt signalling pathways (16), and repressor of various transcription factors (including E2F and steroid receptors), and has tumour suppressor, anti-proliferative and cell-cycle regulation activities (17, 18). PHB has been shown to repress E2F via recruitment of the repressive proteins HDAC1, N-CoR and the chromatin condensing proteins BRG1/Brm, (19, 20). PHB can also repress steroid-activated nuclear receptors e.g. the androgen receptor (AR) (14) and estrogen receptor (ER) (21), and conversely is capable of activating p53 (22). PHB is a potent transcriptional corepressor of ER $\alpha$  and associates with estrogen-regulated promoters in the absence of hormone, dissociating after hormone treatment. Interestingly, PHB knockdown reduces the anti-proliferative actions of estrogen antagonists (23) and PHB recruits BRG1-containing chromatin remodelling complex to antagonist-bound androgen receptor (24). Additionally, PHB associates with HP1 proteins (25), involved in the compartmentalisation of chromatin into heterochromatin and euchromatin (26), and may have a role in facilitating the DNA structural changes required for gene activation and silencing.

Previously we have shown that PHB can repress AR activity and androgen-stimulated growth of LNCaP prostate cancer cells and conversely that RNAi-mediated knockdown of PHB increased AR activity and increased LNCaP cell growth in response to testicular androgens such as testosterone, both *in vitro* and *in vivo* (27). Therefore we aimed to determine the mechanism of AR repression by PHB, and what effects the loss of such a repressor would have on AR activity and tumour growth under conditions mimicking those in patients undergoing androgen ablation therapy. Circulating at relatively high levels and unaffected by chemical castration, adrenal androgens are a significant source of androgens in these patients. Therefore, we aimed to study whether the reduction of a repressor such as PHB could indeed cause or contribute towards the apparent “androgen independence” observed in CRPC and the failure of hormonal therapies.

## Results

### PHB influences the rate and extent of AR binding to chromatin

A pair of doxycycline-inducible LNCaP prostate cancer cell lines was established, one ectopically expressing PHB cDNA (LNCaP/Luc/PHB-cDNA) and one expressing PHB-siRNA (LNCaP/Luc/PHB-RNAi) (27), each with its respective empty vector or scrambled control line. Upon removal of soluble nuclear proteins, PHB was evident in nuclear foci (Figure 1a), number and intensity of which altered with PHB modulation. Fractionation demonstrated that PHB is present in both the cytoplasmic fraction and also the nucleus – both the soluble nuclear fraction but to a greater extent the chromatin-associated fraction (Figure 1b). Micrococcal nuclease digestion of chromatin released some of the associated PHB into the soluble nuclear fraction, supporting the PHB-chromatin association. This nuclear pattern is reminiscent of known PHB-interacting proteins HP1 and HDAC1, which colocalise closely with PHB (Figure 1c). Chromatin association of PHB was reduced in cells grown in full serum compared to hormonally-starved cells (Figure 1d), with a concomitant increase in soluble nuclear PHB, while total and cytoplasmic PHB levels were unchanged. Increased chromatin association of PHB was also seen in serum-starved HeLa cells (Figure 1e), which neither express AR nor are steroid responsive. Returning these cells to full serum reduced the levels of PHB co-purifying with chromatin within 24hr.

Treating hormone-starved LNCaP cells with androgen revealed increased AR chromatin association over time with a concomitant reduction in PHB co-purification (Figure 2a, left hand side). Doxycycline-induced PHB-RNAi reduced the amount of PHB co-purifying with the chromatin as expected, but accelerated AR binding, resulting in increased binding at shorter time-points of androgen treatment (Figure 2a right hand side). Conversely, ectopic expression of PHB cDNA resulted in increased PHB-chromatin association, with a dose-dependent reduction in AR co-purification, even in full serum (Figure 2b). Total levels of AR remained unchanged in each case.

To quantify the effects of PHB loss on the chromatin-binding rate of AR, hormone-starved LNCaP cells were treated with either the potent DHT or the weak androstenedione (both at 10nM). Without doxycycline, DHT treatment resulted in maximal AR chromatin co-purification at 120-240 minutes, whilst doxycycline-induced PHB-RNAi accelerated this, with maximal AR chromatin co-purification at 30-60 minutes. Similarly with androstenedione treatment: AR chromatin binding was lower in the absence of doxycycline, whilst PHB-RNAi resulted in a statistically significant increase in AR binding at 120-240 minutes (Figure 2c). No change in rate of AR binding to chromatin was evident in the scrambled RNAi control line.

### PHB is displaced from the PSA promoter with androgen treatment

Chromatin purification experiments (Figures 1 & 2) showed global chromatin binding patterns of PHB and AR to be in dynamic opposition. Liganded AR binds to AREs in the promoter and enhancer regions of the well-characterised androgen target gene *PSA*, and PHB can bind strongly to the *PSA* promoter in the presence of anti-androgens (24), but little data is available on the effects of co-repressors on AR binding. To address this, chromatin immunoprecipitation assays (ChIP) were performed.

In hormone-starved LNCaP cells, DHT treatment (2 hours) resulted in AR binding to the promoter and enhancer regions as expected, with minimal binding to non-ARE containing regions (Figure 3a). In the absence of hormone, PHB binds across the 8kb *PSA* promoter region with no apparent regional specificity. DHT treatment led to reduced PHB across all *PSA*-promoter regions (Figure 3a), i.e. this was not limited to ARE-containing regions. Also PHB was recruited to both ARE-containing and ARE-negative regions under hormonally

starved conditions as compared to full serum, and was displaced from all three regions by androgen treatment (Figure 3b), coincident with increased AR binding in the case of the enhancer and promoter regions.

### PHB knock down increases AR binding and binding rate to the PSA promoter

Since PHB knockdown increased global AR chromatin-binding, the effects upon AR binding to AREs in the *PSA* promoter were studied. DHT increased AR binding to the enhancer and promoter regions as expected, however PHB-RNAi increased AR binding still further (Figure 3c). DHT treatment reduced PHB binding across all regions, confirming previous results, and PHB-RNAi knockdown reduced this further (Figure 3d). No change was seen in total AR levels in these cells within this timeframe (see Figure 2a) and no change in AR or PHB recruitment was seen in the scrambled siRNA control cell line (Supplemental Figure 1a). Similar data was obtained for the similarly organised androgen-responsive gene *KLK2* (Supplemental Figure 1b).

