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Regulation of the transcriptional coactivator FHL2 during cancer progression, a mechanism for androgen receptor activation in castrate resistant prostate cancer

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Prostate cancer progression to incurable castrate resistant prostate cancer (CRPC) is driven by continued androgen-receptor (AR) signaling and the suppression of AR activity remains the single most important therapeutic target. 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 during CRPC are unclear, particularly pathways which potentiate mutant AR function. Here we identify FHL2 as a novel coactivator of ligand-independent AR variants important in CRPC. We demonstrate that the nuclear localization of FHL2 and coactivation of the AR is driven by calpain-cleavage of the cytoskeletal protein filamin, a pathway which shows differential activation in prostate epithelial versus prostate cancer cell lines. We further identify a novel FHL2-AR-filamin transcription complex, revealing de-regulation of this axis promotes the constitutive, ligand-independent activation of AR variants 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. This study therefore provides mechanistic insight into the enhanced AR activation, most notably of recently identified AR variants including AR-V7 that drives CRPC progression. Importantly, this study is also the first to identify a disease-specific mechanism for the de-regulation of FHL2 nuclear localization during cancer progression. This is a significant finding given the enhanced nuclear FHL2 that is characteristic of several cancers, which can alter the activation of oncogenic transcription factors, as we have shown here for the AR in prostate cancer.
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 tumors (CRPC) develop within 2-3 years (1). There are no curative treatments for CRPC and the current survival rate is 1-2 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 which 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 are a double-zinc finger motif which facilitates protein-interaction and FHL2 can scaffold multi-protein transcription complexes (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 wildtype 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 CPRC has not been examined.

We identify a novel mechanism regulating FHL2 nuclear localization and transactivation function, through interaction with the actin cross-linking 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 co-represses 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 which is largely unknown (16, 17). Therapeutic targeting of nuclear filamin is a recently suggested treatment for CPRC (17). We demonstrate that the nuclear localization of FHL2 and subsequent AR activation is driven by calpain-cleavage of filamin, a pathway which shows differential activation in prostate epithelial versus prostate cancer cells. Critically, de-regulation 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 that 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 ATCC and PNT1a were purchased from Sigma Aldrich (European Collection of Cell Cultures; ECACC), and all cultured cultured in RMPI-1640 containing 10 % FCS and 2mM L-glutamine. ATCC and ECACC test the authenticity of these cell lines using short tandem repeat (STR) 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, morphological inspection and tested negative for mycoplasma by PCR (November 2012). M2_{FIL-} and A7_{FIL+} cell lines were from T. Stossel (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA), cultured as previously (11) and routinely validated by immunoblotting lysates with a filamin antibody (Fig1B). The authenticity of non-cancer COS1 cells (cultured in DMEM 10 % NCS and 2 mM L-glutamine), was not validated.

Luciferase Assays
Luciferase assays were performed by plating $5 \times 10^4$ cells/12-well plate. For Gal-FHL2 assays, cells were co-transfected with 500 ng pG5E1b-luciferase, 20 ng phRL-TK (Renilla), 50 ng of pCMXGal-vector or pCMXGal-FHL2, 500 ng of GFP-vector, GFP-Filamin(wildtype), GFP-Filamin(Δ1762-1764) or HA-vector, HA-calpastatin. COS1, M2_{FIL-} and A7_{FIL+} cells were transfected using lipofectamine (Invitrogen) and 24 hours post-transfection were serum starved or serum starved then treated with LPA, ionomycin or calcimycin. For AR transcriptional assays PNT1a, LNCaP and DU145 prostate cancer cells were co-transfected 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 Myc-Filamin(R16-23; 90kDa filamin fragment) or Myc-vector; 20 ng phRL-TK (Renilla) and 2 μg of ARE3-luciferase or PSA-luciferase. For androgen-
depletion versus androgen-stimulation cells were cultured in phenol-red free RPMI media containing 10% charcoal-stripped serum (CSS) (Gibco; Invitrogen) +/- treatment with 10 nM DHT for 24 hours. Enzalutamide (Selleck Chemicals) was used at 10 μM 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 was 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 BioResource (Cabrini Human Research Ethics Committee 03-14-04-08; Monash University Human Research Ethics Committee 2004/145MC). CRPC samples were provided by Dr David Pook, Southern Health (10247A). Antigen retrieval was performed as previously (19) and incubated with primary antibodies overnight at 4°C; FHL2 (1:50, rabbit), filamin (1:1000, N- or C-terminal), AR-V7 (1:500), cytokeratin 5 (1:200), cytokeratin 8 (1:200) and normal rabbit or mouse IgG (5 μg/ml). DAB staining performed using Envision+ HRP-conjugated antibodies with DAB detection (Dako) and hematoxylin nuclei counterstaining. For immunofluorescence, sections were incubated with Alexa Fluor-conjugated secondary antibodies (1:500), with nuclear TO-PRO®-3 iodide. A Fisher’s exact test determined the statistical significance of FHL2 and filamin nuclear staining in CRPC.

**For other detailed methods;** refer Supplementary Methods
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 library and identified several interacting clones encoding the Ig-like repeat 23 of filamin C (amino acids 2500-2558) (Fig1A). The three filamin isoforms, A, B and C share significant amino acid homology in repeats 22-24, to which FHL2 binds (SuppFig1) (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 (SuppFig2A). Co-immunoprecipitation of HA-FHL2 and truncated Myc-filamin(Repeats 22-24) was demonstrated, also delineating the FHL2-binding site to this C-terminal region of filamin (SuppFig2B).

