Human heterochromatin protein 1 isoforms regulate androgen receptor signaling in prostate cancer

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Abstract

Androgen receptor (AR) signaling is critical for the tumorigenesis and development of prostate cancer, as well as the progression to castration-resistant prostate cancer. We previously showed that the heterochromatin protein 1 (HP1) β isoform plays a critical role in transactivation of AR signaling as an AR coactivator that promotes prostate cancer cell proliferation. However, the roles of other HP1 isoforms, HP1 α and HP1 γ , in AR expression and prostate cancer remain unclear. Here, we found that knockdown of HP1 γ , but not HP1 α , reduced AR expression and cell proliferation by inducing cell cycle arrest at G1 phase in LNCaP cells. Conversely, overexpression of full-length HP1 α and its C-terminal deletion mutant increased AR expression and cell growth, whereas overexpression of HP1 γ had no effect. Similarly, HP1 α overexpression promoted 22Rv1 cell growth, whereas HP1 γ knockdown reduced the proliferation of CxR cells, a castration-resistant LNCaP derivative. Taken together, HP1 isoforms distinctly augment AR signaling and cell growth in prostate cancer. Therefore, silencing of HP1 β and HP1 γ may be a promising therapeutic strategy for treatment of prostate cancer.

Key Words

- ▶ androgen receptor
- ▶ castration-resistant prostate cancer
- ▶ heterochromatin protein 1
- ▶ prostate cancer

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Introduction

Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer-related death of men in developed countries (Siegel *et al.* 2012). Prostate-specific antigen (PSA) screening has improved early detection of prostate cancer and has recently been proved to reduce the risk of death by prostate cancer (Schröder *et al.* 2009, 2012, Hugosson *et al.* 2010, Andriole *et al.* 2012). However, a quarter of early-stage prostate cancer patients still suffer from relapse of the disease despite surgical and/or radiation therapy. In addition, many patients with prostate cancer are diagnosed at an

advanced stage of the disease. Although most prostate cancers are originally androgen dependent and respond well to androgen-deprivation therapy (ADT), they eventually become castration-resistant prostate cancer (CRPC) during ADT, which is thought to remain dependent on androgen receptor (AR) signaling for growth in a low-androgen milieu (Ryan & Tindall 2011).

The AR is a member of the class I subgroup of the nuclear receptor superfamily and a ligand-dependent transcription factor. The androgen/AR signaling pathway is thought to play a central role in the development and

progression of prostate cancer including CRPC. The mammalian heterochromatin protein 1 (HP1) family plays a critical role in a variety of cellular processes including centromere stability, telomere stability, regulation of gene expression, DNA repair, cellular senescence, and cancer progression through their activities in DNA and its interacting proteins (Zhang & Adams 2007, Dialynas *et al.* 2008, Ayoub *et al.* 2009). Three mammalian HP1 isoforms, HP1 α (CBX5), HP1 β (CBX1),

and HP1 γ (CBX3), have been identified in humans. Each of these heterochromatins contains two conserved domains, a chromodomain (CD) interacting with dimethylated Lys9 of histone H3 and a chromoshadow domain (CSD) interacting with the PxVxL motif of its partner, which are separated by a flexible hinge region (Kwon & Workman 2011). HP1 isoforms are known to exhibit different subnuclear localizations in interphasic nuclei. HP1 α is mainly centromeric, HP1 β is also centromeric, but to a lesser extent, and HP1 γ is located in both euchromatic and heterochromatic compartments (Dialynas *et al.* 2007). HP1 heteromers are associated with nucleosomal core histones (Zhao *et al.* 2000) and reduce the transcription of nearby promoters when tethered to DNA (Cryderman *et al.* 1999). Furthermore, HP1 isoforms in mice and humans interact directly with the transcriptional corepressor TIF β , supporting the hypothesis that HP1 isoforms play a role in gene silencing (Nielsen *et al.* 1999). However, it has been recently reported that HP1 isoforms may work at euchromatic regions and as a gene activator despite their name, localization, and suggested function (Piacentini *et al.* 2003, Hediger & Gasser 2006, de Wit *et al.* 2007, Fanti & Pimpinelli 2008). On the other hand, in several cancers, HP1 α expression has been reported to be decreased compared with that in the corresponding normal tissues (Pomeroy *et al.* 2002, Wasenius *et al.* 2003). In addition, reduction of HP1 α expression has been observed in various cancers including cancers undergoing progression (Dialynas *et al.* 2008). However, several recent reports show that HP1 isoforms are upregulated in various tumor tissues, compared with

