FBXW2 Inhibits Prostate Cancer Progression and Metastasis Via Promoting EGFR Ubiquitylation and Degradation

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Abstract

Background: The process of ubiquitylation, carried out by a three-step enzymatic cascade, maintains the balance of intracellular proteins and the stability of their functions. FBXW2 is a novel F-box protein, and acts as a tumor suppressor gene in lung cancer by directly degrading substrates or regulating the other E3 ligases. However, whether or how FBXW2 regulates prostate cancer (PCa) tumorigenesis and progression is still unclear.

Methods: The expression levels of FBXW2 in PCa tissues and matched non-tumor tissues were detected by immunoblotting and qPCR. Cell counting kit-8, wound-healing assay, invasion assay, apoptosis and cell cycle assays, and animal experiments were performed to identify the function of FBXW2 in PCa cells. Half-life analysis, immunoprecipitation, and the *in vivo* ubiquitylation assay were performed to determine how FBXW2 regulates EGFR and its downstream signaling pathway.

Results: FBXW2 expression was lowly expressed in highly-progressive PCa tissues and PCa cell lines. Augmented FBXW2 expression suppressed PCa cell progression and metastasis by repressing EGFR downstream proteins, while FBXW2 depletion had opposite effects. Overexpression of FBXW2 inhibited tumor growth, and even osteolytic bone destruction. Furthermore, FBXW2 and EGFR protein levels were inversely correlated in PCa cell lines and patient tumor tissues. EGFR protein level and its half-life was extended by FBXW2 depletion. While the basal level of EGFR was decreased, and its half-life was shortened upon overexpression of FBXW2-WT but not its dominant negative mutant. Intriguingly, FBXW2 promoted EGFR ubiquitylation and degradation via its binding motif (TSNNST) on EGFR, thereby elevated FBXW2 abrogated the PCa cell growth and metastasis caused by EGF stimulation.

Conclusions: Collectively, our data shows that FBXW2 inhibits PCa cell progression and metastasis by promoting EGFR ubiquitylation and degradation, and would be a novel tumor suppressor for PCa.

Background

Prostate cancer is the most common non-skin cancer and the second leading cause of cancer death in men[1]. Prostate specific antigen (PSA) has greatly improved the early diagnosis of prostate cancer, and the five-year survival rate of early prostate cancer has reached nearly 100%[2]. However, the five-year survival rate is low (28%) in progressive prostate cancer, manifesting as bone metastasis and accompanying hypercalcemia, intractable pain, fracture and nerve compression[3]. Androgen receptor (AR), the first effector of testosterone action, activates certain genes (such as G3BP2, TACC2 and UBE2C) to promote tumor growth and metastasis[4]. Androgen deprivation therapy (ADT) becomes the main treatment for metastatic prostate cancer by reducing the level of androgen in the blood circulation[3]. However, 2 to 3 years post-ADT treatment, patients progressed to castration-resistant prostate cancer (CRPC) with limited treatment choices[5]. In patients with CRPC, more than 80% of deaths have been found to have bone metastases[6, 7], which is the main factor causing poor prognosis in advanced prostate cancer.

EGFR signaling plays an important role in prostate cancer progression and could be a crucial treatment target. EGFR can bind to EGF to induce the formation of dimers and promote the phosphorylation and internalization, leading to the activation of multiple oncogenic intracellular signaling pathways[8]. EGFR is overexpressed in prostate cancer and associated with poor prognosis[9]. By reducing tumor suppressor miR-1 and activating oncogene TWIST1, EGFR promotes prostate cancer progression and bone metastasis[10]. Furthermore, EGFR promotes the growth of tumor-initiating cells (TIC) and circulating tumor cells (CTC)[11]. c-Cbl (Casitas Blineage lymphoma) has been reported as an E3 ligase of EGFR by promoting EGFR ubiquitylation and endocytosis-based degradation upon EGF stimulation[12, 13]. However, a recent study indicated that c-Cbl down-regulation had no effect on EGFR expression [14], which suggests that the degradation mechanisms of EGFR merit further investigation.

FBXW2, one of the 69 F-box proteins, functions as a substrate recognition receptor in the SCF (SKP1-Cullin1- F-box protein) ubiquitin ligase complexes[15]. By forming a SCF ubiquitinated E3 ligase complex with CUL-1/SKP1, FBXW2 plays an important role in ubiquitin-dependent protein degradation. GCM1 (Glial cell missing homolog 1), as an important transcription factor that regulates placental cell fusion, is the first substrate protein of FBXW2. The SCF-FBXW2 E3 ligase complex recruits phosphorylated GCM1 through its WD40 domain, thereby regulating the stability and activity of GCM1 in the placenta[16]. We previously confirmed that FBXW2 served as a tumor suppressor gene to inhibit the tumor growth and metastasis of lung cancer cell lines by promoting ubiquitylation and degradation of β -catenin and SKP2 (S phase kinase-associated protein 2)[17, 18]. The biological functions of FBXW2 in prostate cancer are still unknown.