FHL2 localization was examined in filamin-deficient (M2FIL-) cells compared to the filamin-replete A7FIL+ subline (20), which both express FHL2 (Fig1B). 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, co-localizing with both filamin and actin and was also present at focal adhesions, co-localizing with paxillin (Fig1C, a-l). 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 cross-linking (Fig1C, m-t) (20). The nuclear localization and transactivation activity of FHL2 was examined in M2FIL- versus A7FIL+ cells following stimulation with lysophosphatidic acid (LPA), 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 LPA stimulation and in contrast, was constitutively nuclear in M2FIL- cells (Fig1D). 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 (Fig1E). Quantification of the nuclear:cytoplasmic ratio for FHL2 immunostaining (representative images Fig1D), further revealed a constitutively higher ratio in M2FIL- cells, than A7FIL+.
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cells under non-stimulated conditions (Fig1F). A Gal-FHL2 fusion protein which 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 following LPA stimulation, but constitutively activated transcription in M2FIL- cells irrespective of LPA treatment (Fig1G). 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 Ca2+-dependent calpain proteases, with two cleavage sites; within hinge I, between repeats 15 and 16, and within hinge II between repeats 23 and 24 (Fig1A) (16). Calpain-cleavage of filamin generates two fragments; N-terminal 170 kDa (repeats 1-15) and a C-terminal 90kDa fragment (repeats 16-23) (Fig1A). The effect of filamin-cleavage on FHL2 nuclear localization and transactivation function was examined. Calpain activation was induced in COS1 cells by ionomycin (Ca2+ ionophore) treatment, resulting in filamin cleavage (SuppFig2C) and FHL2 nuclear localization (SuppFig2D) 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 (SuppFig2E) or the calpain-inhibitory protein calpastatin (SuppFig2F) (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 demonstrated (SuppFig2G). Furthermore, expression of a calpain-resistant GFP-filamin (Δ1762-1764) mutant (23), in M2FIL- cells inhibited the increase in transactivation activity observed for Gal-FHL2 following ionomycin-stimulated calpain activation (SuppFig2H). 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 affect 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 (90kDa) (16). A 90kDa filamin fragment was present in prostate cancer cell lines (LNCaP and DU145) and was partially reduced following calpain inhibition using calpeptin (100 μM for 24 hours), while PNT1a cells expressed only full-length filamin (Fig2A). 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 170kDa cleaved fragment (SuppFig3A). Co-immunoprecipitation revealed endogenous FHL2 bound only full-length filamin in PNT1a cells and to both full-length filamin and the 90kDa fragment in LNCaP and DU145 cells (Fig2B). The latter interaction was confirmed by co-immunoprecipitation of the 90kDa filamin fragment (Myc-filamin_R16-23) with HA-FHL2 (Fig2C).

Intact filamin localizes to the cytoskeleton, however, following calpain cleavage the 90kDa filamin fragment translocates to the nucleus and the 170kDa fragment is retained in the cytoplasm (16). In PNT1a cells filamin was not-cleaved, and both FHL2 and filamin (C-terminal antibody) co-localized at the cytoskeleton (Fig2D). In LNCaP and DU145 prostate cancer cell lines, which exhibited significant filamin-cleavage, nuclear co-staining of FHL2 and filamin was observed, which was reduced by calpain inhibition using calpeptin. Ectopic expression of the 90kDa filamin fragment (Myc-Filamin_R16-23) in PNT1a cells induced cytoskeleton to nuclear translocation of FHL2 (Fig2E), evidence that the 90kDa filamin fragment promotes nuclear FHL2. FHL2 coactivation of the AR was examined under conditions of calpain inhibition, using two AR-regulated reporters p(ARE3)-luciferase (Fig2F) and PSA-luciferase (SuppFig4A). Cells were left untreated or treated with the calpain inhibitor calpeptin, followed by
androgen (DHT) stimulation. In the absence of calpain inhibition HA-FHL2 coactivated AR-transcription of both p(ARE3)- and PSA-reporters to a greater extent in prostate cancer cells, relative to the PNT1a cells. Treatment of LNCaP and DU145 cells with calpeptin reduced FHL2 coactivation of AR to basal levels observed in PNT1a cells. Calpain-dependent activation of endogenous AR-target genes by FHL2 was examined using qRT-PCR. In LNCaP cells, expression of HA-FHL2 increased PSA (Fig2G), hK2 and TMPRSS2 (SuppFig4A and 4C) mRNA levels relative to HA-vector control transfected cells, which was abrogated by calpain inhibition. Collectively, these studies demonstrate that the enhanced susceptibility of filamin to calpain cleavage exclusively in prostate cancer cells results in enhanced FHL2 nuclear localization and AR activation. Filamin phosphorylation at ser2152 protects against calpain cleavage (25). The Ca\textsuperscript{2+}/calmodulin-dependent protein phosphatase calcineurin, dephosphorylates filamin to promote calpain cleavage (26) and calcineurin expression is increased in prostate cancer (27). We show here that filamin dephosphorylation by calcineurin in prostate cancer cell lines was responsible for the enhanced susceptibility of filamin to calpain cleavage, a pathway which initiates FHL2 nuclear localization and increased AR activation (SuppFig5).

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

We investigated if 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 cytokeratin (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 (Fig3A). Co-immunostaining 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 (SuppFig6), indicating FHL2 and filamin co-expression 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 (Fig3A), 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 (SuppFig3B), where filamin was not cleaved (Fig2A, SuppFig3A). Our data is 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 (Fig3A). 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 (Fig3B, Table1). A correlation was also observed between the CRPCs which exhibited both nuclear FHL2 and filamin (Table2), 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-naïve prostate cancer (30). We examined whether FHL2 promotes ligand-independent coactivation of two AR variants, trAR (31) and AR-V7 (AR3) (30, 32), which have truncation of the ligand-binding domain 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 (Fig4A). In DU145 cells, FHL2 coactivated full-length AR under androgen-stimulated conditions and significantly, FHL2 coactivation of trAR was ligand-independent (Fig4B). The nuclear 90kDa C-terminal filamin fragment, which we show binds FHL2, also represses AR activity (33). Therefore we determined if the FHL2-filamin complex co-regulates AR activity. In contrast to FHL2, the 90kDa filamin fragment (Myc-filaminR16-23) bound full-length AR, but not trAR (Fig4C). Competitive-binding studies revealed Myc-filaminR16-23 disrupted HA-FHL2 binding to full-length AR, but not trAR (Fig4D). Furthermore, FHL2 coactivation of full-length AR (Fig4E), but not trAR (Fig4F) was repressed by the 90kDa 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 (Fig4G).

