Regulation of the Transcriptional Coactivator FHL2 Licenses Activation of the Androgen Receptor in Castrate-Resistant Prostate Cancer

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Abstract

It is now clear that progression from localized prostate cancer to incurable castrate-resistant prostate cancer (CRPC) is driven by continued androgen receptor (AR), signaling independently of androgen. Thus, there remains a strong rationale to suppress AR activity as the single most important therapeutic goal in CRPC treatment. Although the expression of ligand-independent AR splice variants confers resistance to AR-targeted therapy and progression to lethal castrate-resistant cancer, the molecular regulators of AR activity in CRPC remain unclear, in particular those pathways that potentiate the function of mutant AR in CRPC. Here, we identify FHL2 as a novel coactivator of ligand-independent AR variants that are important in CRPC. We show that the nuclear localization of FHL2 and coactivation of the AR is driven by calpain cleavage of the cytoskeletal protein filamin, a pathway that shows differential activation in prostate epithelial versus prostate cancer cell lines. We further identify a novel FHL2-AR–filamin transcription complex, revealing how deregulation of this axis promotes the constitutive, ligand-independent activation of AR variants, which are present in CRPC. Critically, the calpain-cleaved filamin fragment and FHL2 are present in the nucleus only in CRPC and not benign prostate tissue or localized prostate cancer. Thus, our work provides mechanistic insight into the enhanced AR activation, most notably of the recently identified AR variants, including AR-V7 that drives CRPC progression. Furthermore, our results identify the first disease-specific mechanism for deregulation of FHL2 nuclear localization during cancer progression. These results offer general import beyond prostate cancer, given that nuclear FHL2 is characteristic of other human cancers where oncogenic transcription factors that drive disease are activated like the AR in prostate cancer.

Cancer Res; 73(16); 5066–79. © 2013 AACR.

Introduction

The androgen receptor (AR) is activated by androgens and recruits transcriptional coregulatory proteins to activate genes required for the survival and proliferation of prostate epithelial cells. Prostate cancer begins as a slowly growing localized disease that can be treated by surgery or radiation. Androgen deprivation therapy is used to treat locally advanced, recurrent or metastatic prostate cancer; however, in many cases, castrate-resistant prostate tumors (CRPC) develop within 2 to 3 years (1). There are no curative treatments for CRPC and the current survival rate is 1 to 2 years following tumor relapse (1). The molecular events underlying the transition from hormone-sensitive prostate cancer to CRPC are unclear; however, the resurgence of AR activity is fundamental and allows cancer cells to evade androgen ablation. AR reactivation may occur via AR gene mutations or alternate splicing, resulting in expression of ligand-independent AR variants (2). Altered expression or activity of AR coregulatory proteins also correlates with more aggressive cancer and a deregulated AR transcriptional network (3), suggesting that a shift in the AR–coregulator relationship may also contribute to CRPC. The challenge of current research is to identify pathways involved in the transition to CRPC, notably those that potentiate ligand-independent AR function and the activity of mutant AR.

The role of four and a half LIM protein 2 (FHL2) in cancer is dependent upon its function in the nucleus as a coactivator or corepressor of multiple transcription factors important in cancer, including the AR (4, 5). LIM domains form a double-zinc finger protein structure that has a role in protein-binding. FHL2 comprises four and a half LIM domains and can bind to multiple...
proteins that regulate transcription (6, 7). FHL2 can also shuttle between the cytoplasm and nucleus (8). The expression of FHL2 is deregulated in many cancers; however, more recent studies have identified significant nuclear accumulation of FHL2 in several cancers including lung (9), colon (10) and prostate cancer (8, 11), which is absent in benign tissue and correlates with disease progression and poor patient prognosis. Therefore, FHL2 nuclear localization may be important during cancer progression. In fibroblasts, stimulation with lysophosphatidic acid or activation of Rho GTPase increases FHL2 nuclear localization (8); however, the mechanism promoting FHL2 nuclear accumulation during cancer progression is unknown.

