Abstract

Purpose: Flightless I (FLII), member of the gelsolin superfamily of actin-remodeling proteins, functions as a transcriptional coregulator. We aim to evaluate a tumor-suppressive function of FLII in regulating androgen receptor (AR) in prostate cancer progression.

Experimental Design: We examined FLII protein and mRNA expression in clinical prostate cancer specimens by immunohistochemistry. Kaplan–Meier analysis was conducted to evaluate the difference in disease-overall survival associated with the expression levels of FLII and AR. Prostate cancer cells stably expressing FLII or shRNA knockdown were used for functional analyses. Immunoprecipitation, Luciferase reporter, and immunofluorescence staining assays were performed to examine the functional interaction between FLII and AR.

Results: Our analysis of the expression levels of FLII in a clinical gene expression array dataset showed that the expression of FLII was positively correlated with the overall survival of prostate cancer patients exhibiting high levels of AR expression. Examination of protein and mRNA levels of FLII showed a significant decrease of FLII expression in human prostate cancers. AR and FLII formed a complex in a ligand-dependent manner through the ligand-binding domain (LBD) of AR. Subsequently, we observed a competitive binding to AR between FLII and the ligand. FLII inhibited AR transactivation and decreased AR nuclear localization. Furthermore, FLII contributed to castration-sensitive and castration-resistant prostate cancer cell growth through AR-dependent signaling, and reintroduction of FLII in prostate cancer cells sensitized the cells to bicalutamide and enzalutamide treatment.

Conclusions: FLII plays a tumor-suppressive role and serves as a crucial determinant of resistance of prostate cancer to endocrine therapies.

Introduction

Prostate cancer is one of the most common malignancies and the second most common cause of cancer-related deaths in men (1, 2). The progression of prostate cancer normally goes from castration-sensitive to castration-resistant, inevitably developing highly metastatic properties (3, 4). Androgen deprivation therapy (ADT) is the main therapeutic approach for patients with metastatic prostate cancer (5). Prostate cancer patients respond initially to ADT, but in later stages the tumor becomes hormone refractory and more aggressive, eventually leading to poor prognosis (5).

Arrays of treatments, including secondary hormonal therapies, are available for the treatment of metastatic prostate cancer and castration-resistant prostate cancer (CRPC), which show efficacy when administered with ADT (6). Continuation of ADT is recommended for CRPC treatment as therapies are added. New secondary hormonal therapies include abiraterone, targeting the CYP17 enzyme family, and enzalutamide (Xtandi), an androgen receptor inhibitor with heightened binding specificity (6–8). The optimal decision-making process for metastatic CRPC treatment option remains unclear, pending further research and experience.

Androgen receptor (AR), a member of nuclear transcription factor family, promotes tumor growth and survival-related gene transcription via androgen-mediated signaling pathways in castration-sensitive prostate cancer (CSPC; ref. 9). The ligand-independent activation of AR may be initiated by various biologic alterations including gene mutation, gene amplification, AR coactivator overexpression, and persistent intraprostatic androgens, which often leads to the failure of ADT in CRPC (9, 10). In CRPC, prostate cancer cells still rely on intracellular androgens and, to a greater extent, on active AR for growth and survival (11). Therefore, potent antiandrogens that efficiently disrupt the function(s) of AR are envisioned to be effective drugs for all types of prostate cancers (11). Blocking AR expression and activation has become one of the most effective therapies in clinical management of this disease (12).

FLII gene, originally identified in Drosophila melanogaster, encodes a protein containing an N-terminal leucine-rich repeat (LRR) domain and a C-terminal gelsolin-like domain (GLD;
Translational Relevance

Prostate cancer is the second leading cause of cancer-related death in men worldwide. Castration-resistant prostate cancer is an untreatable form of prostate cancer, which bypassed the normal dependence on androgens for growth and survival. Here, we identified a biochemical and functional link between FLII and androgen receptor (AR). Reduced expression of FLII in advanced prostate cancer may lead to AR overactivation, tumor growth, and development of castration-resistant phenotype of late-stage prostate cancer. This study provides, for the first time, compelling evidence, which rationalizes the targeting strategy to reestablish the functional interaction between FLII and AR signaling in clinical practice to treat prostate cancer.

ref. 13. Gelsolin protein family has previously been identified as a hormone nuclear receptor coactivator (14). LRR of FLII is implicated in protein–protein interaction and many have identified that these binding partners and GLD of FLII have some unique regulatory function (13). PI3K and small GTPases may regulate the subcellular localization of FLII between cytoplasm and nucleus (15). FLII has been found to interact with various proteins important for cellular signaling (15). FLII may function as a transcriptional coregulator which positively or negatively regulates the activity of transcription factors (15). FLII can be recruited by hormone-activated nuclear receptors to the promoters of target genes to serve as a coactivator of the nuclear receptor transcription complex (14, 16, 17). FLII also negatively regulates PPARγ, β-catenin, and ChREBP-mediated transcription in cancer cells (15, 18, 19). It is currently unknown whether FLII plays a role in AR function during prostate cancer development.

In this study, we investigated FLII expression and found it to be inversely correlated with PSA levels in prostate cancer. Patients with higher FLII expression in their tumor have improved overall survival. In human prostate cancer cells, FLII formed a complex in a ligand-dependent manner through the ligand-binding domain (LBD) of AR. Our results indicated competitive relationship between FLII and ligand in binding to the AR. FLII repressed the AR expression, inhibited AR transactivation and modulated AR cytoplasm/nucleus translocation. Furthermore, FLII contributes to the human CSPC and CRPC cell growth, invasion, and migration through AR-dependent signaling. Reintroduction of FLII in castration-sensitive and castration-resistant cells sensitized the cells to bicalutamide and enzalutamide treatment. Our findings revealed that FLII functions as a novel AR repressor and inhibits prostate cancer progression.