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-filaminR16-23) (Fig5A) and endogenous FHL2 and AR-V7 bind in the 22Rv1 prostate cancer cell line (Fig5B). Ligand-independent coactivation of AR-V7 by FHL2 was constitutive and not repressed by the 90kDa filamin fragment (Fig5C and 5D). AR-V7 is frequently expressed 20-fold higher in CPRC than hormone-naïve prostate cancer (30), and correlates with poor survival (34). In serial sections of human CRPC FHL2 and AR-V7 co-localized in the nucleus of cancer cells (Fig5E) and 75% of CRPCs exhibited both nuclear FHL2 and AR-V7 (Table1, Table2). Therefore FHL2 nuclear localization in CRPC may promote constitutive, ligand-independent activation of this clinically important AR variant.

The anti-androgen enzalutamide promotes tumor regression in a mouse xenograft model of CRPC (18) and has progressed rapidly towards approval for treating metastatic CRPC. Enzalutamide acts on the full-length AR by directly binding the ligand-binding domain (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 which do not express endogenous AR. AR-V7 (Fig5F) and trAR (SuppFig7A) 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 (SuppFig 7B). Surprisingly, co-expression of FHL2 was sufficient to restore the transcriptional activity of full-length AR in the presence of this anti-androgen (SuppFig 7B). 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 cancer cell lines, calpain-cleavage of filamin drives nuclear FHL2 and enhanced AR activation. Additionally, 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 under-expression 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 (Fig5G). 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 co-express 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 is our data showing FHL2 activates AR-V7 and both proteins co-localize in the nucleus of CRPC.

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

We show the nuclear 90kDa filamin fragment competes with FHL2 for binding full-length AR and represses ligand-dependent AR coactivation by FHL2. This is supported by the finding that Ig-repeats 22-24 of filamin bind AR (33), a region which 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 activation of the prototype AR is mediated by a balance between the coactivator FHL2 and the corepressor filamin (Fig4G-a). We demonstrated how loss in this balance due to the absence of filamin repression, results in constitutive activation of pathological AR variants notably AR-V7, by FHL2 (Fig4G-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 CPRC. 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 Phosphatidylinositol 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 which lack all or part of the LBD (43). 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 tumours (44). Calpain inhibition also decreases the androgen-independent proliferation of Rv1 cells (31) and prostate cancer invasiveness (45). 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 90kDa 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 (46) and the oncogenic EpICD transcription complex (47). Calpain expression and activity is increased in human colorectal cancer and in colorectal polyps, where it may be an early tumorigenesis event (48) 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 (49). Although this latter study did not examine the presence of the 90kDa 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.
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REFERENCES

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Table I Frequency of nuclear localization of FHL2, Filamin and AR-V7 in human prostate samples (* p = 0.001).

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<tr>
<td>Filamin</td>
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<td>0/8</td>
<td>6/8*</td>
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<tr>
<td>AR-V7</td>
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**Table II** Castrate resistant prostate cancer samples exhibiting nuclear localization of FHL2, Filamin and AR-V7.

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FIGURE LEGENDS

Figure 1: FHL2 localizes constitutively to the nucleus in filamin-deficient cells.

(A) Upper; FHL2 comprises four and a half LIM domains. LIM domains 3 and 4 were used as “bait” in a yeast two-hybrid. Lower; Filamin domain structure comprising an N-terminal actin-binding domain followed by 24 immunoglobulin (Ig)-like repeats. Two hinge regions are cleaved by calpain (arrows). Using yeast two-hybrid we identified FHL2 binds repeat 23 of filamin C. (B) Filamin and FHL2 expression in M2FIL- versus A7FIL+ cells. β-tubulin loading control. (C) A7FIL+ versus M2FIL- cells stained for FHL2 and either filamin, focal adhesions (paxillin) or phalloidin (actin). Scale bar 100 μm, 25 μm in high mag. images. (D) A7FIL+ and M2FIL- cells were serum starved or LPA stimulated before staining for FHL2 and nuclei (propidium iodide). Scale bar 100 μM. (E) Nuclear and cytoplasmic subcellular fractions prepared from unstimulated A7FIL+ and M2FIL- cells and immunoblotted for FHL2. GAPDH and lamin A/C control cytoplasm or nuclear proteins. Ponceau Red staining of Western blot membranes (loading control). (F) The nuclear:cytoplasmic ratio of FHL2 immunostaining was quantified in unstimulated A7FIL+ and M2FIL- (serum starved) or following LPA stimulation, by measuring fluorescence intensity (G) A7FIL+ and M2FIL- cells were co-transfected with a Gal-luciferase reporter and Gal-FHL2 (or Gal-vector, not shown). Cells were LPA stimulated and luciferase activity measured. Data represents mean from n=6 ± S.E.M. * p = 0.01.