FHL2 coactivates wild-type AR (4), promotes the proliferation of prostate cancer cells (12), and is integral for prostate cancer cell survival via repression of the transcription factor FOXO1, which functions downstream from the tumor suppressor PTEN (7). FHL2 integrates a key regulatory network through binding CBP/p300 and β-catenin, which function synergistically to coactivate AR (6). The FHL2 gene is also androgen responsive, providing a feed-forward mechanism for robust AR activation (12). siRNA depletion of FHL2 decreases proliferation of androgen-independent prostate cancer cells (12), suggesting a role in ligand-independent AR activation. However, to date, all studies report FHL2 activation of AR is hormone-dependent (4, 6, 8) and a mechanism for ligand-independent AR activation is not known. FHL2 localizes to the nucleus in high-grade, localized prostate cancer, correlating with cancer recurrence following radical prostatectomy (8, 11). However, despite being a well-defined AR coactivator, a role for FHL2 in CRPC has not been examined.

We identify a novel mechanism regulating FHL2 nuclear localization and transactivation function, through interaction with the actin crosslinking protein filamin (13). In the cytoplasm, filamin promotes actin fiber formation and is implicated in cell motility and prostate cancer invasiveness (14). In the nucleus, a truncated filamin fragment directly corepresses the AR (15). Nuclear filamin is also required to maintain the androgen-dependent growth of prostate cancer cells and sensitizes CRPC cells to androgen-deprivation therapy by a mechanism that is largely unknown (16, 17). Therapeutic targeting of nuclear filamin is a recently suggested treatment for CRPC (17). We show that the nuclear localization of FHL2 and subsequent AR activation is driven by calpain cleavage of filamin, a pathway that shows differential activation in prostate epithelial versus prostate cancer cells. Critically, deregulation of the FHL2-AR–filamin complex facilitates ligand-independent activation of AR variants present in CRPC, hence providing mechanistic insight into the enhanced AR activation that drives CRPC progression. Moreover, this is the first study to identify a disease-specific mechanism for the enhanced FHL2 nuclear localization, which is associated with the progression of an increasing number of different cancers.

Materials and Methods

Cell lines

COS1, LNCaP, DU145, and 22Rv1 cells were purchased from American Type Culture Collection (ATCC) and PNT1a were purchased from Sigma Aldrich (European Collection of Animal Cell Cultures; ECACC), and all cultured in RPMI-1640 containing 10% fetal calf serum and 2 mmol/L l-glutamine. ATCC and ECACC test the authenticity of these cell lines using short tandem repeat analyses. PNT1a and 22Rv1 cells were used immediately following receipt. Bulk frozen stocks of LNCaP and DU145 cells were prepared immediately following receipt and used within 3 months following resuscitation; during this period, cell lines were authenticated by AR immunostaining, morphologic inspection, and tested negative for mycoplasma by PCR (November 2012). M2FIL and A7FIL cell lines were from T. Stossel (Brigham and Women’s Hospital, Harvard Medical School, Boston, MA), cultured as previously described (20), and routinely validated by immunoblotting lysates with a filamin antibody (Fig. 1B). The authenticity of noncancer COS1 cells (cultured in Dulbecco’s Modified Eagle Medium 10% Newborn Calf Serum and 2 mmol/L l-glutamine) was not validated.

Luciferase assays

Luciferase assays were conducted by plating 5 × 10^4 cells per 12-well plate. For Gal-FHL2 assays, cells were cotransfected with 500 ng pG5E1b-luciferase, 20 ng phRL-TK (Renilla), 50 ng of pCMXGal-vector or pCMXGal-FHL2, and 500 ng of GFP-vector, GFP-filamin (wild-type), GFP-filamin (Δ1,762–1,764) or HA-vector, HA-calpastatin. COS1, M2FIL, and A7FIL cells were transfected using Lipofectamine (Invitrogen) and 24 hours posttransfection were serum starved or serum starved and then treated with lysophosphatidic acid, ionomycin, or calcimycin. For AR transcriptional assays PNT1a, LNCaP, and DU145 prostate cancer cells were cotransfected using Lipofectamine 2000 (Invitrogen); 1 μg HA-FHL2 or HA-vector; flag-vector, flag-AR full-length, or trAR; His-vector or His-AR-V7; 2 μg Muc-filamin full-length or (R 16–23; 90 kDa filamin fragment) or Muc-vector; 20 ng phRL-TK (Renilla), and 2 μg of ARE3-luciferase or prostate-specific antigen (PSA)-luciferase. For androgen-depletion versus androgen-stimulation cells were cultured in phenol-red–free RPMI media containing 10% charcoal-stripped serum ( Gibco; Invitrogen) ± treatment with 10 nmol/L dihydrotestosterone (DHT) for 24 hours. Enzalutamide (MDV3100; Selleck Chemicals) was used at 10 μmol/L for 24 hours (18). Cells were lysed in passive lysis buffer and luciferase activity measured using the Dual Luciferase Reporter Kit (Promega). All luciferase data were normalized for transfection efficiency by correcting for constitutive Renilla luciferase activity and is presented relative to the relevant control cells.

