Enhancing the effectiveness of androgen deprivation in prostate cancer by inducing Filamin A nuclear localization

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Abstract

As prostate cancer (CaP) is regulated by androgen receptor (AR) activity, metastatic CaP is treated with androgen deprivation therapy (ADT). Despite initial response, patients on ADT eventually progress to castration-resistant CaP (CRPC), which is currently incurable. We previously showed that cleavage of the 280 kDa structural protein Filamin A (FlnA) to a 90 kDa fragment, and nuclear localization of the cleaved product, sensitized CRPC cells to ADT. Hence, treatment promoting FlnA nuclear localization would enhance androgen responsiveness. Here, we show that FlnA nuclear localization induced apoptosis in CRPC cells during ADT, identifying it as a treatment tool in advanced CaP. Significantly, the natural product genistein combined polysaccharide (GCP) had a similar effect. Investigation of the mechanism of GCP-induced apoptosis showed that GCP induced FlnA cleavage and nuclear localization and that apoptosis resulting from GCP treatment was mediated by FlnA nuclear localization. Two main components of GCP are genistein and daidzein: the ability of GCP to induce G2 arrest was due to genistein whereas sensitivity to ADT stemmed from daidzein; hence, both were needed to mediate GCP’s effects. FlnA cleavage is regulated by its phosphorylation; we show that ADT enhanced FlnA phosphorylation, which prevented its cleavage, whereas GCP inhibited FlnA phosphorylation, thereby sensitizing CaP cells to ADT. In a mouse model of CaP recurrence, GCP, but not vehicle, impeded relapse following castration, indicating that GCP, when administered with ADT, interrupted the development of CRPC. These results demonstrate the efficacy of GCP in promoting FlnA nuclear localization and enhancing androgen responsiveness in CaP.

Endocrine-Related Cancer (2012) 19 759–777

Introduction

Prostate cancer (CaP), both localized and involving distant metastases, depends on the androgen receptor (AR) for growth and survival. Therefore, metastatic CaP is treated with androgen deprivation therapy (ADT; Catalona 1994); however, patients on this treatment eventually relapse, indicative of the development of castration-resistant CaP (CRPC). Although a number of FDA-approved treatments for CRPC are currently available, the condition remains essentially incurable, with high mortality rates. Response rate to ADT and time to progression, however, vary among
patients, and therefore, the goal of the present project was to conduct preclinical studies to identify therapy that ensures sensitivity to ADT in the majority of patients, in order to increase the survival rate in this population.

Previously, we demonstrated that the structural protein Filamin A (FlnA), which promotes actin stress fiber formation in the cytoplasm, is localized to the nucleus of androgen-dependent cells, where it impeded CRPC development (Wang et al. 2007, Bedolla et al. 2009). 88.4% of metastatic deposits from CRPC patients expressed cytoplasmic FlnA, whereas 75% of hormone-naive localized tumors expressed nuclear FlnA (Bedolla et al. 2009). In support of these observations, in vitro studies showed that cytoplasmic localization of FlnA was associated with increased cell motility and invasion (Bedolla et al. 2009) whereas nuclear localization was associated with castration-sensitive growth (Wang et al. 2007). Our results indicated that therapies that promoted the induction of FlnA nuclear translocation would enhance the effectiveness of ADT. The overall goal of these studies was to identify a clinically safe drug that can promote FlnA localization to the nucleus.

Full-length FlnA (280 kDa) is mainly cytoplasmic and consists of an N-terminal actin-binding domain followed by 24 repeats, each 96-amino acids long, interrupted by two hinge domains H1 and H2 (van der Flier & Sonnenberg 2001). Proteolysis of FlnA by cleavage at H1 between repeats 15 and 16 created a 170 kDa N-terminal fragment and a 110 kDa C-terminal fragment, which was further cleaved at H2 between repeats 23 and 24, to yield a 90 kDa fragment, which can translocate to the nucleus. Surprisingly, FlnA was also found to localize to the nucleolus, where it inhibited rRNA production (Deng et al. 2012), but the mechanism of transportation to that organelle is currently unknown. The hinge domain of the AR binds to the C-terminal domain of FlnA at repeats 18–19 and co-localizes to the nucleus (Ozanne et al. 2000, Loy et al. 2003). Phosphorylation of FlnA at Ser 2152 prevented cleavage of the protein (Gorlin et al. 1990), whereas dephosphorylation of FlnA induced nuclear localization and promoted sensitivity to ADT (Wang et al. 2007, Bedolla et al. 2009). Despite the attractiveness of using FlnA to enhance androgen sensitivity, the translational potential of these studies, until now, was impeded by a lack of clinically safe drugs that prevent FlnA phosphorylation and promote its cleavage.

Here, for the first time, we show that genistein combined polysaccharide (GCP), a natural product, induces FlnA nuclear localization. GCP consists of 9% genistein, 6% daidzein, 2% glycitin, 3% equol, 15% lipid, 5% protein, and 60% carbohydrate (deVere White et al. 2010). An initial case study reported that 1.5 g GCP daily for 6 weeks caused tumor regression in a 63-year-old man presenting with T3, Gleason grade 6 (3+3) CaP, and reduced serum levels of prostate-specific antigen (PSA) from 19.4 to 10.2 ng/ml in 3 weeks, indicative of reduction in AR activity (Ghafar et al. 2002). Following this report, the effect of GCP in CaP was investigated in a number of laboratories. In vitro and in vivo studies revealed that GCP reduced cell growth in both androgen-dependent and -independent cells (Bemis et al. 2004, Vinall et al. 2007). GCP markedly suppressed mTOR-p70S6K signaling and attenuated excessive androgen signaling, which is a hallmark of advanced CaP (Tepper et al. 2007, Vinall et al. 2007).

Clinical studies from our institute in men with localized CaP demonstrated the safety of GCP and resulted in no change or decline in PSA (deVere White et al. 2004). In an additional study of GCP alone in men with a diagnosis of CaP but no prior treatment, participants showed no evidence of metastasis; however, serum genistein levels did not correlate with PSA (deVere White et al. 2010). These studies encouraged investigation of GCP in advanced disease. In vitro studies showed that GCP treatment in combination with AR knockdown (Vinall et al. 2007) or co-treatment with the AR antagonist bicalutamide (Burich et al. 2008) had enhanced efficacy. However, the mechanism of enhanced efficacy of GCP in the absence of androgens was unknown. Although GCP is known to inhibit the PI3K/Akt/mTOR pathway, its effect on AR suppression was independent of this pathway (Tepper et al. 2007). The lack of a mediator of GCP action on the AR severely impaired efforts to understand its role in CaP control, until now.