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) Co-immunoprecipitation of endogenous FHL2 and filamin in prostate cancer cell lines. (NonI; non-immune control). (C) Co-immunoprecipitation in COS1 cells co-expressing Myc-FilaminR16-23, (90kDa filamin fragment) and either HA-vector control or HA-FHL2. (D) Cells +/- calpain inhibition using calpeptin were co-stained for FHL2 and filamin (C-terminal) and nuclear TO-PRO®-3 iodide. (E) FHL2
localization in PTN1a cells expressing Myc-vector or Myc-Filamin<sub>R16-23</sub>. TO-PRO®-3 iodide (nuclei). Scale bar is 100 μM. (F) Cells were co-transfected with Flag-AR, HA-FHL2 and p(ARE3)-luciferase and left untreated or pre-treated with calpeptin, prior to stimulation with androgen (10nM DHT). For all luciferase assays data represents the mean from n=5-8 ± S.E.M. * p = 0.01. (G) Expression of the AR-target gene PSA was examined by qRT-PCR in LNCaP cells transfected with HA-FHL2 (or HA-vector) +/- calpain inhibition using calpeptin. Data represents the mean from n = 4 ± S.E.M. * p = 0.05.

**Figure 3: FHL2 and filamin localize to the nucleus in CRPC and not benign prostate tissue or localized prostate cancer.**

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 non-immune rabbit or mouse IgG. (B) Serial sections of human CRPC from two 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.

**Figure 4: FHL2 competes with filamin for binding to wildtype AR, but not a truncated AR (trAR).**

(A) Co-immunoprecipitation in COS1 cells co-expressing HA-FHL2 (or HA-vector) and either FLAG-tagged full-length AR or trAR. (B) DU145 cells were co-transfected with combinations of HA-vector or HA-FHL2; FLAG-vector, FLAG-AR (full-length) or FLAG-trAR and the AR-responsive reporter p(ARE3)-luciferase. Cells were maintained under androgen-depleted conditions (-Androgen) or stimulated with DHT (+ Androgen) and luciferase activity measured. (C) Co-immunoprecipitation in COS1 cells co-expressing Myc-Filamin<sub>R16-23</sub> (or Myc-vector) and either FLAG-tagged full-length AR or trAR. (D) Competitive-binding assay; cells were co-transfected as indicated and also co-expressed Myc-vector or Myc-Filamin<sub>R16-23</sub>. Lysates were immunoprecipitated (IP) and immunoblotted (WB) as indicated. (E-F) Filamin represses FHL2 coactivation of wildtype AR (E), but not trAR (F); DU145 cells
Regulation of nuclear FHL2 in cancer by filamin cleavage

were co-transfected as indicated and cells also co-expressed Myc-vector or Myc-Filamin_{R16-23}. FHL2 coactivation of full-length AR and trAR was measured under androgen-dependent and androgen-independent conditions. For all luciferase assays, data represents the mean from n=8 ± S.E.M. * p = 0.05. (G) AR co-regulation by the FHL2-filamin complex; (a) The 90kDa filamin fragment competes with FHL2 for binding to full-length AR and also represses ligand-dependent AR coactivation by FHL2. ARE, androgen-response element; (b) FHL2, but not the 90kDa filamin fragment, binds to AR variants trAR and AR-V7, such that coactivation of truncated AR variants by FHL2 is constitutive.

Figure 5: FHL2 coactivates an AR variant, AR-V7 present in CRPC.

(A) Co-immunoprecipitation in COS1 cells co-expressing HA-FHL2 (or HA-vector) and His-AR-V7. (B) Co-immunoprecipitation of endogenous FHL2 and AR-V7 from 22Rv1 cells. (NonI; non-immune sera) (C) DU145 cells were co-transfected with HA-vector or HA-FHL2, His-vector or His-AR-V7, and p(ARE3)-luciferase as indicated. Cells were maintained under androgen-depleted conditions and luciferase activity measured. (D) Luciferase assays were repeated as above in cells co-expressing Myc-vector or Myc-Filamin_{R16-23}. (E) Serial sections from two representative CRPCs showing nuclear co-localization of FHL2 and AR-V7. Boxed regions indicate areas shown in high magnification. Scale bar 50 μm for all images. (F) ARE3-luciferase assays were repeated in DU145 cells expressing AR-V7 +/- HA-FHL2 expression as above, +/- enzalutamide treatment. For all luciferase assays, data represents the mean from n= 4-8 ± S.E.M. * p = 0.05. (G) Differential regulation of FHL2 nuclear localization and AR coactivation during prostate cancer progression. In benign prostate tissue, the absence of filamin cleavage results in FHL2 cytoplasmic sequestration and low AR coactivation. In CRPC, filamin cleavage to the 90kDa fragment activates FHL2 nuclear accumulation and FHL2 promotes the constitutive ligand-independent coactivation of the AR variant AR-V7.
Figure 4

A

B

C

D

E

F

G
**Supplementary Materials and Methods:**

**Yeast two-hybrid**

The yeast two-hybrid Matchmaker III system was used as per manufacturer’s instructions (Clontech). To generate the “bait”, LIM domains 3 and 4 of human FHL2 (amino acids 156-279, Accession Q14192), were cloned into the EcoR1 site of the pGBK7 vector (Clontech).