Materials and Methods

Cell culture, plasmid construction, reporter genes, reagents, expression vectors, and DNA transfection

Human prostate cancer LNCaP cells were obtained from ATCC and maintained in RPMI1640 medium (Invitrogen) supplemented with 10% FBS or 10% charcoal/dextran–striped FBS (cFBS). LAPC-4 and C4-2 were maintained in RPMI1640 (Invitrogen) supplemented with 10% FBS. pIREpuro-HA-FLII, pIREpuro-FLAG-FLII, pIREpuro-FLAG-LRR, and pIREpuro-FLAG-FLII-GLD were provided by Dr. Tong (Shanghai Jiao Tong University School of Medicine, Shanghai, China) (15). GST-FLII-LRR, GST-FLII-GLD-A, and GST-FLII-GLD-B were provided by Dr. Stallcup (University of Southern California, Los Angeles, CA) (16). GAL4-AR and truncated mutants were provided by Dr. Balk (Harvard Medical School, Boston, MA) and Dr. Chang (University of Rochester Medical Center, Rochester, MN) (20, 21). Plenti-IREpuro-vector, GIPZ-shFLII-1, and GIPZ-shFLII-4 were purchased from Thermo Scientific. PSA-Luc and ARE4-Luc reporter genes are described in refs. 9 and 22. LNCaP and C4-2 cells infected with GIPZ-Vector, shFLII-1, shFLII-4. Positive cells were selected by FCM.

Tissue samples

Human prostate cancer tissue arrays were purchased from Biomax (PR956B). All the detailed information including Pathology diagnosis, clinical stage, Gleason scores, PSA level, and survival data was directly shown online (http://www.biomax.us/tissue-arrays/Prostate/PR956b).

Overall survival analysis in GSE21034 database

A normalized mRNA expression dataset for prostate adenocarcinoma was downloaded from the cbioPortal for cancer genomics and used to evaluate expression of FLII and AR transcript levels (23). This dataset includes mRNA profiles for 131 primary tumor and 19 metastasis samples. Overall survival analysis was calculated for these transcripts for primary tumor samples. All the detailed information of prostate cancer patients including pathology diagnosis, clinical stage, Gleason scores, and survival data could be directly downloaded as an Excel sheet from the cbioPortal website (http://www.cbioportal.org/).

Immunohistochemistry and immunofluorescence staining

Immunohistochemical (IHC) analysis of human prostate cancer was conducted using a polyclonal FLII antibody. Four-micrometer sections were prepared from paraffin-embedded C4-2 tumor tissues derived from nude mice, and tissues were extracted from paraffin. Tumor tissues were stained with primary antibody including AR (SC-13062, Santa Cruz Biotechnology), FLII (SC-21716, Santa Cruz Biotechnology), and PSA (SC-7316, Santa Cruz Biotechnology), Ki67 (RM-9106-S1, Thermo Scientific). For immunofluorescence staining, cells were fixed in 3.7% paraformaldehyde, permeated by 0.1% Triton X-100, and blocked with 5% goat serum in PBS for 1 hour at 37° C, followed by incubation with FITC and rhodamine secondary antibodies.

RNA isolation and real-time PCR analysis

Total RNAs were extracted with TRIzol reagent according to the manufacturer’s instruction (Invitrogen). To determine the mRNA levels of FLII, AR, and PSA, total RNAs were reversely transcribed by iScript cDNA Synthesis Kit (Bio-Rad Laboratories), respectively, according to the manufacturer’s instructions (2).
**Immunoprecipitation and Western blot analysis**

Immunoprecipitation and Western blot assays were conducted in LNCaP and 293T cells as indicated. Cells were pelleted and lysed in buffer (50 mmol/L HEPES, pH 7.2, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 0.1% Tween 20) supplemented with a Protease Inhibitor Cocktail and 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 0.1% Tween 20) supplemented with a Protease Inhibitor Cocktail (Roche Diagnostics). Antibodies were used for Western blot analysis. Western blot analysis and immunoprecipitation were: anti-AR (sc-13062, Santa Cruz Biotechnology), anti-FLII (sc-21716, Santa Cruz Biotechnology), anti-PSA (sc-7316, Santa Cruz Biotechnology), anti-TMPRSS2 (ab92323, Abcam), anti-HA (Ssc-17121, Santa Cruz Biotechnology), anti-FLAG (M2 clone, Sigma).

**Protein–protein interaction by GST pull-down assays**

In vitro protein–protein interactions were performed as described previously (24, 25). In vitro translated proteins were prepared by coupled transcription–translation with a TNT-coupled reticulocyte lysate kit (Promega) using plasmid DNA (1.0 mg) in a total of 50 mL. GST fusion proteins were prepared from E. coli. In vitro translated protein (15 mL) was added to 5 mg GST fusion protein of GST as control in 225 mL binding buffer [50 mmol/L Tris-HCl, 120 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5% NP40, 1 mmol/L EDTA, 2 mg/mL leupeptin, 2 mg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 mg/mL pepstatin] and rotated for 2 hours at 4°C. Glutathione–sepharose bead slurry (50 mL) was added and the mixture was rotated for a further 30 minutes at 4°C. Beads were washed 5 times with washing buffer (1 mL) and binding buffer (30 mL) was added after the final wash. Sepharose beads were washed 5 times with lysis buffer and boiled in SDS sample buffer, and the proteins released were resolved by SDS-PAGE followed by Western blot analysis.

**Luciferase assays**

Cells were seeded at a density of 1 × 10^5 cells in a 24-well culture plate on the day prior to transfection with Superfect transfection reagent according to the manufacturer’s protocol (Qiagen). For reporter gene assays, a dose response was determined in each experiment with 50 and 200 ng of expression vector and promoter reporter plasmids (0.5 μg). Luciferase activity was normalized for transfection efficiency using β-galactosidase reporter as an internal control. The fold effect of expression vector was determined in comparison with the value of the empty expression vector cassette and statistical analyses were performed using the t test (22, 26).

**Colony formation assays**

Cells were plated in triplicates in 3 mL of 0.3% agarose (sea plaque) in complete growth medium overlaid on 0.5% agarose base, also in complete growth medium. Two weeks after cell seeding, colonies were visualized after staining with 0.04% crystal violet in methanol for 1 to 2 hours. The colonies more than 50 μm in diameter were counted using an Omnicont 3600 image analysis system.