Herein, we found that FBXW2 serves as a tumor suppressor in PCa by promoting the ubiquitylation and degradation of EGFR, leading to the inactivation of oncogenic pathways (such as AKT and STAT3) and activation of tumor suppressors or apoptosis-inducing substrates (such as p21 and p27). Thus, our study identified FBXW2 as a new tumor suppressor in prostate cancer.

Materials And Methods

Cell culture

All cell lines used in this study were obtained from the Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China) and were authenticated by short tandem repeat profiling and monitoring Mycoplasma contamination. 22RV1, LNCaP, PC3 and DU145 PCa cell lines were routinely cultured in RPMI-1640 medium. 293T cells were grown in DMEM medium. All mediums were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All the medium was purchased from Gibco. All cell lines were incubated at 37°C with 5% carbon dioxide.

Cell counting Kit-8 (CCK-8) assay

The cells concentration was adjusted to around 2×10^3 cells/well, and the cells were seeded into 96-well plates in 100 μ l of culture medium, followed by incubation at 37°C in an atmosphere with 5% carbon

dioxidecultivation. At various time points, 10μL of CCK-8 reagent (MCE, HY-K0301) at a 1:10 dilution with serum-free RPMI-1640 medium was added to each well, followed by a further 2h incubation. The cell viability was determined using the CCK-8 assay, and the optical density (OD) was measured at 450 nm. Each experiment was conducted in triplicates.

Plasmids, siRNA, and transfection

The pEX-1 plasmid vector used in this study carried an enhanced green fluorescence protein (eGFP), and HA-tag or FLAG-tag were added in front of the target gene as needed. The construction and quality inspection of the plasmid was completed by Sangon Biotech (Shanghai, China). The targeting plasmids were delivered into PC3 and DU145 cells using the Lipofectamine 2000 transfection reagent (Invitrogen, USA, #11668019) according to the manufacturer's instructions. G418 (PC3 100μg /mL, DU145 120μg /mL, Gibco, USA) was used to screen individual clone expressing FBXW2 or vector control. siRNA designed to target FBXW2 were purchased from Sangon Biotech (Shanghai, China), including siRNA-FBXW2-#970 (5'-CCUCUUAAGUGCAGACAAATT-3'), siRNA-FBXW2-#1014 (5'-GGAGAGAAAUCAACUGUAATT-3') and siRNA-FBXW2-#1174 (5'-CAU CAAGACUCCUGAGAUATT-3'). For transient transfection, control and FBXW2 siRNA (#970, #1014 and #1174) were mixed with Lipofectamine 2000 and then added to cell culture medium in 22RV1 and LNCaP cells according to the manufacturer's instructions. Finally, we used IB to evaluate the transfection efficiency.

RNA isolation, reverse-transcription, and qPCR

Total RNA was isolated by Trizol-Chloroform method and then transcribed into cDNA using reverse transcription kit (Takara, Japan) with an oligo (dT) 20bp primer. RT-qPCR were performed by using the SYBR green reagent (Takara, Japan) on Real-Time PCR System (Thermo Fisher, USA). The $2^{-\Delta\Delta C_t}$ method was used to quantify the data, and the housekeeping GAPDH was used as loading control in the analysis. The sequences of primer sets were 5'-GGACATGCCTGAACACACTC-3' and 5'-CCAGGACTGTGCAAGAGAGA-3' for FBXW2; 5'-ACCCAGAAGACTG TGGATGG-3' and 5'-TCAGCTCAGGGATGACCTTG-3' for GAPDH. Each experiment was conducted in triplicates.

Wound-healing assay

Placed the Culture-Insert (Ibidi, Germany) in the 12-well plate and made the sticky side stick to the bottom of the well. The cells were counted, diluted to $70-100 \times 10^4$ cells/mL, and 70μL suspension was added to the slots on both sides of the Culture-Insert. Vertically removed the Culture-Insert 24h later, and a straight scratch was formed between the cells in the slots. Plate was washed by PBS and the cells were continually incubated in serum free medium. Photographs of the scratch were taken at various time points by inverted microscope (Olympus, Japan). The mean value of each gap width was measured for statistical analysis. Each experiment was conducted in triplicates.

Invasion assay

Matrigel (Sigma, USA) was diluted with 5 times serum-free cell medium, and 50µL of which was added to the bottom of each transwell chamber and incubated for 4h. The matrigel-coated chambers were washed with serum free medium for later use. Placing the chambers in a 24-well plate, in each of them 4×10^4 cells in 200µL medium containing 2% FBS free medium were plated, together with medium containing 10% FBS at the bottom of the well. Cells were incubated for 24-48h, and then fixed with 4% paraformaldehyde for 20 min. After washing with PBS for three times, cells were stained with 0.1% crystal violet blue (Beyotime, China) for 20 min, and then washed with PBS. Cells on the upper surface of the chamber were removed by cotton swab. The positively stained cells were photographed under the microscope, and multiple fields were taken for cell count and statistics. Each experiment was conducted in triplicates.