Prostate sections

Sections of benign prostate tissue and localized prostate cancer were provided by the Australian Prostate Cancer Bio-Resource (Cabrini Human Research Ethics Committee 03-14-04-08; Monash University, Clayton, Victoria, Australia; Human Research Ethics Committee 2004/145MC). CRPC samples were provided by Dr. David Pook, Department of Oncology, Southern Health, East Bentleigh, Victoria, Australia (10247A). Antibody retrieval was conducted as previously described (19) and incubated with primary antibodies overnight at 4°C: FHL2 (1:50, rabbit), filamin (1:1,000, N- or C-terminal), AR-V7 (1:500), and
Figure 1. FHL2 localizes constitutively to the nucleus in filamin-deficient cells. A, FHL2 comprises four and a half LIM domains. LIM domains 3 and 4 were used as “bait” in yeast two-hybrid analysis (top). Filamin domain structure comprising N-terminal actin-binding domain followed by 24 Ig-like repeats (bottom). Two hinge regions are cleaved by calpain (arrows). Using yeast two-hybrid analysis, we identified FHL2 binds repeat 23 of filamin C, B, filamin and FHL2 expression in M2FIL/C0 versus A7FIL+/ cells. β-Tubulin was used as a loading control. C, A7FIL+/ versus M2FIL/C0 cells stained for FHL2 and either filamin, focal adhesions (paxillin), or phalloidin (actin). Scale bar, 100 μm; 25 μm in high magnification images. D, A7FIL+ and M2FIL+ cells were serum starved or lysophosphatidic acid stimulated before staining for FHL2 and nuclei (propidium iodide). Scale bar, 100 μm.
Regulation of Nuclear FHL2 in Cancer by Filamin Cleavage

Results

FHL2 localizes to the nucleus in filamin-deficient cells

To identify new FHL2-binding partners, we used LIM domains 3 and 4 as bait in a yeast two-hybrid screen of a human skeletal muscle cDNA library and identified several interacting clones encoding the Ig-like repeat 23 of filamin C (amino acids 2,500–2,558; Fig. 1A). The 3 filamin isoforms, A, B, and C share significant amino acid homology in repeats 22–24, to which FHL2 binds (Supplementary Fig. S1; ref. 13). Filamin A is expressed in prostate cancer (16) and was used for all subsequent studies. In vitro binding studies confirmed a direct interaction between FHL2 and filamin (Supplementary Fig. S2A). Coinimmunoprecipitation of HA-FHL2 and truncated Myc-filamin (repeats 22–24) was shown, also delineating the FHL2-binding site to this C-terminal region of filamin (Supplementary Fig. S2B).