**Transwell invasion assays**

Biocoat Matrigel invasion chamber inserts (BD Biosciences) were equilibrated for 2 hours at 37°C in serum-free medium. Cells were seeded at a density of 2.5 × 10^4 per well in serum-free medium in the top chamber of the insert and cells were allowed to invade through the Matrigel. Medium containing 10% FBS was added to the bottom chamber as a chemoattractant. After 22 hours, cells were washed with PBS and fixed with 4% formaldehyde/PBS for 15 minutes. After washing with PBS, cells in the top chamber were removed with a cotton swab and cells attached to the bottom surface of the filter were rinsed. Cells were stained with Hoechst 33258 (1 μg/mL) for 30 minutes in the dark and visualized under a fluorescence microscope. Three random fields were captured at 10× magnification (n = 3; ref. 27).

**Wound healing assays**

Cells were grown to confluence and wounded by dragging a 10 μL pipette tip through the monolayer. Cells were washed to remove cell debris and allowed to migrate for 12 hours. Images were taken at time 0 and 12 hours postwounding under a Nikon Diaphot TMD inverted microscope (20×). The relative distance traveled by the leading edge from 0 to 12 hours was assessed using the Photoshop 7.0 software (n = 6; ref. 27).

**In vivo tumor implantation**

A total of 2 × 10^6 cells were injected subcutaneously into 4- to 6-week-old castrated male nude mice purchased from Beijing HFK Bio-technology Co., Ltd. Tumor growth was measured using a digital caliper every 5 days for 4 to 5 weeks. Tumor weight was measured when mice were sacrificed on day 32 after cell implantation. Immunohistochemical staining under the standard procedure was conducted as previously described (2, 22).

**Statistical analysis**

All statistical analyses were performed using Excel 2010 (Microsoft). All in vitro experiments were performed in triplicate and all data were represented as mean ± SD. Statistical analyses were conducted using the Student t test and Pearson correlation coefficient. The significance of each value was determined when P value was less than 0.05.

**Results**

Endogenous expression of FLII is positively correlated to overall survival of prostate cancer patients

FLII, as a transcriptional coregulator, positively regulated estrogen receptor (ER) and thyroid hormone receptor (TR; refs. 14, 16, 17) and suppressed PPARγ, β-catenin, and ChREBP-mediated transcription in cancer cells (15, 18, 19). Currently, it was unknown whether FLII played a role in AR function. We first performed in silico analysis for FLII and AR expression on a gene expression dataset composed of 150 patient samples with follow-up information (Supplementary Fig. S1A; http://www.cbioportal.org/; ref. 23). The Kaplan–Meier analysis was conducted to evaluate the difference in overall survival of patients associated with high versus low expression of FLII and AR gene in their tumor. The median transcript levels of FLII and AR genes were used to assign the patient samples to either high or low expression group (28). According to Kaplan–Meier analysis, patients with primary tumors expressing high levels of FLII (n = 70) had significantly higher overall survival (P = 0.049; Supplementary Fig. S1B). Furthermore, in the primary tumors with high AR expression (n = 58), there was also a significant trend toward improved overall survival of patients with high expression of FLII (n = 37) compared with those with low FLII expression (n = 21) in tumors (P = 0.0076; Supplementary Fig. S1C). However, no statistically significant difference was found between high and
low FLII expression groups in tumors with low AR expression \((n = 74; P = 0.7842; \text{Supplementary Fig. S1D})\), suggesting FLII may interact with AR signaling in prostate cancer.

To further confirm the interaction between FLII and AR, we examined FLII expression using tissue array containing 36 cases of human prostate cancer and 8 adjacent normal tissue specimens (Biomax, PR956B; Fig. 1A). FLII showed whole epithelial cell distribution with stronger staining in adjacent normal tissue \((n = 8)\) compared with prostate cancer tissues \((n = 36; \text{Fig. 1B})\). Furthermore, FLII protein levels were decreased in cancerous tissues with moderate expression in early stages (II) and poor expression in advanced stages (III/IV; Fig. 1B). Kaplan–Meier analysis was conducted to evaluate the difference between disease-overall survival associated with high and low expression levels of FLII. The median FLII expression was used to assign the samples to high or low group. Patients with tumors expressing high FLII \((n = 18)\) had significantly higher disease-overall survival \((P = 0.045; \text{Fig. 1C})\), consistent with the result from in silico analysis. Prostate-specific antigen (PSA), a clinical biomarker of prostate cancer, was mainly induced by androgen and regulated by the AR at the transcriptional level (29). We analyzed the relationship between FLII and PSA. As shown in Fig. 1D, in patients with high PSA levels \((\geq 20 \text{ ng/mL})\), there was a lower FLII expression in tumor, suggesting an inverse correlation between FLII and AR transcriptional activity in clinical prostate cancer specimens.

Next, we analyzed the abundance of FLII mRNA by quantitative real-time PCR (qRT-PCR) in prostate cancer tissues. As shown in Fig. 1B, the mRNA level of FLII was significantly decreased in prostate cancer tissues \((n = 40)\) compared with normal prostate tissue \((n = 20)\). Patients with high PSA levels \((10–20 \text{ or } >20 \text{ ng/mL})\) had a lower FLII expression in tumor \((n = 40; \text{Fig. 1D})\), consistent with the result from tissue array analysis. Furthermore, FLII and AR were inversely correlated in preclinical prostate cancer patients \((r = -0.5745, P = 0.0159, n = 1; \text{Supplementary Fig. S2A})\).