We then analysed AR binding to the *PSA* promoter and enhancer in more detail. LNCaP/Luc/PHB-RNAi cells were grown without steroids ( $\pm$  doxycycline) for 72 hours and then treated with either DHT or androstenedione. DHT treatment promoted AR binding to both regions within 30 minutes, with binding reduced at 1 hour then increasing again at 2 hours. PHB-RNAi changed the pattern of AR binding to a more linear increase in binding with no reduction at the 1-hour timepoint (Figure 4a). Since the kinetics of androstenedione activity were less rapid we extended the study to 4 hours. Similar results were seen: AR recruitment at 1 hour, dropping at 2 hours then peaking again at 4 hours (Figure 4b). Doxycycline-induced PHB knockdown caused increased binding of AR to the promoter and enhancer regions with a peak at 1 hour and no cyclical pattern evident within this timeframe. Confirming previous results, hormone treatment resulted in a rapid reduction (within 15 minutes) of PHB binding at the enhancer and at the promoter, with a slight increase at 30 minutes at the enhancer then a continued reduced level. Unsurprisingly, PHB-RNAi resulted in very low levels of PHB at the enhancer or promoter throughout (Figure 4c). IgG controls showed no changes with either treatment and a similar pattern was observed for recruitment to the *KLK2* enhancer/promoter (Supplemental Figure 2).

### PHB knock down increases androgen receptor activity as measured by endogenous and integrated reporter gene expression, but does not affect ligand binding

Both testicular and adrenal androgens activate androgen-regulated gene expression, but the less efficient adrenal androgens are required at higher concentrations for similar effects. The ability of these to activate the AR in our cell lines was assayed using the expression of both endogenous androgen regulated genes (e.g. *PSA*) and an integrated androgen-responsive luciferase reporter (27). Kinetics of PSA transcript production were studied after treatment with DHT or androstenedione (10nM)  $\pm$  doxycycline. DHT-induced PSA transcript levels were increased at 6 hours post treatment and further thereafter (Figure 5a, left panel), while PHB knockdown resulted in transcript levels peaking earlier (by 2-4 hours) and remaining high thereafter. *KLK2* and *TMPRS2* showed similar trends (Supplemental Figure 3a). PHB knockdown also increased the rate of androstenedione-induced PSA transcript production, which was significantly higher from 4 hours hormone treatment (Figure 5a, right panel).

Similarly we investigated whether the increased AR binding rate caused by PHB-RNAi could increase the efficacy of various androgens. After 16h treatment with 100nM hormone, PSA expression increased 6-fold with DHT (Figure 5b-c left panels), around 3-fold for androstenedione (Figure 5b-c, right panels), while DHEA had no significant effect (data not shown). Overexpression of PHB-cDNA caused strong inhibition of androgen-induced *PSA* expression, whether by DHT or androstenedione (Figure 5b). Conversely, doxycycline-

induced PHB-RNAi knock down increased *PSA* expression in response to both testicular and adrenal androgens (Figure 5c), significant at doses above 1nM.

Using the integrated androgen receptor luciferase reporter, we found increasing PHB reduced luciferase activity as previously reported (27). However, PHB-RNAi resulted in increased luciferase activity at lower doses of DHT, changing the maximal activity concentration (Figure 5d, left panel). Similarly 1nM androstenedione in the presence of PHB-RNAi had equal activation potency to 10nM without (Figure 5d, right panel). To confirm that the observed stimulatory effects of PHB knockdown can be overcome by reintroducing PHB, we transfected LNCaP/Luc/PHB-RNAi cells with a form of PHB not sensitive to silencing. This showed that the increased androgen-dependent (both DHT and androstenedione) luciferase activity seen in the presence of doxycycline-induced PHB knockdown was abolished by increasing PHB levels (Supplemental Figure 3b).

Doxycycline treatment had no effect on the hormone responses of the scrambled RNAi or empty vector control lines (Supplemental Figure 4). Scatchard analysis confirmed that neither ectopic expression of PHB-cDNA nor PHB-RNAi significantly affected the Bmax or dissociation constant (Kd) of ligand binding (Supplemental Figure 5).

### **PHB knock down results in higher baseline levels of acetylated histone H3 in hormonally-starved cells**

PHB is known to recruit HDACs and chromatin remodelling complexes including HP-1 (19, 20, 25). We saw that PHB reduction resulted in a decrease in HP-1 and HDAC1 association with chromatin (Figure 6a), and analysed the chromatin for changes in histone acetylation in response to PHB knockdown. This resulted in an increase in overall acetylation of Histone H3, with increased levels of histone H3-Ac(K9) and Ac(K9) P(S10), even before androgen treatment (Figure 6a). Additionally, androgen induction of these histone H3 modifications was also higher in the doxycycline-treated samples. Histone H3-Ac(18) showed no significant changes (data not shown). Conversely, increasing PHB levels by exogenous PHB-cDNA expression resulted in a dose-dependent reduction in global histone H3 acetylation (Figure 6b).

ChIP analysis of the *PSA* regulatory region showed androgen-induced enrichment of H3-Ac(K9) across most of the promoter region as expected, however when PHB was reduced a further enrichment was observed, most notably at the enhancer and negative regions both before and after DHT treatment (Figure 6c). Interestingly, the RNAi-mediated reduction in PHB also increased levels of acetylated histone H3 at the promoters of the inducible *TAP1* and *Cyclin D1* genes, but had little effect at the constitutively active  *$\beta$ -actin* gene (Figure 6d).

### **PHB knock down increases LNCaP cell growth in response to testicular and adrenal androgens both in vitro and in vivo**

Having previously shown that PHB-RNAi increased prostate cell and tumour growth in response to testosterone (27), we tested whether it could similarly increase the response to adrenal androgens and translate into a tumour growth effect. Interestingly, in the absence of doxycycline we observed no cell growth in response to DHEA at doses up to and including 10nM for 96h (Figure 7a and data not shown). In culture, PHB-RNAi increased cell growth in response to all androgens: DHT, androstenedione and DHEA (10nM) (Figure 7a). PHB-RNAi also increased the percentage of cells in S-phase, under all conditions tested (Figure 7b).

Established prostate cancer therapies do not affect adrenal androgen production. This is confirmed by the fact that serum androstenedione in healthy human males has a normal

range of 1.7-10nM, while samples collected from patients treated with LHRH analogues (Leuprorelin or Goserelin) alone (androgen ablation) or plus bicalutamide (maximal androgen blockade) showed androstenedione values within this normal range (Table 1). Since PHB-RNAi increased AR activity in response to these weaker ligands, we examined in xenograft models whether normal serum levels of these androgens would be sufficient to maintain prostate cancer tumour growth *in vivo* when PHB is reduced.