Collaboration between two independent teams investigating the actions of GCP and FlnA, as shown here, finally led to the demonstration that GCP targets FlnA and cleaves this molecule to the 90 kDa fragment, which can then translocate to the nucleus and impede relapse following ADT. Our results show that nuclear localization of FlnA promotes androgen dependence and that the cooperative effect of GCP and ADT in CaP results from simultaneous effects of these two treatments on this molecule both in vitro and in vivo. Our results therefore identify GCP-induced FlnA nuclear localization as a therapeutic module that enhances the efficacy of ADT.
Materials and methods

Cell culture and materials
LNCaP and CWR22Rv1 (ATCC, Manassas, VA, USA), C4-2 cells (Urocor, Oklahoma City, OK, USA), CWR-R1 cells (Dr Elizabeth Wilson, University of North Carolina), and PC-346C cells (Dr W M van Weerden, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands) were cultured in fetal bovine serum (FBS) or charcoal-stripped serum (CSS) as indicated. All cell lines used here were investigated for the presence of contaminants and their cellular origins were verified before use. The following plasmids were used in the transfections: pCMV-FlnA, FlnA(16–24), and FlnA(1–15) plasmids kindly provided by Dr E W Yong, National University of Singapore, Singapore; PSA-luciferase construct (hPSA-luc) kindly provided by Dr XuBao Shi, University of California Davis. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s specifications based on established protocols.

Materials used
GCP and bicalutamide were kindly provided by AminoUp, Japan, and AstraZeneca, Cheshire, UK respectively. PKI(14–22) was from Calbiochem (Billerica, MA, USA), dihydrotestosterone (DHT) and genistein were from Sigma–Aldrich, and daidzein was from Fisher Scientific (Waltham, MA, USA). PKI, genistein, daidzein, and bicalutamide were dissolved in DMSO, while DHT was dissolved in ethanol. For in vitro studies, GCP was dissolved in a solution of 50% DMSO and 50% ethanol, whereas in vivo, it was provided as a suspension in peanut oil.

Subcellular fractionation
Cells were collected in 0.5 ml Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, and 0.5 mM dithiothreitol) containing 200 μl 10% IGEPAL and protease inhibitors (0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml each phenanthroline, leupeptin, aprotinin, and pepstatin A). Following 10 min of incubation at room temperature, the lysates were transferred to ice and centrifuged at 4 °C at 250 g for 5 min and the supernatant collected as the cytosolic fraction. The pellet containing the nuclei was resuspended in 150 ml Buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, and 10% glycerol) containing the same protease inhibitors and solubilized by vigorous shaking using a sonicator at 4 °C for 2 h. The suspension was then centrifuged at 4 °C as before for 5 min and the supernatant collected as the nuclear fraction.

Antibodies used
Rabbit polyclonal antibodies against β-actin, AR, cyclin A, cyclin B, and cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse MABs against Akt, GAPDH, and α-tubulin along with rabbit polyclonal antibodies against caspase 3, lamin A, poly (ADP-ribose) polymerase (PARP), and phospho-FlnA (S2152) were from Cell Signaling (Beverly, MA, USA). Mouse monoclonal anti-FlnA antibody (C-terminal) was from Millipore (Billerica, MA, USA), rabbit polyclonal anti-FlnA antibody (C-terminal) (for immunofluorescence) was from Abcam (Cambridge, MA, USA), and rabbit polyclonal anti-FlnA (N-terminal) was from Santa Cruz Biotechnology.

RNA inhibition
Anti-FlnA siRNA duplex with the following target sequence was purchased from Dharmacon Research, Inc., Lafayette, CO, USA: 5'-CAACGGTTGTAGTCATTGT-3'. A pool of four duplexes sold as AR siRNA (Santa Cruz Biotechnology) with the following sequences: Strand #1: 5'-CAGUCCACUGUGUCAAATT-3', Strand #2: 5'-CCUGAUCUGUGGAUAUGUATT-3', Strand #3: 5'-GUCGUCAUGGAAUGUATT-3', and Strand #4: 5'-GACACACGCAUUGAAATT-3'. Control was a pool of four scrambled nonspecific siRNA duplex (siCONTROL Non-Targeting siRNA Pool, Dharmacon Research, Inc.).

Analysis of cell proliferation or apoptosis using flow cytometry
Cells were grown under desired conditions in 60 mm dishes at 1 × 10^6 cells/dish. Flow cytometry was conducted on FACSCalibur (Becton Dickinson Immunoctometry Systems, San Jose, CA, USA). Cells were illuminated with 200 mW of 488 nm light produced by an argon-ion laser and 635 nm light produced by a red-diode laser. Fluorescence was read through a 630/22 nm band-pass filter (for propidium iodide) or a 661/16 nm band-pass filter (for Annexin V-Alexa Fluor 647). Data were collected on 20 000 cells as determined by forward and right angle light scatter and stored as frequency histograms; data used for cell cycle analysis were further analyzed using MODFIT (Verity Software, Topsham, ME, USA).
3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay

Cells were cultured in 24-well plates and treated as indicated. Following treatment, each well was incubated with 25 μl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma–Aldrich) for 1 h in a 5% CO₂ incubator at 37 °C, which converted the reactants to formazan in actively dividing cells. Proliferation rates were estimated by colorimetric assay reading formazan intensity in a plate reader at 562 nm.

Western blotting

Whole-cell extracts were prepared by lysing cells in 300 μl cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1% NP-40) containing the protease inhibitors 0.1 mM benzamidine, 1 mM PMSF, and 10 mg/ml each of phenanthroline, leupeptin, aprotinin, and pepstatin A and phosphatase inhibitors 20 mM β-glycerol phosphate, 1 mM Na-orthovanadate, and 10 mM NaF. Proteins were quantitated by BCA assay (Pierce, Rockford, IL, USA) and fractionated on 29:1 acrylamide-bis SDS–PAGE. Electrophoresis was performed at 150 V for 2 h using mini vertical electrophoresis cells (Mini-PROTEAN 3 Electrophoresis Cell, Bio-Rad). The gels were electroblotted for 2 h at 200 mA using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) onto 0.2 μm polyvinylidene difluoride membrane (Osmonics, Westborough, MA, USA). The blots were stained overnight with primary antibodies at 4 °C and detected by enhanced chemiluminescence (Pierce) following incubation with a peroxidase-labeled secondary antibody (donkey anti-mouse IgG or goat anti-rabbit IgG, F₂ specific, Jackson ImmunoResearch, West Grove, PA, USA).