**AR-LBD is required for binding to FLII**

The inverse correlation between PSA and FLII raised a possibility that FLII may function through interaction with androgen/AR signaling. To explore this possibility, we first examined the interaction between FLII and AR. FLAG-FLII wild-type (wt), FLAG-FLII-LRR (residues 1–450), or FLAG-FLII-GLD (residues 451–1268) with HA-tagged AR wt were expressed in HEK 293T cells. HA-AR was immunoprecipitated from cell lysates and analyzed for AR binding by Western blot analysis. We found that FLAG-FLII, FLAG-FLII-LRR, and FLAG-FLII-GLD coimmunoprecipitated with HA-AR \((\text{Fig. 2A})\), suggesting either domain is sufficient for AR binding, and further suggesting that the presence of both decreases binding, compared with either alone. To prove that FLII and AR directly interact, The FLII protein was purified as a GST protein from bacteria and AR was expressed by *in vitro* transcription/translation (IVT). GST pull-down was conducted and the analysis was completed for FLII-LRR (residues 1–494), FLII-GLD-A (residues 495–822), and FLII-GLD-B (residues 825–1268) and their binding for AR. AR bound to FLII-LRR and FLII-GLD-B, but failed to bind the FLII-GLD-A \((\text{Fig. 2B})\).

To identify the domains of AR required for binding to FLII, a series of Gal4-AR-mutant \((\text{residues 11–265, 262–295, 295–550, and 624–919})\) expression vectors were transfected into HEK 293T cells together with vector-expressing HA-tagged FLII \((\text{HA-FLII})\). The N-terminal HA epitope was used to immunoprecipitate HA-FLII. Transfection of HEK 293T cells with a series of AR mutants showed that AR bound to FLII and that the AR-LBD \((\text{residues 624–919})\) was sufficient for binding to FLII \((\text{Fig. 2C})\). To further confirm that FLII only bound AR-LBD, EGFP-AR-ADDBD \((\text{deletion of DNA-binding domain})\), and EGFP-AR-ALBD \((\text{deletion of LBD})\) expression vectors were expressed in HEK 293T cells. The N-terminal HA epitope was used to immunoprecipitate FLII. As shown in Fig. 2D, deletion of the AR-LBD abolish AR binding to FLII. These studies demonstrated that the LBD of AR is required for binding FLII.

**DHT treatment abolishes AR and FLII binding**

When AR was activated by endogenous androgenic ligands, the ligand–receptor complex translated from the cytoplasm into the nucleus, and in association with coregulatory factors, bound to specific androgen-responsive elements in the regulatory regions of AR target genes \((30)\). The AR agonists and antagonists modulated AR function by binding to the AR-LBD \((31)\). Given that AR-LBD was required for binding to FLII, we hypothesized that ligands regulate binding of FLII to AR. To explore this possibility, Gal4-AR wt and Gal4-AR-LBD with FLAG-FLII wt, FLAG-FLII-LRR and FLAG-FLII-GLD were expressed in HEK 293T cells and treated with DHT. FLAG-FLII was immunoprecipitated from cell lysates using an anti-FLAG antibody and analyzed for AR binding by Western blot analysis. As shown in Fig. 3A, AR was coinmunoprecipitated and detected in the absence of DHT, while androgen treatment decreased the binding of FLII to AR, suggesting a potential competition between FLII and DHT for binding AR. Bicalutamide (Casodex), a competitive inhibitor of AR, prevented androgen binding to AR by blocking its binding sites at the target gene promoters \((32)\). To further confirm the potential competition between FLII and DHT in binding to the AR, we cotransfected HA-FLII wt and Gal4-AR wt into 293T cells treated with DHT and/or bicalutamide. HA epitope was used to immunoprecipitate FLII. As shown in Fig. 3B, bicalutamide promoted the binding of FLII on AR and inhibited the DHT-mediated effects.

To determine whether endogenous FLII associated with AR, we performed IP-Western blot assay with an antibody directed toward endogenous AR. AR antibody coprecipitated FLII in LNCap cells in either presence or absence of DHT and/or bicalutamide. DHT decreased binding of FLII on AR \((\text{Fig. 3C})\), while bicalutamide enhanced the binding of FLII and AR and inhibited the DHT-mediated effects \((\text{Fig. 3C})\).

**FLII regulates AR subcellular localization**

The competition between FLII and DHT in binding to AR raised a possibility that FLII may suppress AR transactivation through interrupting AR nuclear translocation. To test the possibility, we performed immunofluorescent staining analyses. PC3 cells were cotransfected with HA-FLII and/or FLAG-AR and treated with DHT. The subcellular localization of AR was examined by detecting FLAG-AR. In the absence of FLII, the unliganded AR was located mainly in the cytoplasm with the ligand-bound AR predominantly in the nucleus \((\text{Supplementary Fig. S3A and S3B})\). In contrast, overexpression of FLII did not alter the cytoplasmic localization of AR in the absence of DHT, while the DHT-induced nuclear localization of AR was decreased \((\text{Supplementary Fig. S3A and S3B})\). Therefore, FLII antagonizes DHT function and promotes cytoplasmic accumulation of AR.
Figure 1.
Expression of FLII was positively correlated to overall survival of prostate cancer patients. A, representative examples of immunohistochemical staining for FLII in each of the clinical stages of prostate cancers as indicated. B, quantification of FLII relative intensity for each clinical stage of prostate cancers. FLII abundance was determined by qRT-PCR between normal (n = 20) and tumorous (n = 40) prostate samples. Data is shown as mean ± SEM for N as indicated in the figure in parenthesis as shown. C, Kaplan-Meier analysis showed a significant trend toward improved survival in cases showing high expression (n = 18) of FLII as opposed to cases showing low expression (n = 17) of FLII (P = 0.045). D, immunohistochemical staining was also conducted to determine FLII expression and PSA levels in the prostate cancer specimens (n = 28). qRT-PCR was conducted to determine FLII expression and PSA levels in the prostate cancer specimens (n = 40).
Figure 2.