Adrenal androgen production in rodents is maximal post-natally but drops to undetectable levels post-puberty (28), as adult males lack 17 $\alpha$ -hydroxylase in the adrenal cortex (29). Androgen synthesis is mainly testicular, from multiple precursors e.g. corticosterone, pregnenolone or acetate, produced in the liver or peripheral tissues (30-32). Orchidectomised male mice showed undetectable levels of both testosterone and androstenedione (Table 1), thus specific androgens could be supplemented to study individually. Androstenedione supplementation increased serum androstenedione to 0.3-2.1nM, coincident with the normal range (0.3-2.0nM), and no significant effect was seen on serum testosterone, although minimal peripheral tissue conversion cannot be ruled out. Androstenedione supplementation, therefore, mimics serum conditions of men undergoing therapy whose circulating testosterone levels are significantly reduced, but who have normal androstenedione levels. LNCaP/Luc/PHB-RNAi xenografts were thus grown under these conditions. Mice were given daily testosterone supplementation (resulting in serum levels of 7-10nM) until tumour burden was confirmed, and the tumours given a relative tumour volume (RTV) of 1. Mice were then separated into treatment groups: continued testosterone treatment, switched to androstenedione, or vehicle only (all  $\pm$  doxycycline).

In testosterone-treated castrated mice, LNCaP tumours grow with a mean doubling time of 20 days, while in vehicle-treated mice tumours stop growing and shrink within 48hr (27). Tumours in androstenedione-treated castrated mice did not show rapid tumour growth as seen for testosterone, but unlike vehicle controls, tumours were maintained with no regression (Figure 7c, dashed line). Under the same conditions plus PHB-RNAi, tumours showed a statistically significant increase in RTV as compared to control (Figure 7c), i.e. PHB reduction promoted androstenedione-induced tumour growth *in vivo*. Figure 7d summarises RTVs for vehicle, androstenedione and testosterone ( $\pm$  doxycycline) at the end of the experiment.

## Discussion

We set out to analyse the mechanisms by which PHB reduction increases AR activity, and its possible contribution to the “androgen-independent” phenotype of CRPC. Although PHB has no specific DNA-binding motifs, a large fraction is chromatin-associated, which alters according to treatment (increased in serum-starved cells compared to either androgen-treated LNCaP cells or serum-stimulated HeLa cells). PHB also associated closely with HP-1 and HDAC1 proteins, which are known to be strongly chromatin-associated. PHB dissociation from chromatin may be a requirement for (or a result of) cell cycle initiation regardless of initiation factor, be it androgens in LNCaP cells or other serum-derived growth factors in HeLa cells. PHB knockdown increased the amount and rate of AR-chromatin association in response to DHT and androstenedione, while overexpression was inhibitory, suggesting AR and PHB have dynamic and opposite roles/activities. PHB was also present upon the promoters of non-AR-regulated genes including the constitutively active  *$\beta$ -actin* and the interferon-inducible gene *TAP1* (33), as well as *Cyclin D1* which is indirectly regulated by androgens (See also Supplemental Figure 6a). PHB knockdown influenced the levels of acetylated histone H3 at these promoters also, indicating a more global phenomenon than simply affecting directly androgen-regulated genes. The widespread increase in histone acetylation did not however cause a global increase in gene expression (Supplemental

Figure 6b). Basal Cyclin D1 expression increased, perhaps in part explaining the increased cell cycle entry seen upon PHB knockdown. Basal TAP1 expression was reduced upon PHB knockdown but upon IFN treatment, induction was moderately increased (Supplemental Figure 7c). Caspase 7 and YY1 expression were oppositely regulated, being reduced and increased respectively upon PHB knockdown, as previously reported by Joshi *et al.* (34). This supports the growing body of evidence that PHB can have diverse effects on many gene promoters, and also that its effects appears to be mainly repressive in the case of androgen-regulated genes.

ChIP at the *PSA* and *KLK2* genes showed DHT-induced binding of AR to ARE-containing regions at 2 hours, while PHB binding decreased across all regions. Reducing PHB protein levels resulted in enhanced AR binding, supporting the theory that PHB and AR binding are in opposition, if not mutually exclusive. The temporal association of AR revealed a complex pattern of association and disassociation such as reported previously (35, 36). PHB was present on all regions in hormonally starved cells but rapidly dissociated in DHT-treated cells. PHB-RNAi changed the pattern of AR binding: AR remained bound, with no fluctuation within the time studied. Work from the Gannon laboratory on ER promoter-binding describes an initial unproductive cycle then subsequent transcriptionally productive cycles (37). A timecourse of DHT-treated LNCaP cells showed that PSA transcripts could be detected much earlier when PHB levels were reduced. Additionally, PHB-RNAi increased histone H3 acetylation (Ac-K9) and increased DNase sensitivity of the chromatin (Supplemental Figure 7) both indicators of chromatin flux (or possible artefacts of the cell cycle-promoting effects of PHB loss). PHB loss may alter histone modifications (such as H3 acetylation, as shown here) such that chromatin is in a more active state, which in the case of androgen-responsive genes may eliminate the unproductive cycle of steroid receptor binding. Alternatively, PHB may be required for hormone starvation-induced chromatin silencing, with its loss preventing the full effects of such starvation on silencing of target genes. Regardless, PHB reduction increases AR efficiency.

Although associated with chromatin and found at AR-regulated regions, PHB protein could not be co-purified with oligonucleotides containing a consensus ARE or larger DNA sequences representing *PSA* regulatory regions (data not shown). Further no DNA-binding sequence has been described for PHB, and it associates with the nuclear matrix (38), suggesting it may bind chromatin with higher-order structure, strongly supported by its recruitment of Brm/Brg chromatin modifiers and association with heterochromatin-binding protein 1 (HP1) (25), as well as its association with many and varied gene promoters. In the context of androgen signalling, PHB may modulate AR binding to AREs via altering chromatin conformation, and/or by recruiting repressive chromatin remodelling complexes to the region in the absence of ligand. Repressive complex recruitment by PHB is essential for antiandrogen effects (24). Since adrenal androgens possess weak anti-androgen activity (39), the loss of a repressor protein such as PHB not only increases the effects of weak androgens but also inhibits their weak anti-androgenic effects, thereby potentially greatly increasing their potency.