Figure 1

HP1 γ knockdown transcriptionally reduces AR expression. (A) LNCaP cells were transfected with 40 nM of the indicated siRNA. At 72 h post-transfection, quantitative real-time PCR was performed using the indicated primers and probes. The target transcript level was normalized to the *GAPDH* transcript level. All values represent at least three independent experiments. The level of each transcript from cells transfected with control siRNA was defined as 1. Boxes, mean; bars, $\pm$ s.d. * P < 0.05 (compared with control siRNA). (B) LNCaP cells were transfected with 40 nM of the indicated siRNA. At 72 h post-transfection, whole cell extracts were subjected to western blotting using the indicated antibodies. (C) LNCaP cells were transfected with 0.5 μ g/ml of the AR-Luc #3 reporter plasmids, 20 nM of the indicated siRNA, and 0.05 μ g/ml pRL-TK and then incubated for 48 h. Firefly luciferase activity was normalized to the corresponding *Renilla* luciferase activity. All values represent at least three independent experiments. The luciferase activity in cells transfected with control siRNA was defined as 1. Boxes, mean; bars, $\pm$ s.d. * P < 0.05 (compared with control siRNA).

those in normal tissues, and increases according to tumor progression (De Koning *et al.* 2009, Takanashi *et al.* 2009).

Recently, we reported that HP1 β plays a tumor-promoting role in prostate cancer through transactivation of AR signaling as an AR coactivator that promotes prostate cancer cell proliferation (Shiota *et al.* 2010a). However, except for HP1 β , the functions of the HP1 family in AR signaling and prostate cancer cell proliferation remain unknown. Additionally, the roles of each domain of HP1 in AR signaling and cancer cell proliferation remain unclear. Therefore, the aim of this study was to determine the functions of the HP1 family in association with the AR and prostate cancer cell proliferation.

Materials and methods

Cell culture

LNCaP and 22Rv1 human prostate cancer cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum. Passage 10–40 LNCaP cells were used in experiments. A castration-resistant derivative of LNCaP cells, LNCaP-CxR cells (referred to as CxR cells), was established and maintained as described previously (Shiota *et al.* 2010b). The cell lines were maintained at 37 °C with 5% CO₂.

Antibodies

Antibodies against the AR (sc-815) and green fluorescent protein (GFP) (sc-8334) were purchased from Santa Cruz Biotechnology. Anti-HP1 α (#2623) and anti-HP1 γ (#2619) antibodies were purchased from Cell Signaling Technology (Cambridge, MA, USA). Anti-HP1 β (ab49938), anti-PSA, anti- β -actin, and anti-HA antibodies were purchased from Abcam (Cambridge, MA, USA), Epitomics (Burlingame, CA, USA), Sigma, and Roche Applied Science respectively.

Plasmid construction

The AR reporter plasmid (AR-Luc #3) was constructed as described previously (Shiota *et al.* 2010b). Construction of pCMV-HA-HP1 α , pCMV-HA-HP1 β , and pCMV-HA-HP1 γ plasmids expressing N-terminal HA-tagged HP1 α , HP1 β , and HP1 γ proteins respectively has been described previously (Shiota *et al.* 2010a). To prepare GFP-HP1 α , GFP-HP1 β , and GFP-HP1 γ plasmids expressing N-terminal GFP-tagged HP1 α , HP1 β , and HP1 γ proteins, respectively, EcoRI-SalI fragments of HP1 α , HP1 β , and HP1 γ cDNAs