AR-LBD is required for binding to FLII. A, schematic representation of AR, FLII, and FLII mutation expression vectors. Immunoprecipitation (IP)–Western blot analysis was conducted of 293T cells transfected with expression vectors encoding either N-terminal FLAG-tagged AR or HA-tagged FLII expression vectors. B, schematic representation of AR, FLII, and FLII mutation expression vectors. GST pull-down conducted with GST-FLII proteins (LRR encoding AA 1-494, GLD-A encoding 495-822, and GLD-B encoding 825-1268) and IVT-AR. C, schematic representation of FLII, AR, and AR mutation expression vectors. Immunoprecipitation–Western blot analysis was conducted of 293T cells transfected with expression vectors encoding either N-terminal HA-tagged FLII or Gal4-tagged AR expression vectors. D, schematic representation of FLII and AR mutation expression vectors. Immunoprecipitation–Western blot analysis was conducted of 293T cells transfected with expression vectors encoding either N-terminal HA-tagged FLII or EGFP-tagged AR mutation expression vectors. All the data are representative of N = 3 separate experiments.
Figure 3.
DHT treatment abolishes AR and FLII binding. A, Gal4-AR wt and Gal4-AR-LBD with HA-FLII wt, HA-FLII-LRR, and HA-FLII-GLD were expressed in 293T cells and treated with DHT. HA-FLII was immunoprecipitated from cell lysates by an anti-HA antibody and analyzed for AR binding by Western blotting analysis. B, HA-FLII wt and Gal4-AR wt were expressed in 293T cells treated with DHT and/or bicalutamide. HA epitope was used to immunoprecipitate FLII. Relative binding of AR to FLII is shown as mean ± SEM for three separate experiments. C, Immunoprecipitation (IP)—Western blotting in LNCaP cells treated with 10 nmol/L DHT and/or 10 μmol/L bicalutamide. Immunoprecipitation was conducted with an AR antibody to detect endogenous AR. Relative binding of AR to FLII is shown as mean ± SEM for three separate experiments.
To further confirm the immunofluorescent staining results, we performed Western blot analyses using cytoplasmic and nuclear fractions of the cell lysates in PC-3 and LNCaP cells. PC3 cells were cotransfected with HA-FLII and/or FLAG-AR and treated with DHT. LNCaP cells stably transduced with lentiviral shRNAs targeting FLII. As shown in Supplementary Fig. S3C, following DHT treatment, the distribution of AR in the nucleus was decreased when FLII was transfected (lane 8) compared with vector control (lane 6). Conversely, depletion of endogenous FLII in LNCaP cells significantly increased the distribution of AR in the nucleus and decreased the distribution of AR in the cytoplasm (Supplementary Fig. S3D). Taken together, these results demonstrated that FLII regulated AR cytoplasm/nucleus localization and may play a role in DHT-regulated AR activation.

**FLII suppresses AR expression and transactivation activity**

To determine whether FLII regulated AR signaling, we conducted PSA-luc and ARE4-luc luciferase reporter assays. As shown in Fig. 4A, overexpression of FLII in LNCaP cells reduced DHT-induced PSA-luc and ARE4-luc activity, and enhanced the effects of bicalutamide on AR. Conversely, knockdown of FLII increased DHT-induced PSA-luc and ARE4-luc activity in LAPC-4 and LNCaP cells (Fig. 4B and Supplementary Fig. S4A). Analysis of FLII effects on basal AR function showed that FLII has significantly decreased basal levels of PSA-luc and ARE4-luc activity (Fig. 4A and B and Supplementary Fig. S4A).

The expression of endogenous PSA and TMPRSS2 gene is controlled by ligand-activated AR. To support the conclusion that FLII impacts AR function, we used shRNA to knockdown endogenous FLII expression in LNCaP cells (Fig. 4C and D) and determined the basal and DHT-regulated PSA and TMPRSS2 gene and protein levels by using Western blot and qRT-PCR analysis. The results show increased basal and DHT-induced PSA and TMPRSS2 gene and protein levels (Fig. 4C and D). Furthermore, we conducted Western blot analysis in LNCaP and C4-2 cells with stable knockdown of FLII to determine the basal and DHT-regulated AR protein levels. As shown in Fig. 4C and Supplementary Fig. S4B, depletion of FLII enhanced AR protein levels in the presence and absence of ligand, suggesting of the idea that FLII may control AR function by regulating stability of the AR protein. Accordingly, LNCaP cells stably knocked down FLII were treated with cycloheximide to inhibit protein synthesis, and AR protein turnover was analyzed over time. Compared with the control cells, AR half-life was considerably increased in LNCaP cells stably knocked down FLII (Supplementary Fig. S4C). Previously, studies indicate the targeting of AR for degradation via the ubiquitin–proteasome pathway is mediated in large part by the RING finger–containing E3 ligases (33–35). We used bortezomib, a proteasome inhibitor, to investigate mechanisms involved in the FLII-dependent AR degradation. The treatment of control LNCaP cells with bortezomib increased accumulation of AR protein (Supplementary Fig. S4D), suggesting the constitutive AR degradation. Similarly, the decreased in AR protein levels (caused by forced overexpression of FLII) was reversed upon treatment with bortezomib (Supplementary Fig. S4D), implying FLII causes the AR degradation in the proteasome. Taken together, these observations suggested that FLII suppresses AR expression and transactivation activity.

**NCoR and SMRT promote the binding and suppressing effect of FLII on AR**

Previous studies showed that NCoR and SMRT were important corepressors of AR and repress AR transcriptional activities (36). Next, we examined the interactions between FLII, AR with NCoR or SMRT. HEK 293T cells were cotransfected with FLAG-tagged NCoR or SMRT vector together with HA-FLII and Gal4-AR. The cells were treated with DHT for 1 hour. HA-FLII was immunoprecipitated from cell lysates using HA antibody and analyzed for FLII and AR binding by Western blot analysis. As shown in Supplementary Fig. S5A and S5B, an increased interaction between FLII and AR in the presence of NCoR or SMRT was observed. These results suggested that NCoR and SMRT interact with FLII and AR. Notably, NCoR and SMRT promoted the binding of FLII on AR.

Furthermore, the effect of NCoR or SMRT on FLII function was examined. As shown in Supplementary Fig. S5C and S5D, overexpression of NCoR or SMRT in LNCaP cells enhanced FLII-repressed PSA and ARE4 reporter activity by 50% following DHT treatment, suggesting NCoR and SMRT promoted the suppressing effect of FLII on AR. Taken together, AR corepressors (NCoR and SMRT) and AR antagonist (bicalutamide) promoted the binding of FLII on AR, while AR agonist (DHT) decreased this binding, indicating a potential competition of FLII in binding to the AR.