Modulating PHB within LNCaP prostate cancer cells influenced AR activity with PHB-RNAi promoting increased androgen-stimulated cell cycle entry, increased endogenous and reporter gene transcription, and increased tumour xenograft growth *in vivo* (27). We examined whether this increased AR activity would cause weak AR agonists to become significant activators. Whereas PHB ectopic expression inhibited all hormone-induced activity (data not shown), PHB-RNAi amplified the response to all androgens, reducing the effective concentration required to give maximum effect approximately 10-fold. It has previously been reported that in the C4-2b prostate cancer cell line, which is derived from LNCaP but more aggressive *in vivo*, PSA is expressed even in the absence of added DHT,

when very small amounts of AR are present at the *PSA* promoter (40). Upon investigation we found that these cells exhibit approximately 20% lower levels of endogenous PHB than LNCaP cells - as do several other more aggressive prostate cancer cells (Supplemental Figure 8). Reducing PHB levels still further in one such line (VCaP) resulted in an increased *PSA* response. It is tempting to speculate that the effective increased *PSA* response in C4-2 cells as compared to LNCaP may reflect, at least in part, the decreased PHB levels and possible that this may also contribute to their more aggressive phenotype.

LNCaP tumour xenografts growing in castrated mice treated with androstenedione mimicked conditions in androgen-ablated prostate cancer patients. Androstenedione increased tumour volume as compared to vehicle control, but this was not statistically significant until PHB was reduced by RNAi. Since we had shown that PHB reduction alters the temporal dynamics of AR activity, facilitating binding of liganded AR to DNA and consequently increasing androgenic activity of weak adrenal androgens, it is likely that this significant tumour growth caused by androstenedione is a consequence of reduced PHB levels. In support of this, reintroducing PHB into the depleted cells reversed the stimulatory effects of PHB knockdown, in the presence of DHT or androstenedione. Adrenal or dietary-derived androgens may therefore become more significant in prostate cancer patients, as they are unaffected by androgen ablation regimens and may become sufficient to promote or maintain tumours under conditions of “androgen ablation”. Further, this supports our hypothesis (Dart et al., 2009) that increasing PHB levels may be of therapeutic benefit in CRPC patients. It is important to note that, while we have also shown that reducing PHB can also increase the stimulatory effects of low levels of potent androgens such as DHT in terms of increasing target gene activity, we have not proven whether this involves dose-dependent alterations in rate of AR chromatin binding or translates into a growth effect. It thus remains an untested possibility that reduced PHB could also exacerbate the effect of any residual low levels of testicular androgens which may be present in CRPC patients.

The AR-mediated downregulation of PHB (14) may be exacerbated during prostate cancer development, especially in tumours with AR ligand binding domain mutations, leading to increased sensitivity to adrenal androgens. This study thus supports the requirement for androgen ablation that additionally targets adrenal androgen synthesis, such as CYP17 inhibitors. PHB loss is a potential mechanism that may at least partially contribute to the increasingly significant burden of castrate-resistant prostate cancer, while co-repressors and co-activators of AR provide new avenues for research into the treatment of recurrent prostate cancer.

## Methods

### Cell Culture

LNCaP and VCaP cells were maintained at 37°C, 5% CO<sub>2</sub> in RPMI medium with 10% foetal bovine serum (First Link UK). All LNCaP/Luc/PHB cells were maintained at 37°C, 5% CO<sub>2</sub> in RPMI medium with 10% dox-free foetal bovine serum (Clontech, Palo Alto, USA), 12µg/ml blasticidin (Sigma), 0.3mg/ml zeocin (Invitrogen, Paisley, U.K.), and 500µg/ml G418 (Sigma). HeLa cells were maintained in DMEM medium (Sigma) with 10% fetal bovine serum (First Link UK, Ltd, Brierley Hill, U.K.). All media was supplemented with 2mM L-glutamine, 100units/ml penicillin, 100mg/ml streptomycin (Sigma). 72 hours before androgen exposure, medium was replaced with ‘starvation medium’ consisting of phenol red-free RPMI (or DMEM) medium, supplemented with 5% charcoal-stripped foetal bovine serum (First Link UK).



### Luciferase assay

Cells were lysed in reporter lysis buffer (Promega). Lysate was mixed with luciferin substrate and light emission measured using the Steadylite luciferase assay kit (Perkin-Elmer, Warrington, Cheshire, UK) in a Topcount luminometer (Packard Instrument Co, Meriden, CT, USA).

### RNA extraction and RT-PCR

Total RNA samples were prepared using commercial kits (Qias shredder and RNeasy, Qiagen) and converted to cDNA using the SuperScript First-Strand Synthesis system (Invitrogen).

### Taqman Quantitative PCR

Reactions were performed in triplicate on cDNA samples in 96-well optical plates on an ABI Prism 7900HT system (Applied Biosystems, Warrington, U.K.). Reactions consisted of 2  $\mu$ l cDNA, 7  $\mu$ l PCR-grade water, 10  $\mu$ l 2 $\times$  TaqMan Universal PCR Master Mix (Applied Biosystems), 1  $\mu$ l Taqman specific assay probes (Applied Biosystems) for PSA, TMPRSS2, KLK2, and L19. Parameters were: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data was recorded using Sequence Detector Software (SDS version 2.3; PE Applied Biosystems). Levels were normalised to GAPDH or L19.

### Chromatin Isolation and In Situ Cell Fractionation

Cells ( $4 \times 10^7$  cells/ml) were suspended in buffer A (10mM HEPES pH7.9, 10mM KCl, 1.5mM MgCl<sub>2</sub>, 0.34M sucrose, 10% glycerol, 1mM DTT, protease inhibitor cocktail (Sigma)). Triton X-100 was added (0.1%), and incubated on ice for 5 minutes. Nuclei were collected by centrifugation for 4 minutes at 1,200  $\times$  g with supernatant collected as the cytoplasmic fraction. Nuclei were lysed in buffer B (3mM EDTA, 0.2mM EGTA, 1mM DTT, protease inhibitor cocktail) and chromatin was collected by centrifugation for 4 minutes at 1,800  $\times$  g, with supernatant collected as a soluble nuclear fraction. The chromatin pellet was washed twice in buffer B and re-suspended in Laemmli buffer.

For *in situ* fractionation for immunofluorescent staining, cells were grown on coverslips, washed in PBS and fractionated by adding buffer A with 0.5% triton X-100, on ice for 5 minutes. Cells were washed twice in PBS and fixed with 1% formaldehyde for 10 minutes.