Apoptosis and cell cycle assays

Cells were synchronized at G0/G1 by serum starvation for 24h and then released by serum addition. For cell apoptosis detection, cells both in adherent and supernatant were collected and were stained in dark with 5µL Annexin v-fitc (Gibco, USA) for 10 min, and then labeled with 10µL FxCycle™ PI/RNase for 5min before flow cytometry. For cell cycle detection, cells were collected when cell density reached 70% to 90%. and fixed in 70% ice-cold ethanol overnight, labeled with 500µL FxCycle™ PI/RNase (Gibco, USA) for 15-30 min in dark at room temperature, and analyzed directly on an LSR II flowcytometer (BD Biosciences, USA). Each experiment was conducted in triplicates.

Immunoblotting (IB) and Immunoprecipitation (IP)

For direct IB analysis, cells were lysed in RIPA buffer with protease inhibitors and phosphatase inhibitors (Roche Life Science, Switzerland). The following primary antibodies were used: rabbit-FBXW2 (ab83467, Abcam; 1:1,000 overnight, 4°C); rabbit-FBXW2 (11499-1-AP, proteintech; 1:500 overnight, 4°C); rabbit-EGFR (#4267, CST; 1:1,000 overnight, 4°C); rabbit-caspase 3 (19677-1-AP, proteintech, 1:1000 overnight, 4°C); rat-HA (#11867423001, Roche Life Science, 1:2000 overnight, 4°C); mouse-FLAG (#F1804, Sigma, 1:2000 overnight, 4°C); mouse-His-Tag (66005-1-Ig, proteintech, 1:1000 overnight, 4°C); rabbit-AR (#5153, CST, 1:1000 overnight, 4°C); rabbit-p-AKT (#4060S, CST, 1:1000 overnight, 4°C); rabbit-AKT (#4691S, CST, 1:1000 overnight, 4°C); rabbit-Cyclin B1 (#12231S, CST, 1:1000 overnight, 4°C); rabbit-Cyclin D1 (#2978, CST, 1:1000 overnight, 4°C); rabbit-Cyclin E1 (11554-1-AP, proteintech, 1:1000 overnight, 4°C); rabbit-p-STAT3 (#9145, CST, 1:1,000 overnight, 4°C); mouse-STAT3 (ab119352, Abcam, 1:1,000 overnight, 4°C); rabbit-BAX (50599-2-Ig, proteintech, 1:1000 overnight, 4°C); rabbit-BCL2 (12789-1-AP, proteintech, 1:1000 overnight, 4°C); rabbit-PARP1 (13371-1-AP, proteintech, 1:1000 overnight, 4°C); rabbit-p27 (25614-1-AP, proteintech, 1:1000 overnight, 4°C); rabbit-p21 (103551-1-AP, proteintech, 1:1000 overnight, 4°C); mouse-GAPDH (60004-1-Ig, proteintech, 1:2000 overnight, 4°C); mouse-β-Actin (60008-1-Ig, proteintech, 1:5000 overnight, 4°C); mouse-α-Tubulin (66031-1-Ig, proteintech, 1:5000 overnight, 4°C). For immune-precipitation, cells were treated by MG132 (10 µg/mL, Beyotime, China) for 4h before lysed in IP-RIPA buffer with protease inhibitors, and Protein A/G plus Agarose (Santa Cruz, USA) were used to pull down the proteins. To immunoprecipitate endogenous proteins, whole cell extracts were pre-cleared with normal IgG-AC (Santa Cruz, USA) followed by overnight incubation at 4°C with antibody against HA-Tag. To

immunoprecipitate exogenously expressed FLAG-Tag proteins, the pre-cleared cell lysates were incubated with HA-Tag antibody in a rotating incubator overnight at 4°C. The Protein A/G plus agarose were washed with NETN-100 and the co-precipitated proteins were assessed by IB. Each experiment was conducted in triplicates.

Half-life analysis

After gene manipulation, 20µg/mL cycloheximide (CHX, MCE, China) was added to the cell medium for inhibiting new protein synthesis. At the indicated time points, cells were harvested, lysed and subjected to IB analysis. Each experiment was conducted in triplicates.

The *in vivo* ubiquitylation assay

Briefly, PC3 cells were co-transfected with HA-FBXW2 and His-Ub to detect ubiquitination of endogenous EGFR. To detect ubiquitination of exogenous EGFR, 293T cells were co-transfected with HA-FBXW2, His-Ub, and FLAG-EGFR-WT, FLAG-EGFR-MU1 or FLAG-EGFR-MU2. Mock vector was used as a control for ubiquitination assay. Cells were lysed in buffer A (6M guanidine-HCl, 0.1M Na₂HPO₄, 0.007M NaH₂PO₄, and 5mM imidazole, 0.1% Triton X-100, 10mM β-mercaptoethanol, pH 8.0) and sonicated. The lysates were incubated with nickel-nitrilotriacetic acid (Ni-NTA) beads (QIAGEN, Germany) at 4°C overnight. The beads were washed respectively with buffer A and buffer B (8M urea, 0.1M Na₂HPO₄, 0.007M NaH₂PO₄, and 5mM imidazole, 0.1% Triton X-100, 10mM β-mercaptoethanol, pH 8.0), and then three times with buffer C (8M urea, 0.025M Na₂HPO₄, 0.075M NaH₂PO₄, and 5mM imidazole, 0.1% Triton X-100, 10mM β-mercaptoethanol, pH 6.3). The beads were boiled and the pull-down proteins were resolved with anti-HA or anti-FLAG antibody by subsequently IB assay. Each experiment was conducted in triplicates.