**FLII contributes to prostate cancer cell growth, migration, and invasion through AR-dependent signaling, and reintroduction of FLII revert castration resistance of prostate cancer cells**

AR has been reported to promote cell proliferation, migration, and invasion and plays a critical role in the development of prostate cancer (37–39). Given that FLII interacts with AR and regulated AR activity, we reasoned that FLII might regulate the growth, migration, and invasion of AR-positive prostate cancer cells. To test our hypothesis, we conducted MTT, colony formation, transwell invasion, and wound healing assays. As shown in Fig. 5A and Supplementary Fig. S6A and S6B, depletion of endogenous FLII in LNCaP, LAPC-4, and C4-2 cells increased the cell growth. We also observed that depletion of endogenous FLII in LNCaP and C4-2 cells increased the cell migration and invasion (Supplementary Figs. S7A and S8A).

To further investigate whether FLII-mediated inhibition of cell growth, migration, and invasion is dependent on AR, we first knocked down FLII in LNCaP cells followed by DHT treatment. Depletion of FLII increased LNCaP cell growth potential in the presence of DHT, while androgen deprivation abolished FLII-mediated inhibition of cellular proliferation (Fig. 5A and Supplementary Fig. S6C). Furthermore, overexpression of FLII with simultaneous depletion of AR in LAPC-4 and C4-2 cells inhibited cell growth in the presence of endogenous AR (Fig. 5B and Supplementary Fig. S6D), while AR knockdown abolished the FLII-mediated repression. Next, similar observation was made in transwell invasion and wound healing assays. Depletion of FLII increased LNCaP cell invasion and migration potential in the presence of DHT, while androgen deprivation abolished FLII-mediated inhibition (Supplementary Figs. S7A and S8A). Conversely, overexpression of FLII with simultaneous depletion of AR in C4-2 cells inhibited cell invasion and migration in the presence of endogenous AR, and AR knockdown abolished the FLII-mediated repression (Supplementary Figs. S7B and S8B). Taken together, these data indicate that FLII inhibits prostate cancer cell growth, migration, and invasion through AR-dependent signaling.
FLII suppresses AR expression and transactivation activity. A and B, androgen-responsive luciferase reporter genes (PSA-Luc, ARE4-Luc) were assessed for AR activity. LNCaP cells with FLII overexpression were treated with 10 nmol/L DHT and/or 10 μmol/L bicalutamide. LAPC-4 cells with FLII knockdown were treated with 10 nmol/L DHT and/or 10 μmol/L bicalutamide. C, FLII, AR, PSA, and TMPRSS2 protein levels were determined by Western blot analysis in LNCaP cells with stable FLII knockdown. LNCaP cells were treated with 10 nmol/L DHT. D, FLII, PSA, and TMPRSS2 mRNA levels were determined by qRT-PCR in LNCaP cells with stably FLII knockdown. LNCaP cells were treated with 10 nmol/L DHT.

Figure 4.

**FLII** Represses Androgen Receptor

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FLII contributes to prostate cancer cell growth through AR-dependent signaling and reintroduction of FLII revert castration resistance of prostate cancer cells. A, LNCaP cells with stable FLII knockdown were treated with or without 10 nmol/L DHT. LAPC-4 cells with stable overexpression of FLII and AR knockdown. Cells were analyzed for cell proliferation by MTT assay. B, C4-2 cells with stable overexpression of FLII and AR knockdown. Cells were analyzed for cell proliferation by MTT assay. C, LNCaP cells with stable knockdown of FLII followed by treatment with bicalutamide (10 μmol/L) or enzalutamide (5 μmol/L). LAPC-4 cells with stable overexpression of FLII and AR knockdown followed by treatment with bicalutamide (10 μmol/L) or enzalutamide (5 μmol/L). Cells were analyzed for cell proliferation by MTT assay. D, C4-2 cells with stable overexpression of FLII followed by treatment with bicalutamide (10 μmol/L) or enzalutamide (5 μmol/L). Cells were analyzed for cell proliferation by MTT.

Figure 5.

FLII contributes to prostate cancer cell growth through AR-dependent signaling and reintroduction of FLII revert castration resistance of prostate cancer cells. A, LNCaP cells with stable FLII knockdown were treated with or without 10 nmol/L DHT. LAPC-4 cells with stable overexpression of FLII and AR knockdown. Cells were analyzed for cell proliferation by MTT assay. B, C4-2 cells with stable overexpression of FLII and AR knockdown. Cells were analyzed for cell proliferation by MTT assay. C, LNCaP cells with stable knockdown of FLII followed by treatment with bicalutamide (10 μmol/L) or enzalutamide (5 μmol/L). LAPC-4 cells with stable overexpression of FLII and AR knockdown followed by treatment with bicalutamide (10 μmol/L) or enzalutamide (5 μmol/L). Cells were analyzed for cell proliferation by MTT assay. D, C4-2 cells with stable overexpression of FLII followed by treatment with bicalutamide (10 μmol/L) or enzalutamide (5 μmol/L). Cells were analyzed for cell proliferation by MTT.

cancer cell growth, migration, and invasion through AR-dependent signaling.