Immunofluorescence

Cells were rinsed with PBST (PBS with 0.05% Tween 20) and fixed with ice-cold methanol for 15 min at room temperature. Fixed cells were washed three times with PBST and blocked with 10% BSA for 30 min at room temperature. Primary antibody, prepared in 1% BSA, was applied to the cells, which were incubated at 4 °C overnight. Cells were then washed three times with PBST and FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch), diluted 1:1000 in 1% BSA, was added and incubated for 30 min at room temperature in the dark. After washing three times with PBST, slow fade mounting medium with DAPI (Invitrogen) was applied to the slides before mounting of the coverslips.

Quantitative real-time PCR

Total cellular RNA was prepared from cells using RNeasy mini kit (Qiagen, Inc.) based on the manufacturer’s protocol. cDNA was synthesized from 1 μg RNA using QuantiTect reverse transcription kit based on manufacturer’s protocol. cDNAs were diluted 1:4 in ddH₂O and 2 μl diluted cDNA were added to 5 μl EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen) and 200 nM of each primer. GAPDH was used as the endogenous expression standard. PCR conditions were 20 s of initial denaturation step at 95 °C, 40 cycles at 95 °C for 3 s, and 60 °C for 30 s, followed by additional 95 cycles starting at 60 °C with 0.5 °C increase per cycle for melt curve analysis. GAPDH forward: 5'-TGACACCCACACTGCTTA and reverse 5'-AGGCAAGGATGTGTT; FlnA forward 5'-AAGTGACCCCAATAACGAC and reverse 5'-GGCGTCAACCCTGTGACTTAT.

Evaluation of mouse retrovirus

Cell lysates were prepared using RIPA Buffer containing protease and phosphatase inhibitors and normalized to 2 μg/μl using BCA Protein Assay (Pierce). Lysate (50 μg) was loaded per lane, run on a 12% SDS–PAGE gel, and transferred using semidry transfer unit (Bio-Rad) at 15 V for 70 min. Gag antibody (1:250) and β-actin antibody (1:10 000, Sigma A-1978) were diluted in 5% Milk Protein TBS Tween and incubated overnight with rocking at 4 °C. Membranes were washed 4× TBS Tween for 10 min at room temperature and then incubated with goat anti-mouse secondary antibody (1:5000, Santa Cruz SC-2005). Membranes were washed again 4× TBS Tween for 10 min at room temperature and incubated for 1 min in SuperSignal West Pico Chemiluminescent Substrate (Pierce 34080). Membranes were drained of excess fluid and images were taken using FluorChem E Imager and analyzed using AlphaView SA software (Protein Simple, Santa Clara, CA, USA).

Mouse xenograft studies

Four- to five-week-old athymic nude male mice (n = 24, Harlan, Indianapolis, IN, USA) were implanted with 12.5 mg, 90-day release testosterone pellets (Innovative Research of America, Sarasota, FL, USA) before injection of CWR22 prostate tumor suspension s.c. in the right flank. These experiments were conducted under an IACUC approved protocol. Cells were mixed 1:1 with 50% Matrigel (BD Biosciences, San Jose, CA, USA) before s.c. injection (2.5 × 10⁶ cells per injection). When palpable tumors were observed, their pellets were removed, and the
animals were left untreated \((n=6)\) or treated with vehicle \((n=8)\) or GCP \((1.2 \text{ mg/kg per day}) (n=10)\) as a suspension in water dispersed in peanut oil by esophageal gavage for 14 consecutive days, after which drug treatment was discontinued. Control animals were given water droplets dispersed in peanut oil only \((n=8)\). Uniformity of the suspension was maintained by vigorous shaking before gavage for each animal. Twenty-one days after start of experiment, the animals were castrated \((n=22)\) and/or left intact (sham operated, \(n=2)\). Sham-operated mice were opened up but then closed again without castration. Two mice from each group (sham castrated, castrated-untreated, castrated-vehicle treated, castrated-GCP treated) were killed 3 days post-castration for molecular analysis. Four others were killed due to large tumor size. The remaining 12 mice (six on GCP and six on vehicle) were used as described in the Results section. Tumor dimensions were measured twice a week using calipers. At the end of the study, tumor-bearing mice were killed using CO\(_2\) gas followed by cervical dislocation.

**Immunohistochemistry**

Mouse tumors were fixed in 10% buffered formalin (Medical Industries, Richmond, IL, USA) at room temperature. The tumor was paraffin embedded and processed based on established protocols. Paraffin-embedded cell blocks were then sectioned and sections were heated to 60 °C, cleared, and rehydrated in xylene and graded alcohols. Antigen retrieval was performed with 10 mM citrate buffer at pH 6.0 for 10 min in a pressure chamber at 121 °C and 10 more minutes without pressure. Slides were allowed to cool for another 20 min, followed by sequential rinsing in TBS-T \((50 \text{ mM Tris–HCl, pH 7.4, 150 mM NaCl, and Tween 20 (0.1%)}\)). Endogenous peroxidase activity was quenched by incubation in TBS-T containing 3% hydrogen peroxide. Slides were then blocked with 10% BSA. Each incubation step was carried out at room temperature and was followed by three sequential washes \((5 \text{ min each})\) in TBS-T. Sections were incubated in mouse monoclonal anti-FlnA antibody against the C-terminal (Millipore), diluted in TBS-T containing 1% BSA \((1 \text{ h})\), followed by incubations with biotinylated secondary antibody for 15 min, peroxidase-labeled streptavidin for 15 min \((\text{LSAB-2 Dako Corp., Carpenteria, CA, USA})\), and diaminobenzidine and hydrogen peroxide chromogen substrate \((\text{Dako Corp.})\). Slides were counter-stained with hematoxylin and mounted. Negative controls were incubated with Universal Mouse Negative Control \((\text{Dako Corp.})\) in place of primary antibody.