### Histone Extraction

Cells were lysed for 10 minutes (4°C) in lysis buffer (PBS, 0.5% TritonX-100, 2mM PMSF, 5mM NaButyrate, protease inhibitor cocktail) at  $10^7$  cells/ml. Nuclei were pelleted by centrifugation at 2000  $\times$  g for 10 minutes and washed in lysis buffer. Nuclei were extracted in 0.2N HCl overnight at 4 °C. Proteins were precipitated by TCA (100%) and pelleted at 16,000  $\times$  g for 10 minutes, acetone washed and air dried, before dissolving in lysis buffer.

### Immunoblotting

Immunoblotting was carried out as described (27). Primary antibodies were;  $\beta$ -actin (Abcam, Cambridge, USA @1:5000), PHB (Thermo Fisher Scientific, Cheshire, U.K. @1:1000), AR (Dako, UK), Histone H3 (Sigma @ 1:2000), Histone H3-Ac(K9), H3 Ac(K9) P(S10), H3 Ac(K18), (New England Biolabs (UK) Ltd @1/500), HP1 (Active motif, USA, @ 1: 500), HDAC1 (Sigma, @1:1000). Peroxidase-labelled secondary antibodies (Dako) were used at 1:2000. The membrane was then incubated in chemiluminescent substrate (Amersham, U.K.) and light emission detected by autoradiography.

### Chromatin Immunoprecipitation (ChIP)

Cells ( $1 \times 10^7$ ) were grown in starvation medium for 3 days then treated with hormone (10nM MB) for 0-2hr. ChIP was performed on formaldehyde cross-linked cell samples essentially as described (41). 5 $\mu$ l of DNA was used for the PCR with 30-35 cycles of 94°C for 30 sec, 57°C for 45 sec and 70°C for 45 sec.

A full list of primers used for ChIP is given in supplemental table 1.

### Confocal microscopy

Cells grown on glass coverslips were fixed in 1% formaldehyde for 10 minutes. For *in situ* fractionation cells were permeabilised in buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 3mM MgCl<sub>2</sub>, 1mM EGTA, 0.5% Triton-X100) and washed several times in PBS before fixation.

Coverslips were blocked with 10% rabbit serum (Dako UK Ltd, Ely, U.K.) for 30min. Mouse anti-human PHB antibody (Neomarkers) was applied for 1 hour followed by PBS washes. FITC labeled secondary antibody (Alexa @ 1:50) was then applied for 1 hour and then washed in PBS, mounted on slides with Vectorshield/DAPI (Vector Labs Inc.) and visualized on Zeiss Meta 512 confocal microscope.

### Sulphorhodamine B (SRB) Assay for cell growth

Cell growth was measured by sulphorhodamine B assay as previously described (14).

### Cell-cycle analysis

Cells were fixed in 70% ethanol at 4°C and stained with 5mg/ml propidium iodide and treated with 50mg/ml RNaseA. Analysis was carried out using a Beckton-Dickinson FACS Calibur, using linear scale representation of forward and side scatter during flow analysis, as well as fluorescence for DNA content. Single cells were gated and the cell cycle profiles measured. A total of 10,000 events were measured per sample.

### Growth of xenografts

Xenografts growth assays were carried essentially as described (27) with the addition of androstenedione at 50 $\mu$ g in 90% propylene glycol and 10% ethanol. All procedures on mice were approved by the local ethical research board and performed under an appropriate Home Office licence.

### Measurement of serum hormones

Serum samples from normal individuals or patients undergoing prostate cancer therapy were collected with appropriate ethical approval (Hammersmith & Chelsea Hospitals LREC, project registration number 2000/5816). Mouse serum was collected from anesthetized mice. Red blood cells were removed by centrifugation at 2000 x g, and serum stored at -80°C. Total testosterone or androstenedione from serum was measured using ELISA kits (Calbiotech, USA).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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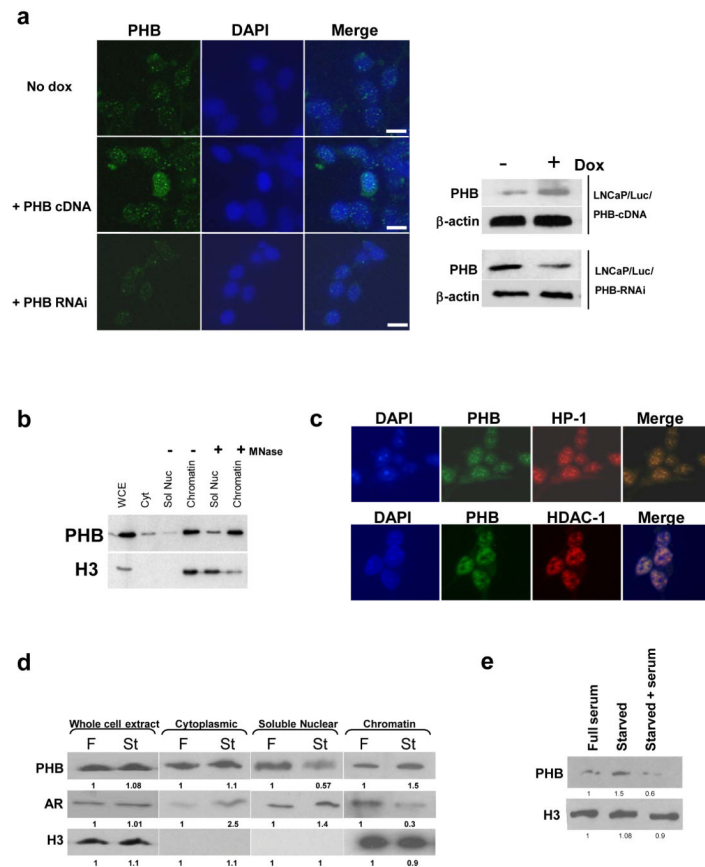
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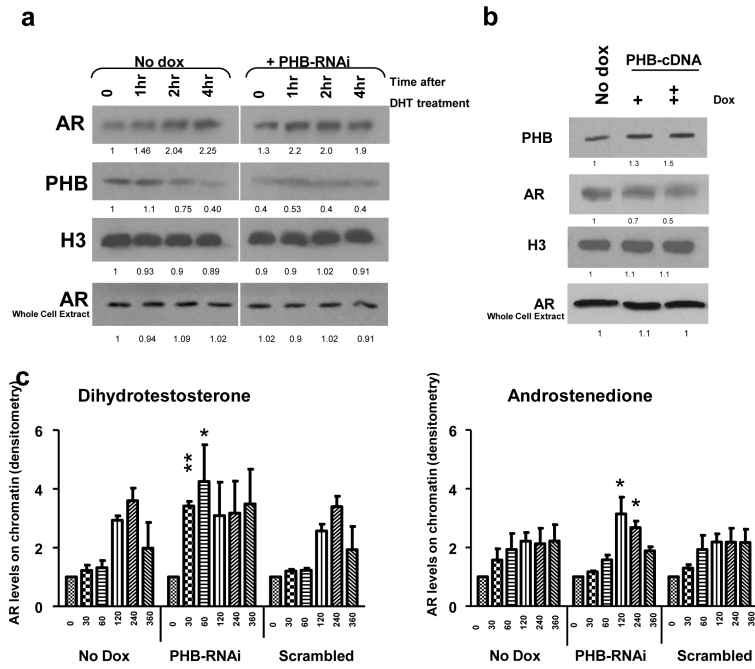
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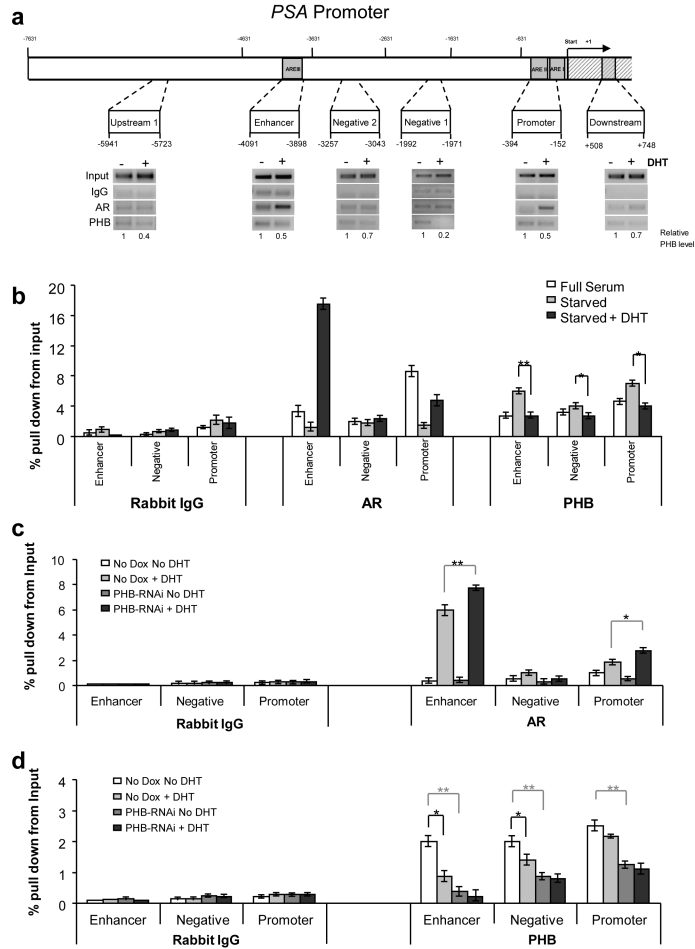