Animal experiments

All experimental procedures using mice were performed in accordance with protocols approved by Laboratory Animal Welfare and Ethics Committee of Third Military Medical University of China. For xenograft model, 1×10⁶ PC3 stable cells (Vector and HA-FBXW2) were mixed 1:1 with matrigel in a total volume of 0.2mL and were injected subcutaneously into both flanks of BALB/c athymic nude mice (nu/nu, male; 4–6wk old; 13-15g). The size of tumors and the weight of the mice were measured twice a week. Mice were killed by carbon dioxide asphyxiation on day 30 after tumor cell injection, when some of the tumors reached the size limit set by the Institutional Animal Care and Use Committee. Tumors were weighed and fixed with 4% paraformaldehyde after resection.

For intratibial injection model, 2.0×10⁶ PC3 stable cells (Vector and HA-FBXW2) were resuspended in 100µL PBS and 10µL of cell solution was slowly injected NOD/SCID mice (male; 4–6wk old; 13-15g) with a 28.5-G needle into the tibia using a drilling motion. Osteolytic lesions were identified on radiographs as radiolucent lesions in the bone. Each bone metastasis was scored as follows [19]: 0, no metastasis; 1, bone lesion covering less than 1/4 of the bone width; 2, bone lesion involving 1/4 to 1/2 of the bone width; 3, bone lesion across 1/2 to 3/4 of the bone width; and 4, bone lesion more than 3/4 of the bone

width. The bone metastasis score of each mouse was the sum of all bone injuries. Mice were sacrificed dependent on survival time. Two hind limbs were dissected and fixed with 4% paraformaldehyde, which were subsequently used for H&E staining.

Clinical specimens and tissue microarray

All 9 prostate samples were collected from the urological specimen bank of the second affiliated hospital of the army medical university. The establishment of the specimen bank met the ethical requirements, and each sample had complete case information and pathological results. Three prostate hyperplasia samples were in the control group, three prostate cancer samples with Gleason score ≤ 7 without metastasis were in the PCa group, and three prostate cancer samples with Gleason score of > 7 and metastasis were in the M-PCa group. Tissue microarray was purchased from Servicebio (Wuhan, China), which provided chip information and completed immunohistochemical staining of the chips.

Immunohistochemistry (IHC)

IHC staining of Ki-67, EGFR, p-AKT, p-STAT3, p27 and p21 proteins in tumors of the nude mice was performed as described previously[17, 20]. The tissues were paraffin-embedded and sectioned after formalin-fixation. Tissue sections were subjected to IHC by incubation with anti-Ki67 (#9449, CST), anti-EGFR, anti-p-AKT, anti-p-STAT3, anti-p27 and anti-p21 antibodies respectively after deparaffinizing and antigen repairing. The sections were then incubated sequentially with biotinylated secondary antibody and HRP (horseradish peroxidase)-conjugated streptavidin. Antigenic detection was performed using chromogenic substrate DAB (3,3-diaminobenzidine tetrachloromethane) and the sections were counter stained further by hematoxylin. The positively stained cells were photographed under the microscope, and multiple fields were taken for cell count and statistics.

Statistical methods and chart

All data were expressed as Mean $\pm$ SD, SPSS 16.0 software was used for statistical analysis, and GraphPad Prism 7.0 software was used to make statistical charts. The proliferation curve was analyzed by repeated measures of variance, and the other data were analyzed by independent sample t-test or one-way analysis of variance, followed by Newman-Keuls test for evaluating the significance of differences between groups. *P*-values less than 0.05 were defined as statistically significant.

Results

FBXW2 is lowly expressed in highly-progressive PCa and augmented FBXW2 expression inhibits cancer progression and metastasis

FBXW2 is a novel F-box protein, which acts as a tumor suppressor gene in lung cancer by directly degrading substrates or by regulating other E3 ligases[17, 18]. The function of FBXW2 in PCa is still elusive. In this study, we firstly screened 9 clinical samples and divided them into three groups: prostatic hyperplasia (Nor), non-metastatic prostate cancer (PCa) and metastatic prostate cancer (M-PCa). Using