from pCMV-HA-HP1 α , pCMV-HA-HP1 β , and pCMV-HA-HP1 γ were inserted into the EcoRI-SalI sites of pEGFP plasmids (C3; Invitrogen) respectively. The pCMV-HA-HP1 β Δ C (aa 1–116) plasmid expressing the N-terminal HA-tagged C-terminally deleted HP1 β protein was created by deletion of the XhoI fragment from the pCMV-HA-HP1 β plasmid. Other deletion mutants were constructed from GFP-HP1 α , GFP-HP1 β , GFP-HP1 γ , and pCMV-HA-HP1 β plasmids using a KOD Mutagenesis Kit (Toyobo, Osaka, Japan) with the following primer pairs: 5'-GTCGACGGTACCGCGGGCCCGGGAT-3' and 5'-ATCATTGCTCTGCTCTCTCTTTTTTTAG-3' for GFP-HP1 α Δ C (aa 1–112); 5'-ATCGCTCGGGGCTTTGAGAGAGGAC-3' and 5'-AATTCGAAGCTTGAGCTCGAGATCTG-3' for GFP-HP1 α Δ N (aa 113–190); 5'-GGTTTGGAGCCGGAGCGGATTATTG-3' and 5'-GTACTTGACAGCTCGTCCATGCCG-3' for GFP-HP1 β Δ N (aa 116–185); 5'-GTCGACGGTACCGCGGGCCCGGGAT-3' and 5'-AGCAGCATCTCTTTTCTTCTTTTGAT-3' for GFP-HP1 γ Δ C (aa 1–110); 5'-GCTGACAAACCAAGAGGATTTGCCAG-3' and 5'-AATTCGAAGCTTGAGCTCGAGATCTG-3' for GFP-HP1 γ Δ N (aa 111–182); and 5'-GGTTTGGAGCCGGAGCGGATTATTG-3' and 5'-AAGAGCGTAATCTGGAA-CATCGTATGGG-3' for pCMV-HA-HP1 β Δ N (aa 116–185). The integrity of mutated plasmids was confirmed by sequencing. To construct pCMV-HA-HP1 α Δ C (aa 1–112), pCMV-HA-HP1 α Δ N (aa 113–190), pCMV-HA-HP1 γ Δ C (aa 1–110), and pCMV-HA-HP1 γ Δ N (aa 111–182) plasmids, XhoI-SalI fragments from GFP-HP1 α Δ C, GFP-HP1 α Δ N, GFP-HP1 γ Δ C, and GFP-HP1 γ Δ N were inserted into XhoI-SalI sites of pCMV-HA plasmids respectively.

Western blotting

The preparation of whole cell extracts and western blotting were performed as described previously (Shiota *et al.* 2010a,b, 2011a,b).

Knockdown analysis using siRNA

Knockdown analysis using siRNA was performed as described previously (Shiota *et al.* 2010a,b, 2011a,b). Briefly, the following double-stranded RNA oligonucleotides were commercially generated: 5'-CAU-AUCCUGAGGAUGCGGATT-3' (sense) and 5'-UCCGC-AUCCUCAGGAUAUGTT-3' (antisense) for HP1 α siRNA (Sigma); 5'-AUUUAUCAGGAACAUGAGCUCU-CC-3' (sense) and 5'-GGAGAGCUCAUGUCCUGAU-GAAAU-3' (antisense) for HP1 β siRNA (Invitrogen); and 5'-CCAAGAGGAUUUGCCAGAGTT-3' (sense) and

Figure 2

HP1 γ silencing reduces prostate cancer cell proliferation by inducing cell cycle arrest at G1 phase. (A) LNCaP cells were transfected with 40 nM of the indicated siRNA and then cultured. The number of cells was counted at the indicated times. The results were normalized to the number of cells at 0 h. All values represent at least three independent experiments. Boxes, mean;

bars, $\pm$ s.d. * $P < 0.05$ (compared with control siRNA). (B) LNCaP cells were transfected with 40 nM of the indicated siRNA and then cultured. At 72 h post-transfection, cells were stained with propidium iodide and analyzed by flow cytometry. The cell cycle distribution is shown in the upper right of each graph.

5'-CUCUGGCAAUCCUCUUGGTT-3' (antisense) for *HP1* γ siRNA (Sigma). LNCaP and CxR cells were transfected with the indicated amounts of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Luciferase reporter assay, RNA isolation, RT-PCR and quantitative real-time PCR, cell proliferation assay, and flow cytometry

These procedures were performed as described previously (Shiota *et al.* 2010a,b, 2011a,b).

Statistical analysis

A *t*-test was used for statistical analysis. *P* values < 0.05 were considered to be statistically significant.