**Figure 1.**

Analysis of PHB levels within cells. (a) Immunofluorescent staining for PHB foci within the nuclei of *in situ* fractionated LNCaP/Luc expressing either PHB-cDNA or PHB-RNAi, with anti-PHB antibody detected with TRITC-labelled secondary and stained with DAPI for DNA. Bar = 20µm. Corresponding comparison of PHB protein levels shown Western blot alongside. (b) Western blot analysis of PHB and H3 from cellular fractionation of LNCaP cells. Where indicated, samples were incubated for 1 min at 37°C ± 0.2 U of micrococcal nuclease. (c) Immunofluorescent staining of LNCaP cells for PHB (TRITC detection), HP1 and HDAC1 (FITC detection), also DNA (DAPI). (d) Western blot analysis of PHB, AR and Histone H3 in cell fractions from LNCaP cells, grown either in full serum (F) or charcoal-stripped serum (St). (e) Western blot analysis of PHB and Histone H3 in purified chromatin fraction from HeLa cells, grown in full serum or serum starved. Densitometry data for each blot are given underneath.

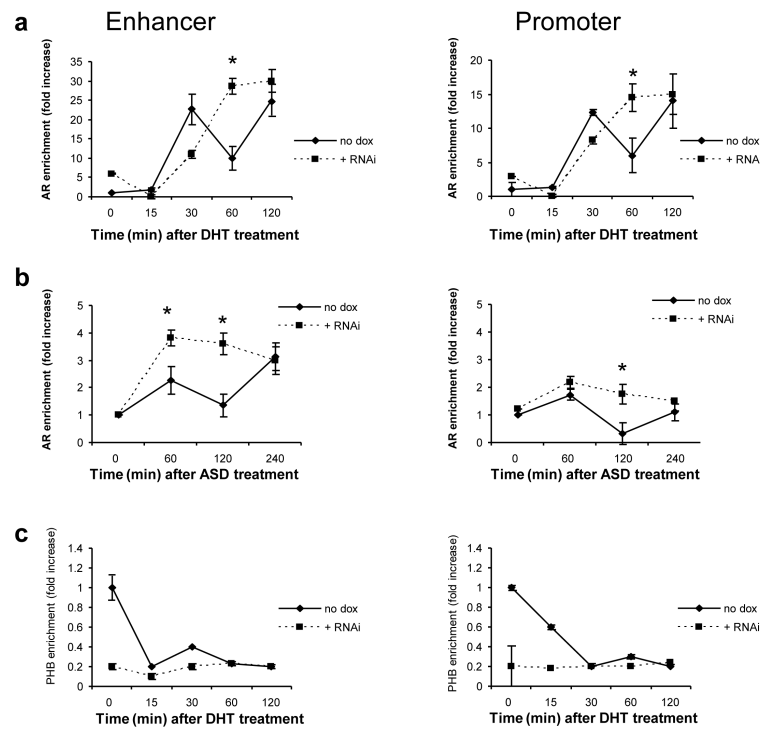


**Figure 2.** Effects of PHB modulation on AR recruitment to chromatin. (a) Western blot analysis for PHB, AR and Histone H3 on chromatin fractions (or whole cell extract, bottom panel) of starved LNCaP/Luc/PHB-RNAi cells ± doxycycline treated with DHT for 0 – 240 minutes. (b) Western blot analysis for AR, Histone H3, and PHB on chromatin fractions or whole cell extract taken from LNCaP/Luc/PHB-cDNA cells (± doxycycline). Densitometry data for each blot are given underneath. (c) Densitometry analysis of AR band density from western blots of chromatin fractions taken from LNCaP/Luc/PHB-RNAi cells (± doxycycline) and the scrambled control line treated with hormone for 0 – 360 minutes. Bars represent the mean from three western blots normalised to histone H3 levels and then plotted normalized to time = 0 for the appropriate dataset. \*\* = P<0.01, \* = P<0.05 (t-test analysis). Left hand side shows response to DHT, right hand side shows response to androstenedione. Data are from a representative LNCaP/Luc/PHB-RNAi clone.