**Constructs**

pCGN-FHL2 (HA-FHL2) was cloned previously (1). The human FHL2 cDNA was cloned into the XbaI site of the pSVTf vector to generate pSVTf-FHL2 to be used for in vitro transcription/translation of FHL2. The filamin truncation construct pEFBOS-Myc-Filamin_R22-24 was described previously (2) and the cDNA encoding filamin_R22-24 was also subcloned into the pGEX-1λT vector (EcoRI/HindIII) from Amersham Biosciences to generate the GST-Filamin_R22-24 fusion protein. For luciferase assays, the pCMXGal-vector, pCMXGal-FHL2 and pG5E1b-luciferase (Firefly luciferase) constructs are described previously (3, 4) and were provided by Professor Roland Schüle, Department of Urology/Women’s Hospital and Centre for Clinical Research, Freiburg, Germany. The renilla expression vector phRL-TK was from Promega. pcDNA-I-HA-calpastatin described in (5) was a gift from Dr Masatoshi Maki, Nagoya University, Japan. The pEGFP-filamin full-length and calpain-resistant pEGFP-filaminΔ1762-1764 mutant were cloned previously (6) by Dr Donald Ingber, Vascular Biology Program, Harvard Medical School and Children’s Hospital, Boston, MA, USA. The ARE3-luciferase construct (7) was from Assoc. Prof. Daniel Gioeli, Department of Microbiology and the Cancer Centre, University of Virginia, Charlottesville, USA. PSA-luciferase and the CMV-FLNa16-24 construct reported in (8) were obtained from Dr E. L. Yong, Department of Obstetrics and Gynecology, National University of Singapore, Singapore. The CMV-FLNa16-24 construct was used to generate the pEFBOS-Myc-Filamin_R16-23 plasmid by PCR amplification of the filamin A cDNA repeats 16-23 and cloning into the MluI site of the pEFBOS-Myc vector supplied by Tracy Willson, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. The Flag-AR(full-length) and Flag-trAR constructs (9) were from Dr Maria Mudryj at Department of Medical Microbiology and Immunology, University of California Davis,
California, USA and the AR-V7 cDNA (10) from Dr J. Luo at Departments of Urology and Oncology, The Johns Hopkins University School of Medicine, Maryland, USA.

Antibodies

Antibodies were supplied as follows; GST (Amersham Biosciences); FHL2 (Abcam, ab6639 and ab58069); Myc, β-tubulin (2128), calcineurin and phosphoFilaminA$^{S2152}$ (Cell Signaling); HA (Covance); filamin A (N-terminal, MAB1678) and (C-terminal, MAB1680) (Chemicon); paxillin (610052, BD Transduction Laboratories); GAPDH (Ambion, AM4300), Lamin A/C (SAB4200236), propidium iodide, FLAG, and m-calpain (large subunit; C0728) (Sigma Aldrich); Phalloidin (Alexa Fluor-594) and To-PRO®-3 (Molecular Probes, Life Technologies); AR-V7 (Precision Antibody); cytokeratin 5 (CK5, RM-2106) and cytokeratin 8 (CK8; 2032-1) (Neomarkers, ThermoScientific); Rabbit and mouse IgG (Upstate, Millipore). Secondary antibodies were from Molecular Probes.

In vitro binding studies

Top10 E. coli bacteria were transformed with pGEX-vector or pGEX-filamin C$_{R22-R24}$ and expression of recombinant protein induced using 0.1 mM IPTG at 24°C for 4 hours, which was then extracted overnight at 4°C in PBS containing 1% Triton X-100, plus protease inhibitor cocktail tablet (Roche). GST-recombinant proteins were bound to glutathione sepharose for 6 hours at 4°C, and sepharose-conjugated proteins collected by centrifugation at 13,000 g for 30 seconds. The full-length human FHL2 cDNA was cloned into the $XbaI$ site of the pSVTF vector. The pSVTF-FHL2 construct was linearised using $SalI$ and FHL2 in vitro transcribed and translated using the TNT wheat germ extract system (Promega) according to manufacturer’s instructions. In vitro transcribed and translated FHL2 was incubated with GST-alone or GST-filamin C$_{R22-R24}$-conjugated sepharose overnight at 4°C, then washed extensively with 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA. Bound FHL2 was eluted with SDS-PAGE reducing buffer. Bound proteins were immunoblotted with FHL2 and GST (1:1000) antibodies.
Co-immunoprecipitation

COS-1 cells were transfected with DNA using the standard DEAE Dextran/Chloroquine method and co-immunoprecipitations performed as previously (11) using 5 μg of HA or Myc antibodies. Immunoprecipitates were immunoblotted for HA 1:5000; Myc 1:1000, Flag 1:1000, His 1:3000. For endogenous binding studies; cells were washed in PBS and lysed (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 % Nonidet P-40 plus a protease inhibitor tablet (Roche), for 1 hour at 4°C. Immunoprecipitations were performed as reported (12) using 5 μg of FHL2 antibody or normal mouse IgG, followed by immunoblotting for FHL2 (1:500), filamin (1:1000; C-terminal), m-calpain (1:1000) or AR-V7 (1:1000).

Ionomycin, calcimycin, calpeptin and cyclosporine A treatments

Cells were serum starved overnight, then treated with ionomycin (0.5μM or 1 μM, 1 hour), calcimycin (1 μM or 5 μM, 3 hours) or DMSO vehicle. For calpain inhibition, cells were treated for 24 hours with calpeptin (100 μM; Calbiochem) or DMSO vehicle. For LPA stimulation cells were serum starved overnight, then stimulated with 20 μM LPA for 3 hours (4). Calcineurin inhibition was performed using cyclosporine A (5 μM) treatment for 24 hours. LPA, ionomycin, calcimycin and cyclosporine A were from Sigma Aldrich.

Immunofluorescence staining of cells

Cells were fixed (11) and incubated with primary antibodies, for 1 hour; FHL2 (1:500 mouse or 1: 200 rabbit), filamin (1:1000; N- or C-terminal), paxillin (1:500) and AR-V7 (1:500). Following washing with PBS, cells were incubated with Alexa Fluor-conjugated secondary antibodies (1:600) for 1 hour. Alexa Fluor-594 phalloidin (1:500) staining for 1 hour was used to detect actin. To detect nuclei, propidium iodide or TO-PRO®-3 iodide staining was performed.

Image J 1.43u software (National Institutes of Health, USA) was used to quantify the nuclear:cytoplasmic ratio of FHL2 staining . The mean nuclear:cytoplasmic ratio was calculated from
three independent experiments. For each experiment 10 cells were analyzed, and for each cell the fluorescence intensity of 10 randomly selected points in the nucleus and cytoplasm were measured and the average nuclear:cytoplasmic ratio determined.