FHL2 localization was examined in filamin-deficient (M2fil−/) cells compared with the filamin-replete A7fil+ subline (20), which both express FHL2 (Fig. 1B). Cells were imaged using a confocal laser scanning microscope and a single optical section taken at the ventral cell surface for visualization of FHL2 staining at the cytoskeleton. In this focal plane the nucleus is not visible. In A7fil+ cells, FHL2 localized to the cytoskeleton, colocalizing with both filamin and actin, and was also present at focal adhesions, colocalizing with paxillin (Fig. 1C, a–i). In M2fil−/ cells, FHL2 localized only to focal adhesions, consistent with its association with integrins (21), but not actin stress fibers, which were absent due to loss of filamin actin crosslinking (Fig. 1C, m–t; ref. 20). The nuclear localization and transactivation activity of FHL2 was examined in M2fil−/ versus A7fil+ cells following stimulation with lysosphosphatidic acid, which stimulates FHL2 nuclear localization (8). Confocal imaging of A7fil+ and M2fil−/ cells as a Z-stack was used to compare FHL2 nuclear and cytoplasmic staining. In unstimulated A7fil+ cells FHL2 was at the cytoskeleton, but translocated to the nucleus following lysosphosphatidic acid stimulation and in contrast was constitutively nuclear in M2fil−/ cells (Fig. 1D). Subcellular fractionation in unstimulated cells revealed FHL2 was enriched in the nuclear fraction in M2fil−/ cells, with relatively less FHL2 present in the nucleus of A7fil+ cells (Fig. 1E). Subcellular fractionation in unstimulated cells showed FHL2 was enriched in the nuclear fraction in M2fil−/ cells, with relatively less FHL2 present in the nucleus of A7fil+ cells (Fig. 1F). Quantification of the nuclear/cytoplasmic ratio for FHL2 immunostaining (representative images Fig. 1G) further revealed a constitutively higher ratio in M2fil−/ cells than A7fil+ cells under nonstimulated conditions (Fig. 1F). A Gal-FHL2 fusion protein that transactivates a Gal-luciferase reporter is used to quantify FHL2 transactivation activity in the nucleus (4, 8). In unstimulated A7fil+ cells, Gal-FHL2 exhibited low transactivation of the Gal-luciferase reporter, which increased...
Figure 2. Calpain cleavage of filamin induces FHL2 nuclear localization and AR coactivation. A, lysates from prostate epithelial (PNT1a) and prostate cancer (LNCaP and DU145) cell lines (+/−) calpain inhibition, immunoblotted for filamin (C-term), FHL2, and β-tubulin (loading control). B, coimmunoprecipitation of endogenous FHL2 and filamin in prostate cancer cell lines. Nonl, nonimmune control. C, coimmunoprecipitation in COS1 cells coexpressing Myc-FilaminR1–23 (90 kDa filamin fragment) and either HA-vector control or HA-FHL2. D, cells ± calpain inhibition using calpeptin were costained for FHL2 and filamin (C-terminal) and nuclear stain TO-PRO-3 iodide (Topro).
following lysophosphatidic acid stimulation, but constitutively activated transcription in M2FIL/C0 cells irrespective of lysophosphatidic acid treatment (Fig. 1G). Therefore, in the presence of intact filamin, FHL2 is sequestered at the cytoskeleton and in the absence of filamin, FHL2 localizes to the nucleus and activates transcription.

**Calpain cleavage of filamin induces FHL2 nuclear localization**

Filamin is a substrate for Ca$^{2+}$-dependent calpain proteases, with 2 cleavage sites; within hinge I, between repeats 15 and 16, and within hinge II between repeats 23 and 24 (Fig. 1A; ref. 16). Calpain cleavage of filamin generates 2 fragments: N-terminal 170 kDa (repeats 1–15) and a C-terminal 90 kDa fragment (repeats 16–23; Fig. 1A). The effect of filamin-cleavage on FHL2 nuclear localization and transactivation function was examined. Calpain activation was induced in COS1 cells by ionomycin (Ca$^{2+}$ ionophore) treatment, resulting in filamin cleavage (Supplementary Fig. S2C) and FHL2 nuclear localization (Supplementary Fig. S2D) and both events were reduced by calpain inhibition using calpeptin. Calpain activation also increased transactivation of a Gal-luciferase reporter by Gal-FHL2, an effect also abrogated by calpain inhibition using calpeptin (Supplementary Fig. S2E) or the calpain-inhibitory protein calpastatin (Supplementary Fig. S2F; ref. 22). Therefore, calpain activation induces FHL2 nuclear localization and transactivation activity. To address whether these processes are filamin-dependent, complex formation between endogenous FHL2, filamin, and m-calpain was shown (Supplementary Fig. S2G). Furthermore, expression of a calpain-resistant GFP-filamin (Δ1,762–1,764) mutant (23), in M2FIL/C0 cells inhibited the increase in transactivation activity observed for Gal-FHL2 following ionomycin-stimulated calpain activation (Supplementary Fig. S2H). Therefore, calpain cleavage of filamin drives FHL2 nuclear localization.