One of the hallmarks of prostate cancer treatment failure was the development of castration resistance to antiandrogen therapies. Ligand-independent AR activation contributed to the development of castration resistance (38, 40). Bicalutamide, a pharmaceutical drug commonly used as an antiandrogen therapy to treat recurrent prostate cancer, was a competitive inhibitor of AR. Bicalutamide prevented androgen binding to AR by blocking its binding sites at the target gene promoters (32). Enzalutamide (Xtandi), a novel AR signaling inhibitor, blocked the growth of CRPC in cellular model systems and was shown in a clinical study to increase survival in patients with metastatic CRPC. Enzalutamide inhibited multiple steps of AR signaling: binding of androgens to AR, AR nuclear translocation, and association of AR with DNA (6). In this study, bicalutamide enhanced the binding of FLII and AR and inhibited the DHT-mediated effects. FLII also enhanced the effects of bicalutamide on AR transactivation activity. To explore whether FLII inhibited ligand-independent AR activation and reverted castration resistance, we first conducted MTT assays in LNCaP, LAPC-4, and C4-2 cells treated with bicalutamide or enzalutamide. As shown in Fig. 5C, we observed that knockdown of FLII alleviated inhibition of growth to bicalutamide and enzalutamide in castration-sensitive LNCaP cells and overexpression of FLII increased inhibition of growth to bicalutamide and enzalutamide in castration-sensitive LAPC-4 cells, indicating that expression of FLII could enhance enzalutamide and bicalutamide sensitivity of castration-sensitive prostate cancer cells. As a result, castration-resistant cells were normally refractory to bicalutamide treatment (41). We observed that overexpression of FLII induced inhibition of growth to bicalutamide and enhanced inhibition of growth to enzalutamide in castration-resistant C4-2 cells (Fig. 5D), indicating that expression of FLII could revert castration resistance of prostate cancer cells. Next, transwell invasion and wound healing assays were performed to further characterize these effects of FLII on cell migration and invasion. As shown in Supplementary Figs. S7C and S7D and S8C and S8D, knockdown of FLII alleviated inhibition of...
migration and invasion to bicalutamide in castration-sensitive LNCaP cells and conversely overexpression of FLII induced inhibition of migration and invasion to bicalutamide in castration-resistant C4-2 cells.

FLII inhibits prostate tumor growth in vivo

Encouraged by these observations, we then investigated the role of FLII in inhibiting prostate tumor growth in vivo. C4-2 cells stably transduced with lentiviral shRNAs targeting FLII (C4-2/shFLII) were implanted subcutaneously into immunodeficient nude mice. Tumor size was measured every 5 days with last measuring performed on day 32 (Fig. 6A). We observed that depletion of endogenous FLII in C4-2 cells significantly reduced both the tumor size and the tumor weight (Fig. 6A). To determine the expression of FLII, AR, PSA, and Ki67 in tumors, we conducted qRT-PCR and IHC staining in tumor specimens. As shown in Fig. 6B, tumors with FLII knockdown had increased PSA and Ki67 mRNA expression, except for AR. Nevertheless, IHC staining demonstrated that tumors with FLII knockdown increased AR, PSA, and Ki67 protein expression, suggesting that FLII may control AR expression by regulating stability of the AR protein, consistent with our previous studies. Taken together, these observations indicated that reduction of FLII promoted growth of prostate cancer cells through enhanced AR signaling.

Next, to demonstrate that FLII-mediated inhibition of cell growth is dependent on AR in vivo, we overexpressed FLII with simultaneous depletion of AR in LAPC-4 cells. Cells were implanted subcutaneously into immunodeficient nude mice and monitored for tumor growth by measuring the tumor size and tumor weight. We observed that overexpression of FLII reduced both the tumor size and the tumor weight in the presence of endogenous AR, while AR knockdown abolished the FLII-mediated repression (Supplementary Fig. S9A), suggesting that FLII inhibits prostate cancer cell growth through AR-dependent signaling. Furthermore, to determine the expression of FLII, AR, and PSA in tumors, we conducted qRT-PCR in tumors. As shown in Supplementary Fig. S9B, tumors with FLII overexpression had decreased PSA expression, while AR knockdown abolished the FLII-mediated repression, suggesting that reduction of FLII promotes growth of prostate cancer cells through enhanced AR signaling.

Discussion

AR, a ligand-activated transcription factor, played a critical role in the development of prostate cancer (9). When activated by the endogenous androgenic ligands, testosterone (T) and dihydrotestosterone (DHT), AR was phosphorylated, and translocated from cytoplasm into the nucleus to bind to specific androgen-responsive element (ARE) in the regulatory regions of AR target genes (30). Androgen action involves dissociation of AR from HSP complex, homodimerization, nuclear translocation, and binding to target genes, all these processes can be influenced by AR coregulators (42). AR coregulators, including AR coactivators and corepressors, positively and negatively regulate transactivation of AR. Aberrant coregulator function due to a mutation or altered expression levels may contribute to the progression of AR-mediated diseases (43). To date, many coregulators of AR have been characterized. Compared with the coactivators, the known corepressors are relatively fewer and less well characterized (42). NCoR and SMRT are important corepressors of AR and repress AR transactivation (36). ARA67/PAT1 binds to AR multiple domains and suppresses AR transactivation by interrupting nuclear localization of AR (42). FBI-1 has been reported to suppress the AR transcription activity, which is dependent on the binding to AR (44). Unfortunately the role of AR corepressors during prostate cancer progression was unclear. Herein, we provided evidence showing endogenous FLII expression was positively correlated with overall survival of prostate cancer patients. Specially, in the primary tumors with high AR expression, there was a significant trend toward improved survival of patients with high expression of FLII compared with those with low FLII expression in tumors. However, no statistically significant difference was found between high and low FLII expression groups in tumors with low AR expression. Next, we demonstrated that FLII is dissociated from AR in a ligand-dependent manner, which is similar to that of other corepressors, such as NCoR1 and SMRT. FLII inhibited AR transactivation and modulated AR cytoplasm/nuclear localization through direct binding to AR-LBD. Furthermore, FLII contributes to the human CSPC and CRPC cell growth, invasion, and migration through AR-dependent signaling. Our data indicate that FLII functions as an AR corepressor and inhibits prostate cancer progression.