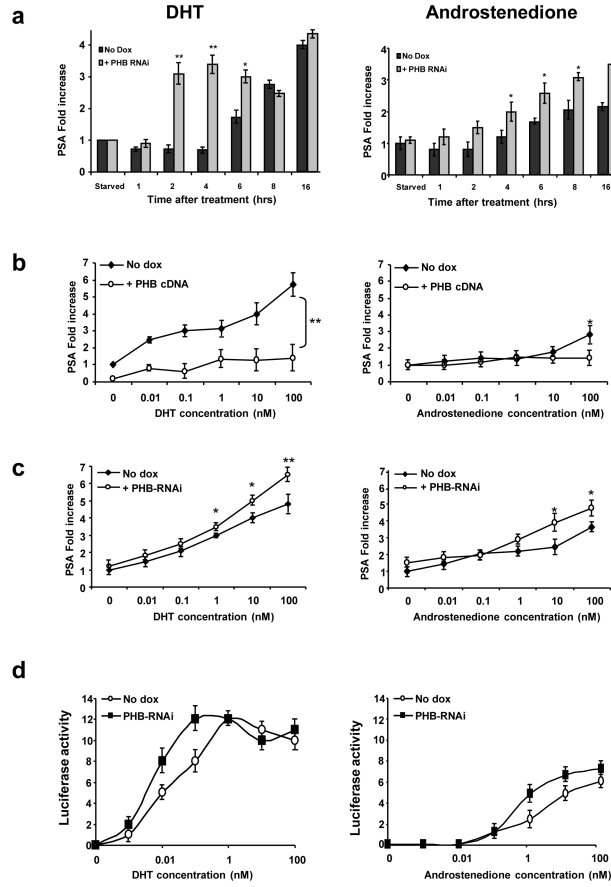


**Figure 3.** ChIP analysis of AR and PHB on the *PSA* regulatory region. (a) Diagrammatic representation of the *PSA* promoter indicating locations of ARE I, II, III and the intervening regions (negative and up/down-stream). Labelling boxes indicate amplification regions of primer pairs used for PCR. DNA immunoprecipitated with either IgG control, AR or PHB antibody was amplified by PCR and the results for each region are shown underneath, compared to their respective input DNA control. Densitometry data for each band for PHB are given underneath. (b) ChIP analysis of AR and PHB binding to the *PSA* promoter of LNCaP cells either grown in full serum, or hormone-starved with  $\pm 10$ nM DHT and for 2hr. Data represents Taqman quantification of immunoprecipitated DNA, from three replicate experiments, normalised to their input DNA controls. (c) ChIP analysis of AR binding to the *PSA* promoter in LNCaP/Luc/PHB-RNAi cells after treatment with DHT for 0-2hrs ( $\pm$  doxycycline). (d) ChIP analysis of PHB binding to the *PSA* promoter in LNCaP/Luc/PHB-RNAi cells after treatment with DHT for 0-2hrs ( $\pm$  doxycycline). Data are from a representative LNCaP/Luc/PHB-RNAi clone. \*\* =  $P < 0.01$ , \* =  $P < 0.05$  (t-test analysis).

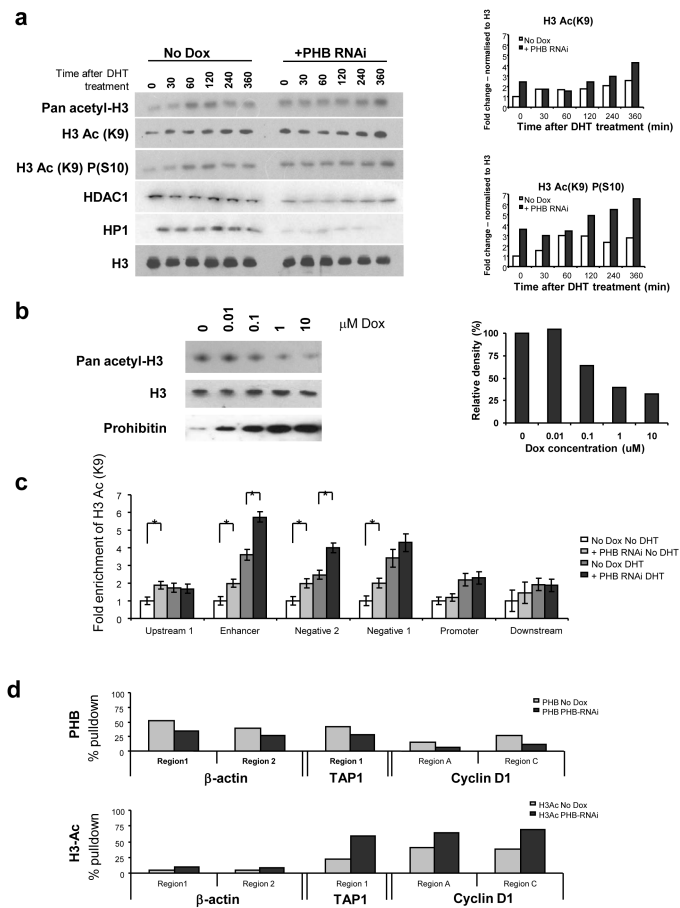




**Figure 4.** ChIP analysis of AR and PHB on the promoter and enhancer regions of the *PSA* promoter. (a) ChIP analysis of AR binding to the *PSA* promoter in LNCaP/Luc/PHB-RNAi cells after treatment with DHT for 0-2hrs ( $\pm$  doxycycline). (b) ChIP analysis of AR binding to *PSA* promoter in LNCaP/Luc/PHB-RNAi cells after treatment with androstenedione for 0-4hrs ( $\pm$  doxycycline). (c) ChIP analysis of PHB binding to the *PSA* promoter in LNCaP/Luc/PHB-RNAi cells after treatment with DHT for 0-2hrs ( $\pm$  doxycycline). Data are mean  $\pm$  SD of 2 independent experiments performed in triplicate on a representative LNCaP/Luc/PHB-RNAi clone. \*\* =  $P < 0.01$ , \* =  $P < 0.05$  (t-test analysis).