RT-qPCR and IB experiments, we found that the mRNA and protein levels of FBXW2 were significantly increased in M-PCa tissue than in Nor and PCa tissues (Fig. 1A). Furthermore, FBXW2 expression in higher metastatic PCa cell lines PC3 and DU145 was significantly decreased compared to that in the less metastatic PCa cell lines LNCaP and 22RV1 (Fig. 1B). We next determined the biological function of FBXW2 against PCa cells. Using *in vitro* assays, we revealed that overexpression of FBXW2 (Fig. S1A) in PC3 and DU145 PCa cells caused a significant reduction in cell proliferation (Fig. 1C and Fig. S1B), migration (Fig. 1D and Fig. S1C) and invasion (Fig. 1E and Fig. S1D). Likewise, knockdown of FBXW2 (#970, #1014, or #1174) (Fig. S2A) in 22RV1 and LNCaP cells promoted proliferation (Fig. 1C and Fig. S2B), migration (Fig. 1D), and invasion (Fig. 1E and Fig. S2C), and which si-FBXW2-#1014 was most. Further analysis showed that FBXW2 overexpression led to accumulation of G1 phase compared to the vector control (Fig. S3A) and had little influence on cell death (Fig. S3B). To further elucidate the mechanism by which FBXW2 suppressed cell progression and metastasis, after manipulation of FBXW2, we measured the levels of few related-proteins. We found that overexpression of FBXW2 induced accumulation of several tumor suppressive proteins, including p21 and p27 (Fig. 1F and Fig. S1E), while knockdown of FBXW2 caused reduction of these proteins (Fig. 1F). Consistent with the cell cycle assay, G1 phase proteins cyclin B1, cyclin D1 and cyclin E1 were decreased by FBXW2 overexpression, but were increased by FBXW2 knockdown (Fig. 1F and Fig. S1E). We also found that FBXW2 overexpression caused a decrease of N-cadherin and an increase of E-cadherin, while FBXW2 knockdown showed the opposite effect (Fig. 1F and Fig. S1E). Furthermore, overexpression of FBXW2 had no effect on the levels of apoptosis-associated proteins, including BAX, BCL-2 and Cas3 (Fig. S3C), which was consistent with the apoptosis assay. Taken together, these results strongly suggested that FBXW2 may function as a tumor suppressor that suppressed to progression and metastasis of PCa cells.

Loss of FBXW2 induces tumor development and promotes osteolytic bone destruction in PCa

To test the tumor suppressor potential of FBXW2 *in vivo*, we performed a xenograft model by inoculating subcutaneously the PC3 stable clones into both flanks of nude mice. We found that overexpression of FBXW2 significantly suppressed the *in vivo* tumor growth, decreased the tumor size and weight compared to the vector control (Fig. 2A-C), but had no effect on the mouse body weight (Fig. 2D). Meanwhile, to determine the effect of FBXW2 on bone metastasis of PCa *in vivo*, the FBXW2 overexpression of PC3 cells were inoculated into the both tibial bone of the mice to monitor development of bone metastatic tumors. Interestingly, we found that FBXW2 overexpression resulted in significant reduction in osteolytic areas compared with control vector (Fig. 2E). H&E staining also showed that FBXW2 dramatically reduced the tumor burden in bone (Fig. 2E). Collectively, the results indicated that FBXW2 significantly inhibited cancer growth and bone metastasis of high metastatic PCa.

FBXW2 is inversely correlated with EGFR, and regulates EGFR protein level

Since EGFR has been demonstrated to stimulate cell proliferation, invasion, and migration, and tumor growth in PCa, and some tumor oncogenes, we further wondered whether this dysregulation are correlated with FBXW2 depressions. Among PCa cell lines tested, FBXW2 protein level was low in higher

metastatic PC3 and DU145, while EGFR protein level was high (Fig. 3A), and vice versa. Furthermore, in the 42 PCa TMA samples, the expression of FBXW2 was negatively correlated with the expression of EGFR, demonstrated by the high FBXW2 staining with low EGFR staining in the same samples ($P < 0.01$, $r = -0.412$), whereas the low FBXW2 staining group is associated with high staining group of EGFR (Fig. 3B). A reduction of endogenous EGFR was also detected when FBXW2 was transfected (Fig. 3C and Fig. S4A). While we performed a siRNA-based knockdown in 22RV1 cells, found endogenous levels of EGFR protein (Fig. 3C). Furthermore, neither FBXW2 overexpression nor depletion had any effect on the level of EGFR mRNA (Fig. S4B). Those results suggested FBXW2 promoted EGFR protein degradation. Furthermore, STAT3 and AKT proteins, known to promote tumors growth as well as known downstream pathways of EGFR [21, 22]. Whose phosphorylation were markedly decreased by FBXW2 overexpression (Fig. 3C and Fig. S4A), while FBXW2 depletion caused it accumulation (Fig. 3C). A similar dose-dependent reduction of endogenous EGFR, p-AKT and p-STAT3 was also detected when FBXW2 was transfected (Fig. 3D and Fig. S4C). Using IB and IHC staining, we further found that the levels of EGFR, p-AKT and p-STAT3 were lower in FBXW2-overexpressing tumor tissues, while p21 and p27 were higher. (Fig. S5). Collectively, the results from both *in vitro* cell culture and *in vivo* xenograft models coherently demonstrated that FBXW2 overexpression significantly inhibited cell proliferation and progression by suppressing EGFR pathway. Then, we determined whether FBXW2 shortened EGFR protein half-life. Indeed, while transfected EGFR remained stable after 4h of cycloheximide treatment, FBXW2 co-transfection significantly reduced EGFR basal level and shortened its half-life (Fig. 3E). Conversely, FBXW2 RNAi silencing (si-FBXW2-#1014) extended protein half-life of EGFR (Fig. 3F). Interestingly, HA-FBXW2-ΔF, a dominant-negative mutant that can bind to the substrates but fails to recruit the other SCF ubiquitin ligase components[18], significantly extended protein half-life of endogenous EGFR (Fig. 3E). These data collectively indicated that FBXW2 negatively regulated EGFR protein levels, and was dependent on the structural integrity of F-box domain.