**Calpain cleavage of filamin is increased in prostate cancer cells, resulting in nuclear FHL2 accumulation and increased AR coactivation**

Calpain expression and activity are increased in prostate cancer (24); therefore, the effect of calpain cleavage of filamin on FHL2 nuclear localization and AR coactivation was assessed in prostate cancer cell lines. Filamin cleavage in a prostate epithelial cell line (PNT1a) versus prostate cancer cell lines (LNCaP and DU145) was compared by
immunoblotting using a C-terminal antibody that recognizes the full-length filamin (280 kDa) and the C-terminal fragment generated following calpain cleavage (90 kDa; ref. 16). A 90 kDa filamin fragment was present in prostate cancer cell lines (LNCaP and DU145) and was partially reduced following calpain inhibition using calpeptin (100 μmol/L for 24 hours), whereas PNT1a cells expressed only full-length filamin (Fig. 2A). Higher doses of calpain inhibitor or longer incubation resulted in cell death and could not be used to further reduce filamin cleavage. Filamin cleavage in LNCaP and DU145 cells was confirmed by immunoblotting with an N-terminal filamin antibody, which recognizes the 170 kDa cleaved fragment (Supplementary Fig. S3A). Coimmunoprecipitation revealed endogenous FHL2 bound only full-length filamin in PNT1a cells and to both full-length filamin and the 90 kDa fragment in LNCaP and DU145 cells (Fig. 2B). The latter interaction was confirmed by coimmunoprecipitation of the 90 kDa filamin fragment (Myc-filaminR16-23) with HA-FHL2 (Fig. 2C).

Intact filamin localizes to the cytoskeleton; however, following calpain cleavage, the 90 kDa filamin fragment translocates to the nucleus and the 170 kDa fragment is retained in the cytoplasm (16). In PNT1a cells, filamin was not cleaved and both FHL2 and filamin (C-terminal antibody) colocalized at the cytoskeleton (Fig. 2D). In LNCaP and DU145 prostate cancer cell lines, which exhibited significant filamin-cleavage, nuclear staining of FHL2 and filamin was observed, which was reduced by calpain inhibition using calpeptin. Ectopic expression of the 90 kDa filamin fragment (Myc-FilaminR16-23) in PNT1a cells induced cytoskeleton to nuclear translocation of FHL2 (Fig. 2E), evidence that the 90 kDa filamin fragment promotes nuclear FHL2. FHL2 coactivation of the AR was examined under conditions of calpain inhibition, using 2 AR-regulated reporters p(ARE3)-luciferase (Fig. 2F) and PSA-

![Figure 3. FHL2 and filamin localize to the nucleus in CRPC and not benign prostate tissue or localized prostate cancer. A, sections of human benign prostate or localized prostate cancer were stained for FHL2, filamin (C-terminal; nuclear fragment repeats 16–23), or filamin (N-terminal; cytoplasmic fragment repeats 1–15). In control studies, benign prostate tissue was stained with nonimmune rabbit or mouse IgG. B, serial sections of human CRPC from 2 representative patients stained for FHL2 and filamin. Boxed regions indicate areas shown in high magnification. Scale bars, 50 μm. Open arrows, basal epithelial cells; closed arrows, luminal epithelial cells; arrowheads, filamin stromal staining. PCa, prostate cancer.](image-url)
Table 1. Frequency of nuclear localization of FHL2, filamin, and AR-V7 in human prostate samples

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<th>Benign</th>
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<td>FHL2</td>
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*P = 0.001.

FHL2 and filamin localize to the nucleus in castrate-resistant prostate cancer

We investigated whether calpain cleavage of filamin regulates FHL2 nuclear localization during prostate cancer progression. Benign prostatic epithelium is composed of multiple cell types including basal and luminal cells, defined by expression of specific CK proteins (28). In benign prostate tissue, FHL2 localized to the cytoplasm of luminal (closed arrow) and basal (open arrow) epithelial cells and filamin was restricted to the cytoplasm of basal cells (Fig. 3A). Coimmunostaining with luminal (CK-8) and basal (CK-5) epithelial cell markers confirmed FHL2 expression in luminal and basal cells and filamin only in basal cells (Supplementary Fig. S6), indicating FHL2 and filamin coexpression occurs in basal cells of benign prostate. The nuclear localization of filamin in benign prostate and localized prostate cancers has been reported (14). In contrast, we observed similar cytoplasmic filamin staining in benign prostate using both N- and C-terminal–specific antibodies (Fig. 3A), indicating that filamin remains intact in the cytoplasm. Similar overlapping cytoplasmic N- and C-terminal filamin antibody staining was observed in the PNT1a prostate epithelial cells (Supplementary Fig. S3B), where filamin was not cleaved (Fig. 2A and Supplementary Fig. S3A). Our data are consistent with the absence of filamin cleavage in benign prostate, correlating with absent nuclear FHL2.