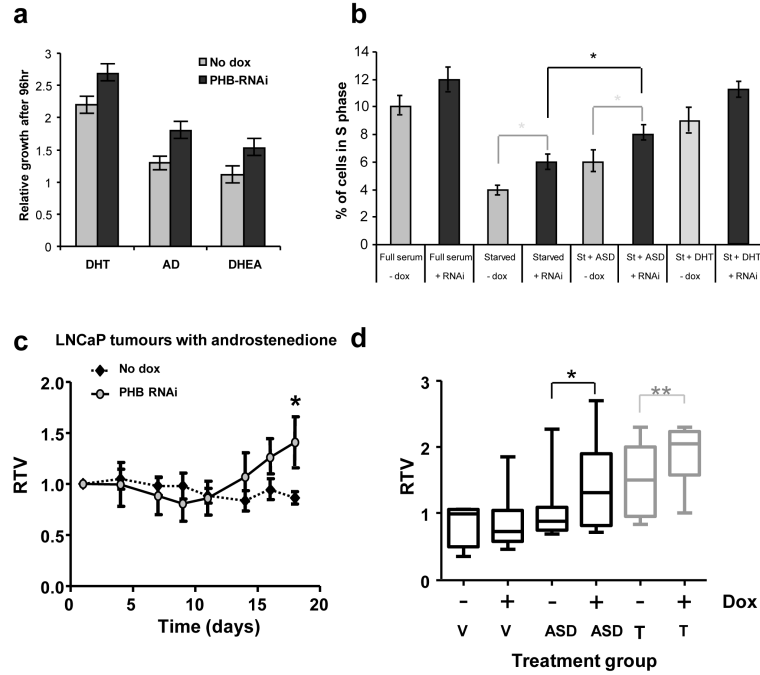


**Figure 5.** Analysis of gene expression in LNCaP cell lines with altered PHB levels. (a) Taqman RT-PCR analysis of PSA transcript levels collected at time intervals (0 – 8hr) from starved LNCaP/Luc/PHB-RNAi cells treated with 10nM DHT (left hand side) or 10nM androstenedione (right hand side) with or without doxycycline. (b) Taqman RT-PCR analysis of PSA transcript levels from starved LNCaP/Luc/PHB-cDNA treated with increasing concentrations of DHT or androstenedione. (c) Taqman RT-PCR analysis of PSA transcript levels from starved LNCaP/Luc/PHB-RNAi cells treated with increasing concentrations of DHT or androstenedione ( $\pm$  doxycycline). (d) Luciferase activity from LNCaP/Luc/PHB-RNAi cells treated with DHT (0-100nM) or Adione (0-100nM) with or without doxycycline. Data are mean  $\pm$  SD of 3 independent experiments performed in triplicate on a representative LNCaP/Luc/PHB-RNAi clone.



**Figure 6.**

PHB knockdown increases global histone H3 acetylation and Histone H3 K9-acetylation of the *PSA* promoter. (a) Western blot analysis of chromatin fraction from a representative clone of LNCaP/Luc/PHB-RNAi cells ( $\pm$  doxycycline) treated with DHT for 0 – 240 minutes. Ac = acetyl, P = Phospho. Densitometry data are given alongside, normalised to Histone H3. (b) Western blot analysis of PHB, H3 and pan-acetyl H3 in LNCaP/Luc/PHB-cDNA cells treated with increasing doxycycline for 24hours. Densitometry data are given alongside, normalised to Histone H3. (c) ChIP analysis of Histone H3-Ac(K9) binding to the *PSA* promoter in a representative clone of LNCaP/Luc/PHB-RNAi cells after treatment with DHT for 0-2hrs ( $\pm$  doxycycline). \* =  $P < 0.05$  (t-test analysis). (d) ChIP analysis of PHB and Histone H3-Ac(K9) binding to the promoters of  $\beta$ -actin, *TAP1* and *Cyclin-D* in LNCaP/Luc/PHB-RNAi cells after treatment with DHT for 0-2hrs ( $\pm$  doxycycline). \* =  $P < 0.05$  (t-test analysis).



**Figure 7.**

Effects of manipulating prohibitin levels within LNCaP/Luc/PHB-RNAi cells. (a) Cells were grown in starvation medium for 72hrs, either with or without doxycycline and then treated with 10nM DHT, Adione or DHEA. Cell growth was determined at 96hr post treatment by SRB assay. (b) FACS analysis of LNCaP cells which were hormonally starved for 72hr with or without dox and then hormone treated (DHT or Adione at 10nM) for 48hours before. The results for the gated S-phase population is shown. \*\* =  $P < 0.01$ , \* =  $P < 0.05$  (t-test analysis) (c) Relative tumour volume measurements of LNCaP/Luc/PHB-RNAi xenografts grown in castrated nude male mice, treated with or without doxycycline and with either vehicle, or androstenedione daily supplementation (d) Boxplots showing relative tumour volumes of LNCaP/Luc/PHB-RNAi tumours at day 20 (n=8), treated with either vehicle (V), or Adione (A). \*\* =  $P < 0.01$ , \* =  $P < 0.05$  (Mann Whitney analysis). Previous data of similarly treated LNCaP xenografts grown with testosterone (T) supplementation is given for comparison (in grey).

**Table 1**

Serum androstenedione (ASD) concentrations in prostate cancer patients and mice.

|                    | <b>Sample</b>   | <b>Serum ASD Concentration (nM)<sup>1</sup></b> |
|--------------------|---|---|
| <b>Human Males</b> | Normal Range  | 1.7 - 10  |
|                    | Patients on GnRH  | 1.7 - 4.2                                       |
|                    | Patients on GnRH and Bicalutamide                                       | 1.6 – 5.5                                       |
| <b>Male Mice</b>   | Intact Adult Mice<br>(normal range for nude mice<br>used in this study) | 0.3 – 2.0<br>0.5 - 20                           |
|                    | Intact Mice all ages <sup>2</sup><br>(inclusive of pubertal phase)      |   |
|                    | Castrated Mice  | <0.03   |
|                    | Castrated Mice + Tes  | <0.03   |
|                    | Castrated Mice + ASD  | 0.3 – 2.1                                       |

<sup>1</sup>ELISA measurement of serum androstenedione levels from human males both normal and patients undergoing treatment regimens for prostate cancer (upper panel). ELISA measurement of serum androstenedione levels from male nude mice either intact or surgically castrated with and without androgen supplementation (lower panel).

<sup>2</sup>Sources: ALPCO Immunoassays (USA), USCN Life Sciences Inc (USA), Calbiotech (USA).