Like the other members of the nuclear receptor superfamily, AR has four functional domains: N-terminal transactivation domain (NTD), the DNA-binding domain (DBD), C-terminal ligand-binding domain (LBD), and hinge region connecting DBD and LBD (45). The LBD of AR is essential for ligand-dependent transcription. The mutation or deletion of LBD dramatically reduces the transcriptional activity of AR in response to androgen. In general, androgen modulates AR cytoplasmic/nuclear translocation and function by binding to its LBD (45). Nuclear localization of androgen bound AR is a prerequisite for its transactivation (42) and decrease of AR nuclear translocation leads to suppression of AR transactivation and androgen-induced cell growth (42). Our study showed that LBD of AR is required for binding FLII. FLII and androgen are mutually exclusive in binding the LBD of AR. In addition, FLII inhibited AR cytoplasmic/nuclear translocation in the presence of its ligand DHT. These results revealed a novel mechanism by which FLII regulates AR transactivation through binding to AR-LBD in prostate cancer. FLII decreased the binding of DHT to AR, which further inhibited androgen receptor cytoplasmic/nuclear localization and its transcriptional activity.

NCoR and SMRT are important corepressors of AR and repress AR transactivation (36). In the presence of AR agonist, NCoR and SMRT can be recruited to the PSA promoter and repress the PSA expression (46). Corepressors of AR that trigger chromatin condensation and/or chromatin modifications typically recruit HDACs to the AR complex (44). The well-characterized corepressors NCoR and SMRT could directly interact with multiple HDACs and recruit them to AR (44, 47). It has been reported that FLII negatively regulated ß-catenin-mediated transcription by disrupting the synergy of FLAP1 with ß-catenin and p300 (19). Furthermore, the negative regulation of ß-catenin, p300, and FLAP1 activity by FLII found that FLII did not bind well to p300 KIX domain and did not appear to inhibit FLAP1 and p300 binding (19). They reasoned FLII may exert its negative influence by squelching the activity of FLAP1 and other essential factors that bind to FLII (19). It was possible that FLII may recruit negative regulators, such as HDACs, to the ß-catenin/LEFI/TCF transcription complex (19). In our studies, an increased interaction between FLII and AR in the presence of NCoR or SMRT was...
Figure 6. FLII inhibits prostate tumor growth in vivo. A, C4-2 tumors with stable FLII knockdown were injected into nude mice. Tumor size was measured every 5 days. The data are shown as mean ± SEM for $N > 6$ separate tumors for each group. Images of tumors dissected from the mice. The tumor size (mm$^3$) was plotted against days post tumor cell implantation. Tumors were weighted after resection at the end of experiment. B, mRNA levels of FLII, AR, PSA, and Ki67 from tumors were determined by qRT-PCR. C, IHC staining detected the protein expression of FLII, AR, PSA, and Ki67 in C4-2 tumor tissues derived from mice. Data for quantified immunohistochemistry are shown as mean ± SEM for $N = 4$ tumors in each group.
observed. Overexpression of NCoR or SMRT in prostate cancer cells enhanced FLII-repressed PSA and ARE-4 reporter activity after DHT treatment. These multiple lines of evidence suggesting NCoR and SMRT enhanced the binding and suppressing effect of FLII on AR. Further studies facilitate to the development of the interaction between FLII and other corepressors (HDAC/NCoR/SMRT).

Hormone-activated nuclear receptors (NR) activate transcription by recruiting multiple coactivator complexes to the promoters of target genes (48). Previous studies showed that FLII directly binds ER-LBD in a hormone-dependent manner in breast cancer (14, 16, 48). FLII enhancement of ER function was observed only when GRIP1 was coexpressed with FLII, regardless the expression of additional coactivators such as CARM1 and p300 (48). The dependency of FLII coactivator function on GRIP1 suggests that FLII is a secondary coactivator of ER (48). In the current study, FLII directly bound LBD of AR. In contrast to FLII/ER interaction where ligand enhanced the binding, DHT deassociated FLII/AR complex, suggesting DHT and FLII may compete with each other for AR binding. Our studies indicate that FLII functioned to repress AR activity in prostate cancer.

ADT is the frontline treatment for patients with advanced prostate cancer including metastatic and castration-resistant diseases (5). As the majority of patients eventually develop castration-resistant cancer despite of initial favorable response, identification of molecules and mechanisms involved in castration resistance is critical for developing effective treatment of this deadly disease (3, 4). We observed that knockdown of FLII alleviated inhibition of growth to bicalutamide and enzalutamide in castration-sensitive LNCaP cells, and overexpression of FLII induced inhibition of growth to bicalutamide in castration-resistant C4-2 cells. This study revealed a leading functional role of FLII on AR antagonist (bicalutamide, enzalutamide) underlying the development of castration and antiandrogen resistance. In the meanwhile, bicalutamide may cooperate with FLII to inhibit AR transactivation activity.

The new paradigm of treating CRPC with enzalutamide and abiraterone provided opportunity to dissect the drug resistance mechanisms to these agents (49). It was now established that a proportion of patients were primary refractory to therapy or develops resistance despite showing an initial response (49). Castration resistance is often marked by a return of AR signaling. The extent to which the spectrum of molecular alterations acquired after first-line therapy contributed to resistance in this new treatment setting remains largely uncharacterized (49). Constitutively active AR-Vs, such as AR-V7, lacking the LBD have been implicated in the pathogenesis of CRPC and in mediating resistance to newer drugs that target the androgen axis (50). In our studies, FLII inhibited AR transactivation and modulated AR cytoplasm/nucleus localization through direct binding to AR-LBD. AR-LBD is required for binding to FLII, suggesting FLII gene therapy would not be effective in AR-Vs patients.

In summary, we identified a biochemical and functional link between FLII and reduced expression in advanced prostate cancer and AR signaling in prostate cancer. Furthermore, loss of FLII expression contributes to prostate cancer growth and survival and reintroduction of FLII sensitizes castration-resistant prostate cancer cells to antiandrogen therapy. This study provides, for the first time, compelling evidence, which rationalizes the targeting strategy to reestablish the functional interaction between FLII and AR signaling in clinical practice to treat particular prostate cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: T. Wang, L. Wang, C. Wang, K. Chen
Development of methodology: T. Wang, W. Song, Y. Chen, L. Wang, K. Chen
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Wang, W. Song, Y. Chen, L. Wang, C. Chen
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Wang, L. Wang, C. Wang, K. Chen
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Flightless I Homolog Represses Prostate Cancer Progression through Targeting Androgen Receptor Signaling

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