**Statistical analysis**

Tumor volumes were calculated as \(V=\text{length} \times \text{height} \times \pi/6\). The change in tumor volume was calculated as percentage of the volume of the tumor at the time of castration. Analyses using unpaired, two-tailed Student’s \(t\)-test were used to compare tumor sizes of the two study groups: untreated vs GCP treated \(P<0.05\) was considered statistically significant. All in vitro experiments using MTT or luciferase data were performed in triplicate. Data are presented as relative luciferase activity using means of untreated controls as standards, normalized to the corresponding reading for β-gal.

**Results**

**Nuclear localization of a 90 kDa FlnA fragment induces apoptosis in CaP cells**

We previously demonstrated that androgen-sensitive and -insensitive cell lines differentially expressed nuclear localization of FlnA, a structural protein (Wang et al. 2007, Bedolla et al. 2009). A castrate-resistant subline of LNCaP cells, C4-2, has been developed from tumors obtained by co-implantation of LNCaP cells with bone stromal cells in castrated nude mice (Thalmann et al. 1994). We previously showed that C4-2 cells express very high AR transcriptional activity compared with LNCaP cells (Ghosh et al. 2005). Unlike LNCaP, C4-2 cells were not growth arrested by the anti-androgen bicalutamide (Casodex) (Fig. 1A, left), a competitive inhibitor of AR ligand binding (Masiello et al. 2002). C4-2 cells expressed lower levels of 90 kDa FlnA compared with LNCaP (Fig. 1A, right) and lower levels of FlnA in the nucleoplasm (Fig. 1B), but not in the nucleolus. Transfection of a plasmid expressing C-terminal FlnA (FlnA 16–24), which resulted in the appearance of the 90 kDa FlnA fragment (Loy et al. 2003, Wang et al. 2007), restored nuclear expression of FlnA in C4-2 cells (Fig. 1B). This effect required the presence of the AR because it was not observed in AR null PC-3 cells (Wang et al. 2007), but in C4-2 cells expressing full-length AR as well as CWR22Rv1 cells expressing a truncated form of AR (Fig. 1C). Transfection of FlnA 16–24, but not full-length FlnA or FlnA 1–15 \((\text{N-terminal})\), induced apoptosis in cells treated with the AR antagonist bicalutamide (Fig. 1D). Taken together, these results demonstrate that nuclear localization of the 90 kDa fragment of FlnA induces apoptosis in CaP cells.
Figure 1 Nuclear localization of a 90 kDa FlnA fragment induces apoptosis in prostate cancer cells. (A) (Left) LNCaP and C4-2 cells were treated with increasing doses of the anti-androgen bicalutamide for 48 h and the percentage of cells in S-phase was analyzed by flow cytometry in PI-stained cells. (Right) Untreated LNCaP and C4-2 cells. Western blotting revealed that both cell lines expressed equal levels of 280 kDa FlnA, but LNCaP cells expressed higher levels of 90 kDa FlnA compared with C4-2. (B) LNCaP, C4-2, and C4-2 cells stably transfected with FlnA 16–24 (C4-2 16–24) were stained by immunofluorescence for FlnA with an antibody specific to the C-terminus of FlnA. Immunofluorescent staining shows nuclear localization of 90 kDa FlnA in LNCaP cells whereas in C4-2 cells FlnA was mainly localized to the cytoplasm but not in the nucleoplasm, although bright staining in the nucleolus was also detected. In contrast, C4-2 16–24 cells expressed this protein equally in the cytoplasm and the nucleus, while nucleolar staining could still be detected. Scale bar: 10 μm. (C) Subcellular fractionation of CWR22Rv1 cells transfected with 1 μg/ml cDNA expressing FlnA 16–24 showing that the 16–24 fragment goes into the nucleus. Some full-length FlnA enters the nucleus when transfected with FlnA 16–24 due to nucleolar localization. (D) Flow cytometric analysis in PI-stained, ethanol-fixed C4-2 cells to determine the % cells undergoing apoptosis. Apoptosis was considered to be the cells staining with Annexin V and showed that transfection with FlnA 16–24 induced apoptosis in C4-2 cells.
Treatment with GCP replicates the effects of FlNA nuclear localization

The above results indicated that restoration of FlNA nuclear localization likely restored hormone-sensitive behavior in CaP. Therefore, pharmaceutical approaches to FlNA cleavage were pursued. Castration-resistant CWR22Rv1 cells derived from a relapsed CWR22 tumor were chosen for these studies because they express multiple forms of AR – a 114 kDa full-length form containing a H874Y point mutation and an in-frame tandem duplication of exon 3, as well as truncated AR species lacking the ligand binding domain (LBD; Tepper et al. 2002). Hence, treatment that promotes hormone sensitivity in these cells would be widely applicable. Transfection of FlNA 16–24, but not full-length FlNA or FlNA 1–15, promoted expression of the 90 kDa fragment both in the nucleus and in the cytoplasm (Fig. 2A), sensitized these cells to bicalutamide (Fig. 2B), and induced apoptosis (Fig. 2C).

The physiological range of testosterone in FBS is 55.1–97.5 pM whereas in CSS is 15.6–19.0 pM (Sedelaar & Isaacs 2009). Hence, culture in CSS results in ADT in vitro. GCP sensitized CWR22Rv1 cells to ADT, resulting in growth arrest and apoptosis (Fig. 2D), which was enhanced in low androgen levels.

This was accompanied by increases in caspase 3 and PARP cleavage, indicative of apoptosis, especially in low-androgen medium (Fig. 2E). These results demonstrated that the effect of GCP was very similar to that induced by FlNA 16–24.

FlNA cleavage to the 90 kDa fragment mediates GCP-induced apoptosis

We previously showed that the AR activated similar pathways in CWR22Rv1 and CWR-R1, two cell lines established from two independent relapsed CWR22 tumors (Chen et al. 2010a). Similar to CWR22Rv1 (Tepper et al. 2002), CWR-R1 cells were refractory to hormonal treatment (Fig. 3A); significantly, these cells were also sensitized to cytostatic effects of GCP in low-androgen media (Fig. 3B). Investigation of the mechanism of this effect indicated that GCP induced G2/M arrest in CaP cells (Fig. 3C), similar to its main component genistein as has been demonstrated by other laboratories in various cell lines (Lian et al. 1998, Schmidt et al. 2008, Zhao et al. 2009, Han et al. 2010). Further, GCP suppressed the levels of 280 kDa FlNA while causing an increase in the levels of the cleaved FlNA levels (110 and 90 kDa) (Fig. 3D), thereby indicating that GCP causes FlNA cleavage.