In low-grade localized prostate cancers, FHL2 was restricted to the cytoplasm and filamin was not detected in cancer cells (Fig. 3A). Loss of basal cells is a hallmark of neoplastic foci in localized prostate cancer (29), therefore, the absence of filamin in localized cancer is consistent with filamin expression exclusively in CK5+8–basal cells in benign prostate. Notably, the majority of CRPC samples examined (75%) showed strong nuclear FHL2 staining and a reemergence of filamin staining in the nucleus of cancer cells (Fig. 3B; Table 1). A correlation was also observed between the CRPCs, which exhibited both nuclear FHL2 and filamin (Table 2), suggesting an association between generation of the nuclear filamin fragment by calpain cleavage and FHL2 nuclear localization in CRPC.

Ligand-independent activation of the AR by FHL2

CRPC is driven by ligand-independent AR activation and is associated with high expression of AR variants, which are absent or expressed at low levels in benign prostate or hormone-naive prostate cancer (30). We examined whether FHL2 promotes ligand-independent coactivation of 2 AR variants, trAR (31) and AR-V7 (AR3; refs. 30, 32), which have truncation of the ligand-binding domain (LBD) and promote androgen-independent growth. Interestingly, trAR is generated by calpain cleavage of the AR (31). HA-FHL2 binds both trAR and full-length AR (Fig. 4A). In DU145 cells, FHL2 coactivated full-length AR under androgen-stimulated conditions and, significantly, FHL2 coactivation of trAR was ligand independent (Fig. 4B). The nuclear 90 kDa C-terminal filamin fragment, which we show binds FHL2, also represses AR activity (33). Therefore, we determined whether the FHL2-filamin complex coregulates AR activity. In contrast to FHL2, the 90 kDa filamin fragment (Myc-filaminR16–23) bound...
Figure 4. FHL2 competes with filamin for binding to wild-type AR, but not a truncated AR (trAR). A, coimmunoprecipitation in COS1 cells coexpressing HA-FHL2 (or HA-vector) and either FLAG-tagged full-length AR or trAR. B, DU145 cells were cotransfected with combinations of HA-vector or HA-FHL2; FLAG-vector, FLAG-AR (full-length) or FLAG-trAR, and the AR-responsive reporter pARE3-luciferase. Cells were maintained under androgen-depleted conditions (-Androgen) or stimulated with DHT (+Androgen) and luciferase activity measured. C, coimmunoprecipitation in COS1 cells coexpressing Myc-FilaminR16–23 (or Myc-vector) and either FLAG-tagged full-length AR or trAR. D, competitive-binding assay; cells were cotransfected as indicated and also coexpressed Myc-vector or Myc-filaminR16–23. Lysates were immunoprecipitated (IP) and immunoblotted (WB) as indicated.
full-length AR, but not trAR (Fig. 4C). Competitive-binding studies revealed Myc-filamin16–23 disrupted HA-FHL2 binding to full-length AR, but not trAR (Fig. 4D). Furthermore, FHL2 coactivation of full-length AR (Fig. 4E), but not trAR (Fig. 4F), was repressed by the 90 kDa filamin fragment. Therefore, FHL2 and filamin compete for binding to full-length AR, but not trAR. Moreover, FHL2 coactivation of full-length AR is repressed by filamin, but the ligand-independent activation of trAR by FHL2 is constitutive (Fig. 4G).

Similar results were obtained using the AR-V7 variant, which is generated by alternate splicing of the AR gene (30, 32) and has generated significant interest as a pathologically-relevant AR variant expressed in CRPC (30, 32, 34, 35). AR-V7 bound to FHL2, but not filamin (Myc-filamin16–23; Fig. 5A) and endogenous FHL2, and AR-V7 bind in the 22Rv1 prostate cancer cell line (Fig. 5B). Ligand-independent coactivation of AR-V7 by FHL2 was constitutive and not repressed by the 90 kDa filamin fragment (Fig. 5C and D). AR-V7 is frequently expressed 20-fold higher in CRPC than hormone-naive prostate cancer (30) and correlates with poor survival (34). In serial sections of human CRPC, FHL2 and AR-V7 colocalized in the nucleus of cancer cells (Fig. 5E) and 75% of CRPCs exhibited both nuclear FHL2 and AR-V7 (Table 1, Table 2). Therefore, FHL2 nuclear localization in CRPC may promote constitutive, ligand-independent activation of this clinically important AR variant.