**Western blot analysis**

Cell were lysed for 1 hour at 4°C using 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 % Nonidet P-40 plus a protease inhibitor tablet (Roche). Lysates were purified by centrifugation at 13,000 g for 10 min at 4°C and 25-50 µg of protein separated by SDS-PAGE followed by immunoblotting for filamin (1:1000; N- or C-terminal), FHL2 (1:500) or calcineurin (1:1000). For phospho-immunoblots, lysates were prepared as above with the addition of phosphoSTOP inhibitor tablets (Roche). For immunoblotting PVDF membranes were blocked with 5 % w/v BSA in Tris saline containing 0.05% Tween-20, and probed with a phosphoFilamin\textsuperscript{2152} antibody (1:500). Re-probing immunoblots for β-tubulin (1:1000) was used as a loading control. Band intensities were quantified by densitometry using Image J 1.43u software (National Institutes of Health, USA) and corrected for protein loading by comparing to β-tubulin.

Subcellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific). 15 µg of nuclear and cytoplasmic protein fractions were immunoblotted for FHL2 (1:200) and GAPDH (1:2000) or Lamin A/C (1:5000) as control proteins for the cytoplasmic and nuclear fractions respectively. Ponceau Red (Sigma Aldrich) staining of Western blot membranes was used to confirm equal protein loading.

All uncropped immunoblots are shown in Supplementary Figure 8.

**RNA Analysis**

Total cellular RNA was extracted using and RNeasy Mini Kit (Qiagen). 50ng RNA was used for first-strand cDNA synthesis with AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies). Quantitative RT-PCR (qRT-PCR) analysis was performed on the Corbett Rotorgene 3000 (Qiagen) using
Brilliant II SYBR Green QPCR Mastermix (Agilent Technologies, Stratagene). Validated RT-PCR primers for hK2, TMPRSS2 and GAPDH were from Qiagen, and PSA reported in (12). qRT-PCR data was normalized using the GAPDH and calculated relative to control cells using the comparative $2^{-\Delta\Delta CT}$ method.

**Microscopy**

Microscopy was performed at Monash Microimaging, Australia, using Leica TCS NT and Nikon C1 confocal microscopes. Prostate cancer sections were imaged using an Olympus Provis Ax70 microscope fitted with an Olympus DP70 camera (DAB) and using AnalySis software. Image processing was performed using Image J software (National Institutes of Health, USA) and was limited to alterations of brightness, subjected to the entire image.

**Statistical Analysis**

Performed using the Microsoft excel or GraphPad 5.0 programs. Unless otherwise stated, p values were calculated using an unpaired student’s t-test, whereby p < 0.05 indicated statistical significance. Individual P values are indicated in the figure legends for each data set.
Regulation of nuclear FHL2 in cancer by filamin cleavage


Supplementary Figure 1: Sequence Alignment of Filamin A, B and C isoforms, indicating minimal FHL2-binding site.

Alignment of the amino acid sequences of the C-terminal Ig-like repeats 23-24 from human filamin A (Accession P21333), filamin B (Accession BAD52434), and filamin C (Accession AAD12245). ↓denotes the first amino acid of each domain or Hinge II which is cleaved by calpain; an asterisk represents identical amino acids in all three sequences; a colon represents conservative substitutions. The filamin C sequence shown in bold represents that encoded by the clones isolated from a yeast two-hybrid skeletal muscle library screening, using FHL2 as bait.

Supplementary Figure 2: Activation of filamin cleavage in COS1 cells promotes the nuclear localization and transactivation function of FHL2.

(A) Direct protein interaction between FHL2 and filamin; The C-terminal Ig-like repeats 22-24 of filamin (GST-filaminR22-R24) or GST-alone were incubated with *in vitro* translated FHL2. Sepharose-bound proteins were eluted and immunoblotted for FHL2 or GST.

(B) FHL2 and filamin interaction in intact cells; COS1 cells were co-transfected with HA-FHL2 (or HA-vector) and Myc-filaminR22-R24, and lysates were immunoprecipitated (IP) with a HA antibody and immunoblotted for Myc or HA.

(C) Filamin is a calpain substrate; COS1 cells were either serum starved (unstimulated) or starved then treated with the Ca$^{2+}$ ionophore ionomycin (1µM for 1 hour) to activate calpain, +/- addition of the calpain inhibitor calpeptin (100 µM) or vehicle (DMSO). Lysates were immunoblotted with a C-terminal-specific filamin antibody which recognizes full-length filamin (280kDa) and the C-terminal 90kDa filamin fragment (repeats 16-23) generated following calpain cleavage. β-tubulin was used as a loading control. The 90kDa filamin fragment was present only upon calpain activation and was reduced by the pre-treatment of cells with calpeptin.

(D) Activation of calpain indues FHL2 nuclear localization; COS1 cells were treated as above (C) and co-stained with FHL2 and filamin antibodies. Arrows (d) indicate nuclear FHL2. Scale bar 100 µM. Nuclear FHL2 induced by ionomycin was completely abrogated by calpeptin, indicating that calpain regulates FHL2 nuclear localization.
(E) Activation of calpain promotes the transactivation activity of FHL2; The transactivation activity of FHL2 was measured using a Gal-FHL2 fusion protein which has been shown previously to activate a Gal-responsive luciferase reporter construct (pG5E1b-luciferase). COS1 cells were co-transfected with Gal-luciferase (pG5E1b-luciferase), renilla luciferase (phRL-TK, transfection efficiency control) and either Gal-vector or Gal-FHL2. Cells were either serum starved (unstimulated) or starved then stimulated with Ca\(^{2+}\) ionophores, calcimycin (2 hours), ionomycin (1 hour) to activate calpain or DMSO vehicle control as indicated. A subset of cells, were pre-treated with calpeptin prior to ionomycin treatment. Luciferase activity was measured and the transactivation activity of Gal-FHL2 presented as the fold increase above Gal-vector control transfected cells for which data is not shown. Data represents the mean from n = 6 independent experiments ± S.E.M. * p = 0.05, ** p = 0.01, ***p = 0.001. Treatment of cells with ionomycin or calcimycin to activate calpain, induced a dose-dependent increase in Gal-FHL2 mediated transcriptional activity which was blocked by calpain inhibition using calpeptin.