Results

HP1 γ knockdown transcriptionally reduces AR expression

First, we functionally analyzed AR expression by knock-down using HP1-specific siRNAs in LNCaP prostate cancer cells. *HP1* β has been shown to affect PSA expression but not AR expression as an AR coactivator in our previous study (Shiota *et al.* 2010a). Similarly, *HP1* α knockdown exerted no effect on AR expression. On the other hand, *HP1* γ silencing reduced the AR transcript level (Fig. 1A). Consistently, AR protein expression was also suppressed by *HP1* γ silencing (Fig. 1B), and also major AR-target gene *PSA* expression was suppressed by *HP1* β and *HP1* γ knockdown. In addition, using an AR reporter

plasmid (AR-Luc #3) in a luciferase reporter assay, we found reduced luciferase activity by *HP1* γ silencing, indicating that *HP1* γ knockdown downregulates AR transcription (Fig. 1C).

Figure 3

Expression of the HP1 family by expression plasmids. (A) Schematic representation of full-length and deletion-mutant HP1 isoforms. (B) Whole cell extracts of LNCaP cells transfected with 1 μ g/ml of the indicated expression plasmid and then incubated for 48 h were subjected to western blotting using the indicated antibodies.

HP1 γ silencing reduces prostate cancer cell proliferation by inducing cell cycle arrest at G1 phase

Next, we investigated the effects of HP1 isoforms on prostate cancer cell proliferation by HP1 knockdown. Previously, we showed that HP1 β knockdown reduces prostate cancer proliferation by suppressing AR transactivation (Shiota *et al.* 2010a). Here, we examined the effect of HP1 α and HP1 γ on cell proliferation. The data showed that HP1 γ silencing reduced LNCaP cell proliferation, whereas HP1 α knockdown did not affect cell

growth (Fig. 2A). To reveal the mechanism of cell growth retardation by HP1 γ silencing, we performed cell cycle analysis using flow cytometry. As shown in Fig. 2B, HP1 γ suppression resulted in an accumulation of LNCaP cells in G1 phase, indicating cell growth suppression by inducing cell cycle arrest at G1 phase.

Overexpression of HP1 affects AR expression and prostate cancer cell proliferation

Next, we examined the effect of HP1 overexpression on AR expression and prostate cancer cell proliferation. To overexpress the HP1 family, we constructed HA-tagged HP1 expression plasmids, as well as deletion mutants to investigate the roles of the CD and CSD (Fig. 3A). The expression of HA-tagged full-length and deletion-mutant HP1 isoforms was confirmed by western blotting using whole cell extracts (Fig. 3B). Next, AR transcript levels were examined after overexpression of various HP1 isoforms. As shown in Fig. 4A, overexpression of HP1 α and HP1 α Δ C increased the AR transcript level, which was supported by the results of luciferase reporter assays using an AR reporter plasmid (Fig. 4B). Furthermore, cell proliferation was enhanced by overexpression of HP1 β , which was in agreement with our previous report, as well as full-length HP1 α and HP1 α Δ C (Fig. 4C).

Furthermore, we examined the role of the HP1 family in prostate cancer cell proliferation using another AR-expressing prostate cancer cell line, 22Rv1. Expression of GFP-HP1 isoforms in 22Rv1 cells was confirmed by western blotting as shown in Fig. 5A. After overexpression

Figure 4

Overexpression of HP1 isoforms affects AR expression and LNCaP cell proliferation. (A) LNCaP cells were transfected with 1 μ g/ml of the indicated expression plasmid. At 72 h post-transfection, quantitative real-time PCR was performed using the indicated primers and probes. The target transcript level was normalized to the GAPDH transcript level. All values represent at least three independent experiments. The level of each transcript from cells transfected with mock plasmid was defined as 1. Boxes, mean; bars, $\pm$ s.d. * P < 0.05 (compared with Mock). (B) LNCaP cells were transfected with 0.5 μ g/ml of the AR-Luc #3 reporter plasmids, 0.5 μ g/ml of the indicated expression plasmid, and 0.05 μ g/ml pRL-TK and then incubated for 48 h. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. All values represent at least three independent experiments. The luciferase activity in cells transfected with mock plasmid was defined as 1. Boxes, mean; bars, $\pm$ s.d. * P < 0.05 (compared with Mock). (C) LNCaP cells were transfected with 1 μ g/ml of the indicated expression plasmid and then cultured. The number of cells was counted at 72 h. The results were normalized to the number of cells at 0 h. All values represent at least three independent experiments. Boxes, mean; bars, $\pm$ s.d. * P < 0.05 (compared with Mock).