The antiandrogen enzalutamide promotes tumor regression in a mouse xenograft model of CRPC (18) and has progressed rapidly toward approval for treating metastatic CRPC. Enzalutamide acts on the full-length AR by directly binding the LBD (18) and expression of AR variants including AR-V7, which lack the LBD, may confer resistance to this therapy (36, 37). Enzalutamide does not directly inhibit the transcriptional activity of AR-V7 (36) and AR-V7–expressing prostate cancer cells are resistant to enzalutamide (37). FHL2 activation of full-length AR, trAR, and AR-V7 following enzalutamide treatment was examined in DU145 cells that do not express endogenous AR. AR-V7 (Fig. 5F) and trAR (Supplementary Fig. S7A) coactivation of the ARE3-luciferase reporter under androgen-depleted conditions was not affected by enzalutamide treatment and was also increased by FHL2 irrespective of enzalutamide treatment. In contrast, activation of the ARE3-luciferase reporter by full-length AR was reduced by enzalutamide (Supplementary Fig. S7B). Surprisingly, coexpression of FHL2 was sufficient to restore the transcriptional activity of full-length AR in the presence of this antiandrogen (Supplementary Fig. S7B). Therefore, FHL2 can not only sustain enhanced activation of AR variants lacking the LBD irrespective of enzalutamide treatment, but can also reverse the inhibitory affects of enzalutamide on full-length AR.

**Discussion**

We report a novel pathway for AR activation in prostate cancer. In a prostate epithelial cell line FHL2, nuclear localization and AR coactivation are tightly regulated, whereby, the absence of filamin cleavage results in FHL2 sequestration at the cytoskeleton and low AR activity. Conversely, in prostate

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**Figure 4.** (Continued) E and F, filamin represses FHL2 coactivation of wild-type AR (E), but not trAR (F); DU145 cells were cotransfected as indicated and cells also coexpressed Myc-vector or Myc-Filamin16–23. FHL2 coactivation of full-length AR and trAR was measured under androgen-dependent and androgen-independent conditions. For all luciferase assays, data represent the mean from $n = 8 \pm$ SEM; $^{*}, P = 0.05$. G, AR coregulation by the FHL2-filamin complex. The 90 kDa filamin fragment competes with FHL2 for binding to full-length AR and also represses ligand-dependent AR coactivation by FHL2. ARE, androgen-response element; FLNa, filamin A (a); FHL2, but not the 90 kDa filamin fragment, binds to AR variants trAR and AR-V7, such that coactivation of truncated AR variants by FHL2 is constitutive (b).
cancer cell lines, calpain cleavage of filamin drives nuclear FHL2 and enhanced AR activation. In addition, FHL2 localization and hence AR activation in prostate cancer cells is driven both by increased calpain activity and the susceptibility of filamin-to-calpain cleavage following dephosphorylation by calcineurin. Over- and underexpression of FHL2 is reported in prostate cancer (8, 11, 38), we revealed FHL2 nuclear localization by the mechanism identified here may also be important. In tumors, which had progressed to CRPC, nuclear localization of FHL2 and calpain-cleaved filamin was consistently observed in the same patient biopsies supporting our contention that calpain...
mediates filamin and FHL2 nuclear association. Critically, FHL2 and filamin were not detected in the nucleus of benign prostate tissue or low-grade localized prostate cancer (Fig. 5G). The reemergence of filamin staining in CRPC cells (absent in localized cancer) is interesting given the prediction that cancer stem cells, which reinitiate CRPC tumor growth following androgen-deprivation therapy, reside in the CK5+/8– basal cell compartment (28) and are shown here to coexpress FHL2 and filamin. Calpain expression and activity are higher under androgen-depleted conditions (39), suggesting that androgen ablation therapy creates an environment that is not only highly selective for expression of ligand-independent variants including AR-V7 (40), but is also conducive for promoting nuclear FHL2. Therefore, of note are our data showing that FHL2 activates AR-V7 and both proteins colocalize in the nucleus of CRPC.