(F) Calpastatin reduces the transactivation activity of FHL2; Luciferase assays were performed as described above (E), however cells were also co-transfected with HA-calpastatin, a calpain-specific inhibitory protein (or HA-vector control). Cells were left unstimulated (serum starved; black bars) or treated with ionomycin (white bars). Data represents the mean from n = 4 independent experiments ± S.E.M. * p = 0.05.

(G) Endogenous FHL2 forms a multi-protein complex with filamin and m-calpain; Lysates from COS1 cells were immunoprecipitated (IP) with a FHL2-specific antibody followed by immunoblotting (WB) with m-calpain, filamin or FHL2 antibodies. In control studies immunoprecipitations were performed with non-immune sera (NonI.).

For all immunoblots, full-length blots are presented in Supplementary Figure 8.
**Expression of a calpain-resistant filamin mutant reduces FHL2 transactivation activity:** M2\textsubscript{FIL-} cells were co-transfected with Gal-FHL2, a Gal-responsive luciferase reporter and either GFP-vector, GFP-filamin(wildtype) or the calpain-resistant GFP-filamin(Δ1762-1764) mutant. Cells were left unstimulated (serum starved) or treated with ionomycin (as above in E) to activate calpain and luciferase activity measured. The transactivation activity of Gal-FHL2 is expressed relative to Gal-vector control. Data represents the mean from n=6 independent experiments ± S.E.M. ** p = 0.001. Expression of a GFP-filamin (Δ1762-1764) mutant, which is resistant to calpain-cleavage, in M2\textsubscript{FIL-} cells, resulted in low transactivation activity of Gal-FHL2 in unstimulated, that did not increase following calpain activation using ionomycin. In contrast, expression of GFP-filamin (wildtype) in unstimulated M2\textsubscript{FIL-} cells reduced the transactivation activity of Gal-FHL2, relative to GFP-vector expressing cells, an effect reversed following calpain activation. Therefore, reconstitution of wildtype filamin in unstimulated M2\textsubscript{FIL-} cells results in FHL2 sequestration at the cytoskeleton, however, upon filamin cleavage FHL2 translocates to the nucleus and activates transcription. In cells expressing the calpain-resistant filamin mutant, FHL2 exhibited low transactivation activity despite calpain activation.
Supplementary Figure 3: Filamin is cleaved by calpain in prostate cancer cell lines resulting in nuclear localization of the 90kDa filamin fragment and FHL2.

(A) The N-terminal filamin cleavage product is present in prostate cancer cell lines; Immunoblot analysis using an N-terminal specific antibody which recognizes the full-length filamin protein and the cytoplasmic 170kDa filamin fragment (Actin-Binding Domain plus R1-15), confirmed that filamin is cleaved by calpain only in LNCaP and DU145 prostate cancer cell lines but not in the PNT1a prostate epithelial cell line. β-tubulin was used as a loading control.

For all immunoblots, full-length blots are presented in Supplementary Figure 8.

(B) In the absence of filamin cleavage, the N-terminal and C-terminal filamin antibodies exhibit overlapping cytoplasmic staining in a prostate epithelial cell line; Prostate epithelial (PNT1a) or the prostate cancer DU145 (or LNCaP, not shown) cell lines were co-stained with an antibody specific for FHL2 and two filamin antibodies which recognize both the full-length filamin protein and either the N-terminal (170kDa; actin-binding domain, plus R1-15) or C-terminal (90kDa; R16-23) filamin fragments generated following calpain cleavage. TO-PRO®-3 iodide was used to detect nuclei. Scale bar 100 μM.

Previous studies have shown that full-length, intact filamin localizes to the cytoskeleton, however upon filamin cleavage by calpain the 90kDa C-terminal filamin fragment translocates to the nucleus and the 170kDa fragment is retained in the cytoplasm. In PNT1a cells both the N- and C-terminal filamin antibodies demonstrated overlapping cytoplasmic staining indicating the absence of filamin cleavage. In DU145 and LNCaP (not shown) prostate cancer cell lines nuclear staining of the C-terminal 90kDa filamin fragment was detected and in contrast the N-terminal filamin antibody exhibited cytoplasmic localization. FHL2 nuclear localization was present only in DU145 and LNCaP (not shown) prostate cancer cell lines which also exhibited nuclear staining of the 90kDa filamin fragment that is generated following calpain cleavage.
Supplementary Figure 4: FHL2 coactivation of the AR is regulated by calpain.

(A) FHL2 coactivation of the AR is reduced following calpain inhibition; Cells were co-transfected with Flag-AR, HA-FHL2 and PSA-luciferase and left untreated or pre-treated with calpeptin (100 μM, 24 hours), prior to stimulation with androgen (10nM DHT). For all luciferase assays data represents the mean from n=5-8 ± S.E.M. * p = 0.01.

(B-C) FHL2 activation of endogenous AR-target genes is reduced following calpain inhibition; Expression of the endogenous AR-target genes hK2 (B) and TMPRSS2 (C) were examined by qRT-PCR in LNCaP cells transfected with HA-FHL2 (or HA-vector) +/- calpain inhibition using calpeptin (or vehicle) (as above). Data represents the mean from n = 4 ± S.E.M. * p = 0.05.
Supplementary Figure 5: Filamin is susceptible to calpain-cleavage in prostate cancer cell lines due to increased dephosphorylation by calcineurin.