Figure 2 Treatment with genistein combined polysaccharide (GCP) replicates the effects of FlNA nuclear localization. (A) Expression of the transfected proteins when CWR22Rv1 cells were transiently transfected for 48 h with 2 μg/ml of an empty vector, full-length FlNA (280 kDa), FlNA 1–15 (170 kDa), or FlNA 16–24 (90 kDa). Actin and lamin A were used as markers of the cytoplasm and the nucleus respectively. (B) As estimated by MTT assay, neither 10 μM bicalutamide nor FlNA 16–24 individually had significant effects on the growth of CWR22Rv1 cells, but in combination, they prevented cell growth. Each point on the graph represents mean ± s.d. of three independent readings. (C) CWR22Rv1 cells underwent apoptosis when transfected with FlNA 16–24 but not when transfected with an empty vector, FlNA 280 or FlNA 1–15 as determined by flow cytometry after 48 h in Annexin V- and propidium iodide-stained cells. (D) (Upper) MTT assay showing effect of CWR22Rv1 cells with GCP or vehicle in FBS- and CSS-containing medium. Each point on the graph represents mean ± s.d. of three independent readings. (Lower) Analysis of apoptosis by flow cytometry in propidium iodide- and Annexin V-stained cells demonstrating that CWR22Rv1 cells treated with 100 μg/ml GCP for 48 h experienced more apoptosis than vehicle-treated cells. Early apoptosis: Annexin V staining, mid-apoptosis: Annexin V + PI staining, late apoptosis: PI staining. Results presented represent numbers after subtraction from untreated cells. Note the increase in apoptosis in cells cultured in CSS vs FBS containing medium. (E) Western blots demonstrating that 100 μg/ml GCP after 48 h increased apoptosis as demonstrated by cleaved caspase 3 and PARP levels.
CWR-R1 and CWR22Rv1 lines had been propagated in mice and were contaminated by mouse retrovirus (Paprotka et al. 2011). On the other hand, LNCaP cells were free from this virus (Sfanos et al. 2011). Similar to CWR22Rv1, GCP induced apoptosis in LNCaP cells, and this effect was more prominent in CSS-cultured cells (Fig. 3E). Therefore, we investigated whether GCP-induced apoptosis was mediated by FlnA. Significantly, FlnA siRNA, but not control siRNA, prevented GCP-induced apoptosis in LNCaP cells (Fig. 3F). These results show that GCP-induced apoptosis is enhanced with ADT and is mediated by FlnA.

GCP’s effects reflect a combination of both genistein and daidzein

Next, we investigated which components of GCP are responsible for its effects. In human patients, the serum levels of genistein and daidzein were determined to be 43 and 51 µmol/l respectively (about 10 µg/ml each in an average adult male), following 6 months of GCP intake at 5 g/day (deVerwe White et al. 2010). At comparable doses, daidzein reduced proliferation in the absence, rather than the presence, of androgens (22.4% decrease in FBS, P = 0.039, vs 69.5% decrease in CSS, P < 0.0001) whereas the effect of genistein remained virtually unchanged in FBS (84.68% decrease, P = 0.002) vs CSS (74% decrease, P < 0.0001) (Fig. 4A). We verified these results in androgen-sensitive PC-346C CaP cells (Fig. 4B; van Weerden et al. 1996, Limpens et al. 2006) that did not express the mouse retrovirus (Fig. 4D). Genistein induced G2 arrest in PC-346C cells, while daidzein growth arrested them at G1 (Fig. 4B), while GCP resulted in growth arrest in both G1 and G2, with very few cells in S-phase. Significantly, genistein, but not daidzein, restored the levels of the 90 kDa FlnA.
fragment (Fig. 4C). Therefore, both genistein and daidzein are needed to replicate the effects of GCP.

**Castration-sensitive LNCaP cells exhibited greater sensitivity to GCP + ADT compared with CRPC C4-2 cells**

LNCaP cells retain the ability to convert testosterone in FBS to effective levels of DHT, a stronger ligand for AR, whereas they are unable to do so in CSS. Culture in CSS induced 55% reduction in LNCaP cell growth ($P=0.0014$) compared with culture in FBS. GCP treatment in FBS also reduced growth by 51% compared with vehicle ($P=0.0006$); however, the combination of ADT and GCP decreased growth by 87% ($P=0.0002$; Fig. 5A), demonstrating greater efficacy of GCP in lower levels of androgens. Flow cytometry showed that GCP induced G2 arrest in LNCaP cells in FBS, whereas in CSS, GCP also depleted cells in S-phase (Fig. 5B). Western blotting of lysates collected from GCP- and vehicle-treated cells in FBS and CSS showed that GCP treatment decreased cyclin D1 levels, completely suppressed cyclin A levels, but increased cyclin B levels, thereby supporting a G2 arrest (Fig. 5C). Further, GCP induced cleavage of PARP, especially in the presence of CSS, indicating the induction of apoptosis (Fig. 5C).

C4-2 cells do not express inherent levels of the 90 kDa fragment, yet GCP induced expression of the 90 kDa fragment in these cells as well (Fig. 5D). Culture in CSS did not affect the ability of these cells to grow, despite the addition of 100 µg/ml GCP (not shown), likely due to aberrant DHT production in C4-2 cells even from castrate levels of testosterone (Cai et al. 2011). In contrast, bicalutamide,
a competitive inhibitor of AR ligand binding, induced growth arrest even in the presence of 20 μg/ml GCP in C4-2 cells (Fig. 5E). The differential effects in FBS and CSS in LNCaP cells, and CSS vs bicalutamide in C4-2, indicate that GCP is ineffective in the presence of ligands, but in the absence of ligands (with bicalutamide) is able to overcome castrate-resistant AR activation.