Figure 5

Overexpression of HP1 isoforms affects 22Rv1 cell proliferation. (A) Whole cell extracts of LNCaP cells transfected with 1 μ g/ml of the indicated expression plasmid and then incubated for 48 h were subjected to western blotting using the indicated antibodies. (B) 22Rv1 cells were transfected with 1 μ g/ml of the indicated expression plasmid and then cultured. The number of cells was counted at 72 h. The results were normalized to the number of cells at 0 h. All values represent at least three independent experiments. Boxes, mean; bars, $\pm$ s.d. * $P < 0.05$ (compared with GFP).

of the HP1 family, 22Rv1 cell proliferation was augmented by HP1 β and more strongly by HP1 α (Fig. 5B).

HP1 expression and function in CRPC

To investigate the relevance of the HP1 family in CRPC, we compared the expression of HP1 isoforms between LNCaP cells and its castration-resistant derivative, CxR cells. We found that HP1 β expression was elevated in CxR cells, as well HP1 β knockdown resulted in decreases of PSA expression and CxR cell proliferation (Shiota *et al.* 2010a). In addition, HP1 α was upregulated, while HP1 γ expression was comparable in CxR cells (Fig. 6A). Next, we examined the effect of HP1 knockdown on AR expression. As shown in Fig. 6B, AR expression was also reduced in CxR cells by HP1 γ silencing, which was similar to that in LNCaP cells (Fig. 6B). As a result, HP1 γ knockdown also suppressed CxR cell proliferation (Fig. 6C).

Discussion

Classically, the HP1 family has been thought to have suppressive effects on cancer growth and progression. For example, it has been reported that HP1 α expression is reduced in invasive human breast cancer cell lines compared with that in non-invasive breast cancer cell lines (Kirschmann *et al.* 1999). Overexpression of HP1 α in invasive cells reduces their *in vitro* invasive potential (Kirschmann *et al.* 2000), whereas reducing its expression increases their invasive potential (Norwood *et al.* 2006). These data suggest that HP1 α acts as a progression suppressor in breast cancer cells. In addition, reduction of HP1 α expression has been observed in metastatic colon cancer cell lines compared with that in non-metastatic cell lines (Ruginis *et al.* 2006), and in papillary thyroid

Figure 6

HP1 expression and function in CRPC. (A) Whole cell extracts of LNCaP and CxR cells were subjected to western blotting using the indicated antibodies. (B) CxR cells were transfected with 40 nM of the indicated siRNA. At 72 h post-transfection, whole cell extracts were subjected to western blotting using the indicated antibodies. (C) CxR cells were transfected with 40 nM of the indicated siRNA and then cultured. The number of cells was counted at the indicated times. The results were normalized to the number of cells at 0 h. All values represent at least three independent experiments. Boxes, mean; bars, $\pm$ s.d. * $P < 0.05$ (compared with control siRNA).

carcinoma compared with that in normal thyroid tissue (Wasenius *et al.* 2003), as reviewed by Dialynas *et al.* (2008). In line with this finding, HP1 isoforms have been reported to be downregulated in mouse prostate cancer (Shapiro *et al.* 2008).

However, recent findings suggest a conceptual shift from a suppressive to promoting role of the HP1 family in cancers. For example, HP1 γ has been shown to be positive in all cancer tissues but is not positive in the matching normal epithelium (Takanashi *et al.* 2009). Consistently, suppression of HP1 γ expression restrains the proliferation of various cancer cell types, but not that of normal cells, suggesting that HP1 γ is a promising therapeutic target (Takanashi *et al.* 2009). Similarly, HP1 α expression is upregulated in various tumor tissues in contrast to the corresponding normal tissues without detectable HP1 α expression, as well as in highly malignant breast cancers with poor patient survival (De Koning *et al.* 2009). In addition, HP1-related methyltransferase G9a has been reported to promote lung cancer invasion and metastasis (Chen *et al.* 2010). In addition, we have recently shown that HP1 β is upregulated with an increased Gleason score in prostate cancer (Shiota *et al.* 2010a).