Fbxw2 Promotes Egfr Ubiquitylation And Degradation

Given that overexpression of HA-FBXW2-WT down-regulated the protein level and shortened the half-life of EGFR, whereas overexpression of HA-FBXW2-ΔF failed to do it, we hypothesized that FBXW2 might be actively involved in the control of EGFR stability. We next determined whether FBXW2 and EGFR bind to each other under physiological conditions. Indeed, using immunoprecipitation (IP), we detected endogenous EGFR in FBXW2 immuno-precipitants (Fig. 4A), indicating the physical interaction between EGFR and FBXW2. Comparing the sequence of EGFR with FBXW2, we found that EGFR actually contained the consensus FBXW2 degron sequence TSXXXS [17, 18]. EGFR has two evolutionarily conserved putative binding sites (TSXXXS and TSXXXST) of FBXW2 on codons 446-452 and 1041-1046. We further generated two EGFR mutants on FBXW2 phosphorylated binding site by changing 'TSXXXS' to 'AAXXA' and 'TSXXXST' to 'AAXXA', which were designated as 'EGFR-MU1' and 'EGFR-MU2' (Fig. 4B), respectively. We found that FBXW2 is bound to EGFR-WT and EGFR-MU1, but not EGFR-MU2 (Fig. 4C). Furthermore, FBXW2 overexpression significantly reduced, in a dose dependent manner, the levels of exogenous EGFR-WT and EGFR-MU1, but not EGFR-MU2 (Fig. 4D). Likewise, FBXW2 overexpression

markedly shortened protein half-life of exogenous EGFR-WT and EGFR-MU1, but not EGFR-MU2 (Fig. 4E). Those data indicated that targeted EGFR-FBXW2 binding is dependent on an evolutionarily conserved FBXW2 consensus binding motif (1041/1042 and 1045/1046) on EGFR. To identify the potential kinase(s) that would mediate EGFR phosphorylation at the FBXW2 binding motif (TSXXXST), we searched computer database (GSP 3.0 <http://gps.biocuckoo.org>) for consensus kinase binding site, and identified CK1 (Casein kinase) as candidates with the highest score (Table 1). We, therefore, added CK1 inhibitor IC261 and found that IC261 significantly extended the protein half-life of endogenous EGFR in PC3 cell (Fig. 4F), indicating that CK1 kinase mediated EGFR phosphorylation at the FBXW2 binding site.

Table 1
Putative kinases for FBXW2 phosphorylation at the EGFR binding motif (TSNNST).

Position	Code	Kinase	Peptide	Score	Cutoff
9	T	CAMK	SATSNNSTVACI***	2.08	1.749
2	S	CK1	*****LSATSNNST	13.174	7.074
4	T	CK1	****LSATSNNSTVA	10.576	7.074
5	S	CK1	***LSATSNNSTVAC	12.035	7.074
8	S	CK1	LSATSNNSTVACI**	11.229	7.074
9	T	CK1	SATSNNSTVACI***	11.715	7.074
2	S	TKL	*****LSATSNNST	4.746	4.148
4	T	TKL	****LSATSNNSTVA	12.197	4.148
5	S	TKL	***LSATSNNSTVAC	5.296	4.148
9	T	TKL	SATSNNSTVACI***	6.31	4.148
2	S	AGC/DMPK	*****LSATSNNST	4.232	2.427
4	T	AGC/DMPK	****LSATSNNSTVA	3.942	2.427
5	S	AGC/DMPK	***LSATSNNSTVAC	2.536	2.427
8	S	AGC/DMPK	LSATSNNSTVACI**	3.449	2.427
2	S	AGC/GRK	*****LSATSNNST	14.034	7.991
4	T	AGC/GRK	****LSATSNNSTVA	13.069	7.991
5	S	AGC/GRK	***LSATSNNSTVAC	14.216	7.991
8	S	AGC/GRK	LSATSNNSTVACI**	18.259	7.991
9	T	AGC/GRK	SATSNNSTVACI***	22.457	7.991
2	S	AGC/PKC	*****LSATSNNST	2.552	1.416
4	T	AGC/PKC	****LSATSNNSTVA	1.742	1.416
5	S	AGC/PKC	***LSATSNNSTVAC	1.965	1.416
9	T	AGC/PKC	SATSNNSTVACI***	1.572	1.416

Position: The position of the site that is predicted to be phosphorylated. **Code:** The residue that is predicted to be phosphorylated. **Kinase:** The regulatory kinase that is predicted to phosphorylate the site. **Peptide:** The predicted phosphopeptide with 7 amino acids upstream and 7 amino acids downstream around the modified residue. **Score:** The value calculated by GPS algorithm (<http://gps.biocuckoo.org>) to evaluate the potential of phosphorylation. The higher the value, the more potential the residue is phosphorylated. **Cutoff:** The cutoff value under the threshold. Different threshold means different precision, sensitivity and specificity.