The FHL2-filamin complex is a novel AR regulatory mechanism that may be deregulated in CRPC

We show that the nuclear 90 kDa filamin fragment competes with FHL2 for binding full-length AR and represses ligand-dependent AR coactivation by FHL2. This is supported by the findings that Ig-repeats 22–24 of filamin bind AR (33), a region that overlaps with the FHL2-binding site (Ig repeat 23) identified here. Therefore, we identify filamin as an important negative AR regulator via modulation of the AR-coactivator relationship, whereby the activation of the prototype AR is mediated by a balance between the coactivator FHL2 and the corepressor filamin (Fig. 4G, a). We showed how loss in this balance due to the absence of filamin repression results in constitutive activation of pathologic AR variants notably AR-V7, by FHL2 (Fig. 4G-b). AR-V7 is a major AR variant present in CRPC that activates a transcriptional program distinct from the prototype AR, including genes, which regulate cell-cycle progression (32, 35). Despite its importance in CRPC, the regulation of AR-V7 is largely uncharacterized, the identification of which may lead to novel therapeutic targets for incurable CRPC. Vav3, a RhoGTPase guanine nucleotide exchange factor, which like FHL2 enhances AR-V7 coactivation, is critical for CRPC growth and survival (41). AR-V7 is also regulated by the phosphoinositide 3-kinase/Akt/FOXO1 signaling pathway, whereby FOXO1 inhibits AR-V7 activity (42). Interestingly, FHL2 represses FOXO1 activity in prostate cancer cells (7). The absence of filamin binding to C-terminally truncated AR variants is consistent with a requirement for the AR–LBD for filamin association (33) and indicates filamin repressive function is limited to the prototype AR. Differential regulation of full-length AR versus truncated AR by the FHL2–filamin complex is an important finding given the prevalence of AR mutations in CRPC (10%–30%), many of which result in AR mutants that lack all or part of the LBD (2). Given that inhibition of calpain activity blocks filamin cleavage, FHL2 nuclear localization, and AR coactivation, we speculate that calpain inhibitors may provide a novel avenue for CRPC treatment. We have further shown FHL2 coactivation of AR-V7 and trAR is resistant to enzalutamide. Expression of FHL2 also restored full-length AR transcriptional activity in enzalutamide-treated prostate cancer cells. Therefore, we predict that FHL2 may reduce the efficacy of enzalutamide treatment of CRPC by maintaining AR function and calpain inhibitors may provide an adjunct to enzalutamide therapy. Targeting calpain activity is suggested for several cancers to limit the development, metastases, and neovascularization of primary tumors (43). Calpain inhibition also decreases the androgen-independent proliferation of Rv1 cells (31) and
prostate cancer invasiveness (44). In contrast, a recent report has suggested that enhancing generation of the nuclear filamin fragment using genistein-combined polysaccharide may be a potential CRPC treatment (17). This comes from observations that the 90 kDa filamin fragment maintains sensitivity of CRPC cells to androgen deprivation (16, 17). Our current results, however, clearly indicate that further studies are required to examine the complex interplay between FHL2, filamin, and the AR in CRPC.

Regulation of nuclear FHL2 by calpain cleavage of filamin may be important in other cancers

Our mechanism for FHL2 nuclear accumulation during cancer progression is likely to have implications for other cancers. FHL2 nuclear localization is increased in human colorectal cancer and during tumor development in the Apc mouse model, where FHL2 suppression inhibits tumor initiation (10). Transcriptional targets for FHL2 in colon cancer include β-catenin (45) and the oncogenic EpICD transcription complex (46). Calpain expression and activity is increased in human colorectal cancer and in colorectal polyps, where it may be an early tumorigenesis event (47) and we predict this may increase filamin cleavage and promote nuclear FHL2. Filamin is a potential diagnostic biomarker for colorectal cancer as it is shed by tumors into patient feces (48). Although this latter study did not examine the presence of the 90 kDa filamin fragment generated by calpain cleavage, of note was the observation that filamin appeared at a lower molecular weight than predicted. Future studies will be directed towards examining calpain-dependent regulation of FHL2 in other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank the Associate Professors John Pedersen and Mark Frydenberg (Departments of Anatomy and Cell Biology, and Surgery, Monash University) for supplying the human tissues and also Dr. Melissa Papargiris (Australian Prostate Cancer Collaboration) for assisting with the acquisition of patient samples.

Grant Support

The Australian Prostate Cancer BioResource is supported by the National Health and Medical Research Council of Australia Enabling Grant (No. 614926) and by a research infrastructure grant from the Prostate Cancer Foundation Australia.

Received December 18, 2012; revised May 30, 2013; accepted June 13, 2013; published OnlineFirst June 25, 2013.

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