**Androgen deprivation phosphorylates FlnA and prevents its cleavage to the 90 kDa fragment**

Next, we investigated the molecular mechanism of the process by which GCP sensitized CaP cells to ADT. Prolonged culture in CSS reduced the levels of 90 kDa FlnA (Fig. 6A), whereas supplementation of cells in CSS with 1 nM DHT restored its levels (Fig. 6B). These results indicated that cleavage of full-length FlnA to the 90 kDa fragment is androgen regulated, consistent with the increase in 280 kDa FlnA with CSS seen in Figs 3D and 5C, D. This increase was not related to transcriptional control; levels of FlnA mRNA were decreased by AR siRNA, but not control siRNA (Fig. 6C), implicating the increase in 280 kDa FlnA levels to posttranscriptional modifications. Interestingly, FlnA levels decreased both from the nucleoplasm and the cytoplasm when LNCaP cells were cultured in CSS for prolonged periods (Fig. 6D), but in the nucleolus remained unchanged. Further studies are required to explain how nucleolar FlnA is protected.
C4-2 cells expressed higher levels of FlnA phosphorylation at Ser 2152 compared with LNCaP (Fig. 6E). In both cell lines, culture in CSS stimulated FlnA phosphorylation, but treatment with low doses, but not high doses, of DHT prevented ADT-induced FlnA phosphorylation (Fig. 6E). These results are consistent with the observation that low doses of DHT stimulate whereas high doses inhibit cell growth (Hofman et al. 2001). Loss of 90 kDa FlnA coincided with increased levels of FlnA phosphorylation by ~60% as determined by qPCR in medium with CSS (AD-). (D) LNCaP cells were cultured in either FBS or CSS containing medium, and FlnA protein expression was determined by immunofluorescence. Scale bar: 10 μm. (E) LNCaP and C4-2 cells were cultured in FBS or CSS with increasing levels of DHT as indicated for 48 h. Western blots demonstrate the levels of expression of various proteins. Note that C4-2 cells actually have much lower levels of FlnA 90 kDa; therefore, the blot shown here for C4-2 was at a higher exposure. (F) (Upper) Scheme showing FlnA cleavage at H1 is regulated by phosphorylation at Ser 2152. When FlnA is phosphorylated, it does not undergo cleavage and remains in the cytoplasm, whereas upon dephosphorylation, FlnA is cleaved to the 90 kDa fragment, which translocates to the nucleus. (Lower) LNCaP cells were cultured in FBS or CSS with increasing levels of the PKA inhibitor PKI 14–22 (PKI) for 48 h. Western blotting demonstrated that culture in CSS depleted levels of the 90 kDa FlnA, but treatment with increasing doses of PKA inhibitor in CSS-containing medium restored its expression.

C4-2 cells expressed higher levels of FlnA phosphorylation at Ser 2152 compared with LNCaP (Fig. 6E). In both cell lines, culture in CSS stimulated FlnA phosphorylation, but treatment with low doses, but not high doses, of DHT prevented ADT-induced FlnA phosphorylation (Fig. 6E). These results are consistent with the observation that low doses of DHT stimulate whereas high doses inhibit cell growth (Hofman et al. 2001). Loss of 90 kDa FlnA coincided with an increase in FlnA phosphorylation or with an increase in 280 kDa FlnA, indicating that the level of 90 kDa FlnA results from cleavage of the 280 kDa form or is negatively regulated by FlnA phosphorylation. As a result, FlnA cleavage to the 90 kDa fragment was also regulated by androgens (Fig. 6E).

Additionally, we verified that FlnA phosphorylation regulates its cleavage, using a regulator of protein kinase A (PKA), known to phosphorylate FlnA (Jay et al. 2000, 2004). As we previously showed that a PKA inhibitor increased FlnA cleavage and nuclear translocation (Bedolla et al. 2009), we treated LNCaP cells cultured in FBS or CSS with increasing concentrations of the PKA inhibitor 14–22 (PKI).

Significantly, the PKI had no effect in FBS, but in CSS medium, loss of expression of the 90 kDa fragment was prevented by treatment with the PKI (Fig. 6F). Taken together, the above data demonstrate that ADT suppresses cleavage to 90 kDa FlnA by enhancing phosphorylation at Ser 2152.

**GCP dephosphorylates FlnA at Ser 2152 and promotes the formation of the 90 kDa fragment and FlnA nuclear localization**

In support of data showing that ADT prevents FlnA cleavage to the 90 kDa fragment, 48 h treatment with bicalutamide depleted CWR22Rv1 cells of the 90 kDa FlnA fragment while the level of full-length FlnA increased. In contrast, GCP increased the levels of 90 kDa FlnA while reducing the levels of the full-length protein (Fig. 7A, upper). Comparison of LNCaP and C4-2 cells with vehicle or GCP showed that GCP treatment in C4-2 cells restored FlnA localization to the nucleus (Fig. 7A, lower). These results prompted us to examine the role of GCP on FlnA phosphorylation.

Androgen-dependent PC-346C and LNCaP cells expressed lower levels of phospho-FlnA compared
with the CRPC lines C4-2, CWR22Rv1, and CWR-R1 (Fig. 7B). Hence, we investigated the effect of GCP in CRPC cells. GCP treatment of C4-2 cells prevented FlnA phosphorylation and cleaved the protein to the 90 kDa fragment (Fig. 7C). In support, by immunofluorescence, C4-2 cells expressed higher levels of phospho-FlnA (Ser 2152) compared with LNCaP (Fig. 7D). Incidentally, phosphorylated FlnA was localized in the cytoplasm, showing that FlnA phosphorylation prevented its nuclear translocation. Transfection with FlnA 16–24 in C4-2 cells prevented its phosphorylation, in support for a role of its effector GCP in suppressing FlnA phosphorylation. Culture in CSS increased the levels of FlnA phosphorylation at Ser 2152, which was suppressed by GCP treatment (Fig. 7E). These results show that GCP sensitizes CaP cells to ADT by preventing ADT-induced FlnA phosphorylation.

GCP treatment prevented relapse in the CWR22 xenograft mouse model following ADT

As GCP sensitized CaP cells to ADT, we investigated whether it impeded development of CRPC following ADT. Athymic nu/nu mice were s.c. implanted with CWR22 tumor extracts. When the tumors were palpable, mice were treated with GCP or vehicle as described in the Materials and methods section. Following 2 weeks of treatment, the animals underwent sham operation or were castrated; initial tumors from each group were collected 3 days post-surgery and showed strong Ki67 staining, indicative of proliferation, in sham-operated but not in castrated animals (Fig. 8A). Sham-operated, untreated tumors showed strong nuclear localization of FlnA, while castrated tumors, either untreated or treated with vehicle only, showed weak cytoplasmic, but no nuclear staining for FlnA (Fig. 8B). In contrast, castrated,