(A) Filamin phosphorylation is decreased in prostate cancer cell lines and correlates inversely with calcineurin expression; Lysates from prostate epithelial (PNT1a) or prostate cancer (LNCaP and DU145) cell lines were immunoblotted with phosphoFilamin2152, total filamin, calcineurin or β-tubulin (loading control) antibodies. Migration of the full-length filamin and the C-terminal 90kDa filamin fragment (repeats 16-23) is shown.

(B-C) PhosphoFilamin2152 and calcineurin expression was quantified using densitometry analysis of immunoblots and are represented relative to levels in the PNT1a prostate epithelial cell line. Data represents the mean from n = 6 independent experiments ± S.E.M. * p = 0.05.

(D) Inhibition of calcineurin decreases filamin cleavage in a prostate cancer cell line; DU145 cells were treated with the calcineurin inhibitor cyclosporine A (5 μM, 24 hours) and lysates immunoblotted as above in (A).

For all immunoblots, full-length blots are presented in Supplementary Figure 8.

(E) Calcineurin inhibition reduces the nuclear localization of FHL2 and filamin in a prostate cancer cell line; DU145 cells were treated with cyclosporine A (CSA) as above in (D) and co-stained with antibodies for FHL2 and filamin, and the nuclear stain TO-PRO®-3 iodide. Scale bar 100 μM.
Calcineurin inhibition reduces FHL2 coactivation of AR in a prostate cancer cell line; DU145 cells were cotransfected with Flag-AR (full-length), HA-FHL2 and the AR-responsive luciferase reporter p(ARE3)-luciferase followed by treatment with cyclosporine A. Cells were stimulated with androgen (DHT; 10nM) and luciferase activity measured. Luciferase data is presented relative to FLAG-vector and HA-vector control co-transfected cells and is the mean from n = 6 ± S.E.M. * p < 0.05.
Supplementary Figure 6: FHL2 and filamin are co-expressed in the cytoplasm of basal cells in benign prostate epithelium.

Benign prostate epithelium is composed of multiple cell types including basal, luminal, intermediate and neuroendocrine cells, based on cell morphology, location and expression of specific cytokeratin protein markers. We determined the specific cell types in which FHL2 and filamin were expressed in benign prostate. Sections of benign prostate tissue were co-stained with antibodies specific for FHL2 or filamin, and markers for either luminal (cytokeratin 8; CK8) or basal (cytokeratin 5, CK5) epithelial cells. TO-PRO®-3 iodide was used to detect nuclei. Representative low and high (inset) magnification images are shown. Scale bar 50 μM. This analysis revealed that FHL2 localized to the cytoplasm of both CK8-stained luminal (a-d) and CK-5 positive basal (e-h) epithelial cells, and cytoplasmic filamin staining was restricted to CK-5 stained basal cells, revealing the coexpression of FHL2 and filamin only in basal cells.

Supplementary Figure 7: FHL2 coactivation of full-length AR and trAR is resistant to the antiandrogen enzalutamide.

(A-B) DU145 cells were co-transfected with p(ARE3)-luciferase, HA-FHL2 (or HA-vector), together with Flag-trAR (A) or Flag-AR full-length (B) (or Flag-vector control). The transcriptional activity of full-length AR was measured under conditions of androgen stimulation (10nM DHT) and trAR examined under androgen-independent conditions. Cells were left untreated for treated with the anti-androgen enzalutamide (10 μM, 24 hours) and luciferase activity measured. For all luciferase assays data represents the mean from n=4 ± S.E.M. * p = 0.01.

Supplementary Figure 8: Uncropped images of all immunoblots.
Supplementary Figure 1

↓ Repeat 23

Filamin C 2476-VGEQSQAGDPGLVSAYGPGLEGGTGTVSNAGSGALSVTID
Filamin A 2420-VGEQPGHGDPGLBSAYGAGLEGGVTGPNPAEFVBNATSAGALSVTID
Filamin B 2364-VGEQPGQAGNPALVSAAYGTGLLEGGTTGIQEFFINTTRAGPGTLSVTID

↓ Hinge II

GPSKVQLDCRECPEGHVTYPMPGNLIAIKYG
GPSKVMDQCQCPEGYRVYTPMAPGNYLISIKYGGPYHGGSPFKVTGPRLSGH
GPSKVMDCQETPEGYKVMYTPMAPGNLISVYGGPGPHGSPFKVTGQRLVSPG

↓ Repeat 24

SLHETSTVLVETVKKSSSRGSSSYSSIPKFDSDASKVTRGPGLSQAFAVGQKNSFTVD
SLHETSSVFVDSTKATCAP--QHGPAPGPDASKVAKGLLSKAYVGQKSSFTVD
SANETSSILVESVTRSSSTET--CYSAPAKSSDSASKVTSGAKLSSKAVGQKSSFLV

CSKAGTMNMVGVHPKTPCEEVYVKHNGNRVNYTVKKEGDDLLKILIVKFWGDESVPG
CSKAGMLMLVGVHPRTCEELIKVHGSRYSVCYLLKDGKETYTLVWKWGEHIPG
CSKAGSNMLLGPHPCEEVSMKHHGNNQVYNHTVVKERGDYLVAKWGEHIPG

SPFKVVP-2705
SPYRVSVP-2647
SPFHVTVP-2591

* * *
Supplementary Figure 7

A

Relative Luciferase Activity (RLU)

Flag-vector
Flag-trAR
HA-vector
HA-FHL2

Untreated
Enzalutamide

B

Relative Luciferase Activity (RLU)

Flag-vector
Flag-AR(full-length)
HA-vector
HA-FHL2

Untreated
Enzalutamide