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 *EcoR*1 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-FilaminR22-24 was described previously (2) and the cDNA encoding filaminR22-24 was also subcloned into the pGEX-1λT vector (*EcoR*I/*HindIII*) from Amersham Biosciences to generate the GST-FilaminR22-24 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-FilaminR16-23 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$_{R22-R24}$-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*

Cells 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 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.


**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). 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-filamin<sub>R22-R24</sub>) or GST-alone were incubated with <em>in vitro</em> 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-filamin<sub>R22-R24</sub>, 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<sup>2+</sup> 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 induces 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.
(II) 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.
(F) 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.