Figure 7 GCP prevents phosphorylation of full-length FlnA and promotes cleavage of FlnA to its 90 kDa fragment. (A) (Upper) Western blot showing expression of FlnA in CWR22Rv1 cells. Treatment with 10 μM bicalutamide depleted levels of 90 kDa FlnA while transfection with FlnA 16–24 and further treatment with 100 μg/ml GCP for 48 h rescued the expression of the 90 kDa FlnA fragment. Note higher exposure of left panel compared with right. (Lower) LNCaP and C4-2 cells treated with vehicle or 100 μg/ml GCP were stained with an antibody specific to the C-terminus FlnA to determine subcellular localization. LNCaP cells and C4-2 cells treated with GCP showed FlnA expression in the nucleus while C4-2 cells treated with vehicle showed expression only in the cytosol. Scale bar: 10 μm. (B) Western blot comparing levels of phosphorylated full-length FlnA (Ser 2152) in various cell lines. (C) Western blot demonstrating that GCP prevented phosphorylation of full-length FlnA and increased expression of 90 kDa FlnA. (D) LNCaP cells and C4-2 cells transfected with vector or FlnA 16–24 were stained with an antibody specific for phosphorylated FlnA (Ser 2152) and FlnA phosphorylation levels determined by immunofluorescence. Scale bar: 10 μm. (E) LNCaP cells were cultured in medium containing either FBS or CSS and treated as indicated. Growth in CSS-containing medium increased the phosphorylation of full-length FlnA while treatment with GCP reduced full-length FlnA phosphorylation.
GCP-treated tumors exhibited strong FlnA staining (brown) both in the nucleus and in the cytoplasm (Fig. 8B). Within the first 7 days following castration, the tumors in GCP-treated mice regressed (mean reduction 1148 mm³, n=6) compared with vehicle-treated mice (mean reduction 772 mm³, n=6) (Fig. 8C). The animals were monitored for up to 7 months (211 days) following castration. During this time, control CWR22 xenografts regressed approximately 38 days post-castration (mean reduction 40%, P=0.0008; n=6), after which the tumors stabilized for several months and then relapsed after 6 months post-castration (179 days, relapse was defined as two or more consecutive increases in tumor volume >5% each time) (one control animal had to be killed due to large tumor size). There was essentially no significant difference between the control (n=5) and GCP (n=6) groups until 6 months post-castration, after which, tumors in vehicle-treated mice remained steady relapsed (P>0.05), whereas GCP-treated tumors continued to regress (P=0.0056) (Fig. 8D). After 211 days (7 months post-castration), tumors in

Figure 8 GCP treatment prevented relapse in the CWR22 xenograft mouse model. (A) The tumors were paraffin-embedded, sectioned, and stained with anti-Ki67 antibody to determine the effect of GCP and castration on proliferation. Note that tumors in sham-operated animals were positive for Ki67 staining whereas those that were castrated, either in the presence or in the absence of GCP lacked this staining. Tumors were collected 3 days after the operation. (B) FlnA and AR staining (brown) in tumors from animals who were i) sham operated, untreated, ii) castrated, untreated, iii) castrated, vehicle treated, and iv) castrated, GCP-treated. Note high FlnA staining in the latter group. (C) Both vehicle- and GCP-treated tumors rapidly underwent tumor regression within 7 days following castration. Waterfall plot showing tumor regression in the animals 7 days post-castration. Note that GCP-treated tumors underwent greater levels of regression compared with those in vehicle-fed mice. Red circle denotes one tumor that did not undergo significant change in volume. (D) After the initial regression (up to ~35 days), tumor volume remained essentially steady until 179 days post-castration after which GCP-treated tumors continued to regress while vehicle-treated tumors began to recover and were distinctly larger than the tumors in GCP-treated mice after 211 days.
vehicle-treated mice were distinctly larger (mean reduction 50% compared with initial tumor volumes; \(P=0.25\)) than the ones in GCP-treated mice (mean reduction 88% compared with initial volumes; \(P=0.002\)) (Fig. 8D). Taken together, these results indicated that GCP treatment prolonged the effects of castration (ADT) in this model.

**Discussion**

CaP patients with metastases, treated with ADT, frequently relapse, leading to CRPC, which essentially remains incurable. Studies found that although proliferation indices were consistently suppressed following ADT (Westin et al. 1995, Matsushima et al. 1999), apoptosis was only partially affected (Murphy et al. 1991, Westin et al. 1995). Surviving cells likely undergo growth arrest and lie dormant following ADT, but revive when androgen-independent stimulants release them from growth arrest (Agus et al. 1999, 2002). Hence, our goal was to develop treatments that promote apoptosis during ADT in CaP cells. Here, we show that proteolysis of FlnA to the 90 kDa fragment and its subsequent nuclear localization induces apoptosis and sensitizes AR-positive CaP cells to ADT.

Full-length FlnA is a well-known mediator of cell migration (Feng & Walsh 2004). FlnA proteolysis has two effects: i) it prevents cell migration mediated by full-length FlnA scaffolding interaction between actin on its N-terminal end and actin-binding proteins on its C-terminal end (Bedolla et al. 2009) and ii) the C-terminal FlnA in the nucleus suppresses AR activity (Loy et al. 2003). FlnA had no effect in AR-negative PC-3 cells but inhibited growth of not only LNCaP and C4-2 cells that express a full-length AR with a mutation in its LBD (T877A) but also CWR22Rv1 and CWR-R1 cells that express both full-length and a truncated AR lacking the LBD (Chen et al. 2010a,b). Hence, FlnA is a likely target that may serve to regulate the ability of CaP cells to respond to androgens, and the objective of these studies was to identify clinically safe pharmaceutical agents that regulate FlnA proteolysis.

We previously showed that FlnA cleavage to the 90 kDa fragment and subsequent nuclear translocation suppresses migration (Bedolla et al. 2009). The repeat structure of FlnA allows it to act as scaffold regulating co-localization of various cell migration regulators such as integrins (Ott et al. 1998, D’Addario et al. 2002, Robertson et al. 2003, Travis et al. 2004) and migfilin (Das et al. 2011). Various studies have reported that FlnA promotes cell migration (Nagano et al. 2002, Gawecka et al. 2010), whereas others show that it suppresses migration (Xu et al. 2010). It is likely that the differential effect is caused by a relative expression of intact vs cleaved FlnA. Surprisingly, a recent study indicated the presence of FlnA in the nucleolus where it inhibits rRNA production (Deng et al. 2012). FlnA expresses a nucleolar localization signal, and the C-terminal end of the molecule (FlnA 16–24) is needed to inhibit rRNA proteins (Deng et al. 2012). Our results reveal that despite the decrease in cytoplasmic and nuclear levels of FlnA with ADT, the expression of nucleolar FlnA remains unchanged, indicating that it is protected from degradation. It is important to note that nucleolar FlnA is not phosphorylated (Fig. 7D), hence reflects the cleaved product as well. Further studies are required to determine whether nucleolar FlnA plays a role in AR signaling.