Takanashi *et al.* (2009) reported that re-expression of HP1 γ by overexpression maintains human pre-adipocytes in an undifferentiated state even with HP1 γ suppression by a differentiation inducer, suggesting a role of HP1 γ re-expression in maintaining the undifferentiated state of cells. Similarly, the HP1 family has been shown to be downregulated in differentiated blood lymphocytes compared with that in their undifferentiated precursors (Gilbert *et al.* 2003, Baxter *et al.* 2004, Ritou *et al.* 2007). Taken together, these results indicate that loss of HP1 γ expression is essential for differentiation, suggesting a function as a safety mechanism for the transition to cellular differentiation. Consistently, our results showed that HP1 γ knockdown suppressed prostate cancer cell growth, whereas overexpression of HP1 γ showed no significant effect on cell proliferation. This result indicates that HP1 γ overexpression is unnecessary for cell proliferation at least in our experimental setting, although it is necessary to maintain cell cycling.

By contrast, HP1 α overexpression promoted prostate cancer cell proliferation, whereas HP1 α silencing exerted no apparent suppressive effect on cell growth, which is consistent with a previous report showing that HP1 α knockdown does not affect cell proliferation (Norwood *et al.* 2006) or cell cycle distribution (De Koning *et al.* 2009). Furthermore, the proliferation-promoting role of HP1 α is consistent with previous findings indicating that

oncogenic E2F (Weinmann *et al.* 2002, Oberley *et al.* 2003) and myc (Li *et al.* 2003, Kim *et al.* 2008) transcription factors positively regulate HP1 α expression. These results appear to indicate a role of HP1 α in cell proliferation as a growth factor, which can be substituted by other factors.

In addition to the tumor-promoting role of HP1 proteins, we revealed a role of HP1 isoforms in AR signaling. We previously reported the role of HP1 β as an AR coactivator in prostate cancer. In addition, we revealed the role of HP1 α and HP1 γ in AR expression: HP1 α overexpression induced AR expression while HP1 γ knockdown reduced AR expression, by overexpression and knockdown methods respectively. Although these findings were not recapitulated by reversing the expression of HP1 α and HP1 γ , they were correlated with cell proliferation, which was consistent with the role of the AR in cell proliferation of prostate cancer. As HP1 β knockdown did not affect cell proliferation in AR-negative PC-3 cells (Shiota *et al.* 2010a), AR signaling may mediate the effect on cell proliferation by HP1, especially HP1 β .

Overexpression of CD- or CSD-deleted HP1 γ partially exerted a similar effect as that of full-length HP1 γ , indicating that both the CD and CSD of HP1 γ can retain partial activity of full-length HP1 γ (Takanashi *et al.* 2009). Similarly, our study showed that HP1 α Δ C exerted similar effects on AR signaling and prostate cancer cell proliferation as those of full-length HP1 α , suggesting a supporting or compensating role of the CD of HP1 α for full-length HP1 α .

Intriguingly, HP1 isoforms are thought to be implicated in the DNA damage response induced by various toxins including oxidative stress (Dinant & Luijsterburg 2009). HP1 is recruited to damaged regions of DNA and facilitates the DNA repair pathway activated by oxidative lesions (Zarebski *et al.* 2009). These findings are interesting because it has been shown that oxidative stress is induced by androgen deprivation (Shiota *et al.* 2010b) and is increased in CRPC (Shigemura *et al.* 2007), which is consistent with upregulation of HP1 α and HP1 β in CRPC cells and their promoting role in AR signaling and cell proliferation in prostate cancer. These results indicate that HP1 isoforms participate in cross talk between oxidative stress and AR signaling in prostate cancer. Moreover, HP1 isoforms may augment therapeutic effects by genotoxic anticancer therapy that exerts oxidative stress, which is supported by the finding that a higher sensitivity to ionizing radiation is also observed in HP1 α - or HP1 β -overexpressing cells (Sharma *et al.* 2003).

In conclusion, HP1 isoforms distinctly promote AR signaling and prostate cancer growth, which may

contribute to progression to CRPC. Therefore, silencing of HP1 β and HP1 γ appears to be a promising therapeutic strategy for treatment of prostate cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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