Position	Code	Kinase	Peptide	Score	Cutoff
9	T	Atypical/PDHK	SATSNNSTVACI***	5.548	4.981
8	S	CAMK/MAPKAPK	LSATSNNSTVACI**	9.753	8.972
2	S	CAMK/PHK	*****LSATSNNST	9.346	3.785
4	T	CAMK/PHK	****LSATSNNSTVA	6.038	3.785
5	S	CAMK/PHK	***LSATSNNSTVAC	5.692	3.785
8	S	CAMK/PHK	LSATSNNSTVACI**	4.5	3.785
9	T	CAMK/PHK	SATSNNSTVACI***	5.885	3.785
5	S	CK1/CK1	***LSATSNNSTVAC	6.546	3.998
8	S	CK1/CK1	LSATSNNSTVACI**	6.992	3.998
2	S	CK1/VRK	*****LSATSNNST	7.5	0.977
4	T	CK1/VRK	****LSATSNNSTVA	8.8	0.977
5	S	CK1/VRK	***LSATSNNSTVAC	7.6	0.977
8	S	CK1/VRK	LSATSNNSTVACI**	2.1	0.977
9	T	CK1/VRK	SATSNNSTVACI***	1.6	0.977
2	S	TKL/RIPK	*****LSATSNNST	2.667	1.75
4	T	TKL/RIPK	****LSATSNNSTVA	2.667	1.75
8	S	TKL/RIPK	LSATSNNSTVACI**	4	1.75
2	S	TKL/STKR	*****LSATSNNST	6.5	5.609
5	S	TKL/STKR	***LSATSNNSTVAC	5.938	5.609
8	S	TKL/STKR	LSATSNNSTVACI**	6.438	5.609
2	S	AGC/GRK/BARK	*****LSATSNNST	11.233	4.78
4	T	AGC/GRK/BARK	****LSATSNNSTVA	11.372	4.78
5	S	AGC/GRK/BARK	***LSATSNNSTVAC	9.581	4.78
8	S	AGC/GRK/BARK	LSATSNNSTVACI**	9.767	4.78

Position: The position of the site that is predicted to be phosphorylated. **Code:** The residue that is predicted to be phosphorylated. **Kinase:** The regulatory kinase that is predicted to phosphorylate the site. **Peptide:** The predicted phosphopeptide with 7 amino acids upstream and 7 amino acids downstream around the modified residue. **Score:** The value calculated by GPS algorithm (<http://gps.biocuckoo.org>) to evaluate the potential of phosphorylation. The higher the value, the more potential the residue is phosphorylated. **Cutoff:** The cutoff value under the threshold. Different threshold means different precision, sensitivity and specificity.

Position	Code	Kinase	Peptide	Score	Cutoff
9	T	AGC/GRK/BARK	SATSNNSTVACI***	17.209	4.78
4	T	AGC/GRK/GRK	****LSATSNNSTVA	3.906	3.858
2	S	AGC/PKC/PKCa	*****LSATSNNST	6.178	4.803
5	S	AGC/PKC/PKCa	***LSATSNNSTVAC	5.578	4.803
2	S	AGC/RSK/MSK	*****LSATSNNST	4.741	4.025
2	S	CAMK/CAMK1/CAMK4	*****LSATSNNST	3.542	2.206
4	T	CAMK/CAMK1/CAMK4	****LSATSNNSTVA	3.208	2.206
9	T	CAMK/CAMK2/CAMK2B	SATSNNSTVACI***	8.718	8.277
2	S	CAMK/CAMKL/AMPK	*****LSATSNNST	2.568	1.777
4	T	CAMK/CAMKL/LKB	****LSATSNNSTVA	2.9	2.821
9	T	CAMK/CAMKL/MELK	SATSNNSTVACI***	1.944	1.36
4	T	CAMK/CAMKL/PASK	****LSATSNNSTVA	6	5.767
2	S	CAMK/MAPKAPK/MK5	*****LSATSNNST	11.727	9.696
2	S	CAMK/MAPKAPK/MNK	*****LSATSNNST	10	8.025
2	S	CAMK/PKD/PRKD1	*****LSATSNNST	2.115	1.5
8	S	CK1/CK1/CK1-A	LSATSNNSTVACI**	3.947	3.887
9	T	CK1/CK1/CK1-A	SATSNNSTVACI***	4.263	3.887
2	S	CK1/CK1/CK1-D	*****LSATSNNST	7.861	4.344
4	T	CK1/VRK/VRK1	****LSATSNNSTVA	5.2	3.159
5	S	CK1/VRK/VRK1	***LSATSNNSTVAC	4.1	3.159
2	S	CK1/VRK/VRK2	*****LSATSNNST	24.5	5.225
4	T	CK1/VRK/VRK2	****LSATSNNSTVA	28.75	5.225
5	S	CK1/VRK/VRK2	***LSATSNNSTVAC	27.5	5.225
8	S	CK1/VRK/VRK2	LSATSNNSTVACI**	17.75	5.225