The data presented show that the AR affects both FlnA levels and proteolysis. Androgens promote FlnA cleavage to the 90 kDa fragment; thus, ADT prevents FlnA cleavage and FlnA nuclear (but not nucleolar) localization. Androgens had a biphasic effect on FlnA expression and cleavage. At low androgen levels, there was very little FlnA cleavage whereas at physiological levels of DHT, FlnA cleavage was resumed, likely caused by decreased FlnA phosphorylation (Ohta & Hartwig 1995). However, at very high levels (100 nM DHT), FlnA was again phosphorylated. Therefore, FlnA is likely cleaved and as a result remains in the nucleus only at physiological levels of androgens and is dispersed when these levels are too high or too low. Loss of 90 kDa FlnA may be one cause for resistance to ADT in advanced CaP.

Interestingly, GCP-induced FlnA dephosphorylation promoted FlnA cleavage and nuclear localization. AR levels regulate FlnA expression, phosphorylation, cleavage, and nuclear localization, while FlnA in turn regulates AR transcriptional activity; hence, it is not surprising that the interaction between these factors play a role in the efficacy of ADT. We show that both genistein and daidzein are needed for effective regulation – neither component of GCP, by itself, could achieve this effect. Therefore, GCP-induced G2 arrest is mediated by genistein, while in CSS (but not in FBS), daidzein induced G1 arrest, explaining why GCP caused androgen responsiveness. Other studies had noted combination effects of genistein and daidzein that may contribute to other effects of GCP noted by us. While multiple reports noted that genistein inhibits metastasis in most cancers (Pavese et al. 2010, Zhang et al. 2010), including CaP (Lakshman et al. 2008), some reports noted that genistein promoted metastasis (Nakamura et al. 2011). Daidzein appears to...
prevent genistein-induced metastasis in some models (Singh-Gupta et al. 2010), but not in others (Martinez-Montemayor et al. 2010). Our data indicate that the effect of dietary isoflavones on tumor promotion and metastasis may be related to the ability of the isoflavones to induce FlnA proteolysis.

The kinase mediating FlnA phosphorylation has not yet been identified. The Ser 2152 site on FlnA is a substrate for p90RSK (Woo et al. 2004), PKA (Jay et al. 2000, 2004), and PKCa (Tigges et al. 2003), which are likely candidates. We previously showed that ADT causes the upregulation of ErbB3, a member of the EGFR family (Chen et al. 2010c, 2011). This family stimulates not only PI3K but also the MAPK family, including JNK, which in turn activates p90RSK (Zhang et al. 2001). On the other hand, p90RSK is inhibited by genistein (Gwin et al. 2011) and by daidzein (Kang et al. 2007). Genistein also inhibits EGFR (Aggarwal & Shishodia 2006) and mTOR (Anastasius et al. 2009) and members of our group had demonstrated earlier that mTOR is one of the primary targets of GCP (Tepper et al. 2007). Previous studies have indicated that MAPK regulates mTOR activity via RSK (Roux et al. 2007, Carriere et al. 2008), which may indicate a prominent role for this kinase in GCP-mediated FlnA proteolysis.

Several lines of evidence indicate that GCP-induced FlnA cleavage prevents ligand-independent AR transcriptional activity. First of all, GCP inhibited growth of androgen-dependent LNCaP cells in CSS but had no effect on C4-2 cells in CSS, whereas in the presence of bicalutamide, GCP induced growth arrest in C4-2 cells. Upon ligand binding, the AR undergoes a conformational change that allows it to enter the nucleus and induce transcriptional activity (Kuil et al. 1995). In some CRPC cells, the AR can be activated by non-specific ligands that allow the cells to propagate in CSS and also by ligand-independent AR conformational change, which induces resistance to anti-androgens such as bicalutamide (Feldman & Feldman 2001). Hence, GCP was able to prevent ligand-independent AR activity and overcome the effects of bicalutamide but was ineffective when aberrant ligands activated the AR. Second, GCP and FlnA 16–24 induced growth arrest in CWR22Rv1 cells expressing a truncated AR lacking the LBD. Our data indicate that this effect can be prevented by the presence of nuclear FlnA, but further studies are needed to elucidate the mechanism by which FlnA promotes this effect. In this respect, FlnA is distinct from other AR co-repressors such as NCoR and SMRT and likely explains results showing the inability of the latter to inhibit AR activity in CRPC lines (Laschak et al. 2011).

Finally, we show that in the CWR22 androgen-dependent xenograft model, relapsed tumors following castration were observed in vehicle-treated but not GCP-treated mice, although both tumors regressed at similar rates. This was accompanied by induction of FlnA in GCP. Our current and previous results (Wang et al. 2007) reveal that nuclear localization of the 90 kDa FlnA fragment sensitizes CaP cells to the effects of ADT. These results indicate that due to increased nuclear FlnA, co-administration of GCP together with ADT may be an important therapeutic strategy to prevent CRPC development.

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.

Funding
This work was supported by a Biomedical Laboratory Research and Development (BLRD) service Merit Award (I01BX000400) from the Department of Veterans Affairs and by R01CA133209 from the National Cancer Institute.

Acknowledgements
The authors wish to thank Christopher B Wee and Honglin Chen for their expert technical assistance in mouse xenograft and qPCR studies respectively. They also thank Dr E W Yong, National University of Singapore, Singapore, for pCMV–FlnA, FlnA(16–24), and FlnA(1–15). Human PSA-luciferase construct (hPSA-luc) was kindly provided by Dr XuBao Shi, University of California Davis, Dept of Urology. CWR-R1 cells were provided by Dr Elizabeth Wilson (University of North Carolina) while PC-346C cells were obtained from Dr W M van Weerden, Josephine Nefkens Institute. Casodex (bicalutamide) was kindly provided by AstraZeneca, Cheshire, UK, while GCP was generously provided by Amino Up, Shin-ei, Kiyota, Sapporo, Japan. The work reported here does not represent the views or opinions of the Department of Veteran Affairs or the United States Government.

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Received in final form 30 August 2012
Accepted 18 September 2012
Made available online as an Accepted Preprint 19 September 2012