Position: The position of the site that is predicted to be phosphorylated. **Code:** The residue that is predicted to be phosphorylated. **Kinase:** The regulatory kinase that is predicted to phosphorylate the site. **Peptide:** The predicted phosphopeptide with 7 amino acids upstream and 7 amino acids downstream around the modified residue. **Score:** The value calculated by GPS algorithm (<http://gps.biocuckoo.org>) to evaluate the potential of phosphorylation. The higher the value, the more potential the residue is phosphorylated. **Cutoff:** The cutoff value under the threshold. Different threshold means different precision, sensitivity and specificity.

Position	Code	Kinase	Peptide	Score	Cutoff
9	T	CK1/VRK/VRK2	SATSNNSTVACI***	18.25	5.225
4	T	CMGC/CDK/CDK9	****LSATSNNSTVA	5.45	4.647
8	S	TKL/IRAK/IRAK4	LSATSNNSTVACI**	7.2	6.78
2	S	TKL/MLK/MLK	*****LSATSNNST	3.909	1.5
4	T	TKL/MLK/MLK	****LSATSNNSTVA	1.727	1.5
5	S	TKL/STKR/STKR1	***LSATSNNSTVAC	8.625	8.6
4	T	TKL/STKR/STKR2	****LSATSNNSTVA	1.75	1.344
2	S	AGC/GRK/BARK/BARK1	*****LSATSNNST	15.405	5.269
4	T	AGC/GRK/BARK/BARK1	****LSATSNNSTVA	8.405	5.269
5	S	AGC/GRK/BARK/BARK1	***LSATSNNSTVAC	10.952	5.269
8	S	AGC/GRK/BARK/BARK1	LSATSNNSTVACI**	21	5.269
9	T	AGC/GRK/BARK/BARK1	SATSNNSTVACI***	28.595	5.269
2	S	AGC/GRK/GRK/GRK	*****LSATSNNST	12.778	9.35
4	T	AGC/GRK/GRK/GRK	****LSATSNNSTVA	10.278	9.35
2	S	AGC/PKC/PKCa/PRKCA	*****LSATSNNST	11.051	7.372
4	T	AGC/PKC/PKCa/PRKCA	****LSATSNNSTVA	13.889	7.372
5	S	AGC/PKC/PKCa/PRKCA	***LSATSNNSTVAC	18.152	7.372
8	S	AGC/PKC/PKCa/PRKCA	LSATSNNSTVACI**	14	7.372
9	T	AGC/PKC/PKCa/PRKCA	SATSNNSTVACI***	13.348	7.372
2	S	AGC/RSK/RSKp70/RPS6KB1	*****LSATSNNST	2.88	2.548
2	S	AGC/RSK/RSKp90/RPS6KA1	*****LSATSNNST	4.375	2.462
5	S	Atypical/PDHK/PDHK/PDK1	***LSATSNNSTVAC	7.459	4.74
9	T	Atypical/PDHK/PDHK/PDK1	SATSNNSTVACI***	6.568	4.74
4	T	CAMK/CAMKL/AMPK/AMPKA1	****LSATSNNSTVA	7.75	7.125

Position: The position of the site that is predicted to be phosphorylated. **Code:** The residue that is predicted to be phosphorylated. **Kinase:** The regulatory kinase that is predicted to phosphorylate the site. **Peptide:** The predicted phosphopeptide with 7 amino acids upstream and 7 amino acids downstream around the modified residue. **Score:** The value calculated by GPS algorithm (<http://gps.biocuckoo.org>) to evaluate the potential of phosphorylation. The higher the value, the more potential the residue is phosphorylated. **Cutoff:** The cutoff value under the threshold. Different threshold means different precision, sensitivity and specificity.

Position	Code	Kinase	Peptide	Score	Cutoff
5	S	CAMK/CAMKL/AMPK/PRKAB1	***LSATSNNSTVAC	5.667	2.525
4	T	CAMK/CAMKL/LKB/STK11	****LSATSNNSTVA	2.793	2.793
2	S	CAMK/CAMKL/QIK/SIK1	*****LSATSNNST	10.688	4.074
4	T	CAMK/CAMKL/QIK/SIK1	****LSATSNNSTVA	8.312	4.074
5	S	CAMK/CAMKL/QIK/SIK1	***LSATSNNSTVAC	7.188	4.074
8	S	CAMK/CAMKL/QIK/SIK1	LSATSNNSTVACI**	4.25	4.074
2	S	CAMK/MAPKAPK/MK5/MAPKAPK5	*****LSATSNNST	11.727	9.696
8	S	CAMK/MAPKAPK/MNK/MNK2	LSATSNNSTVACI**	16.429	9.344
9	T	CK1/CK1/CK1-A/CSNK1A1	SATSNNSTVACI***	3.421	2.971
4	T	CK1/CK1/CK1-D/CK1e	****LSATSNNSTVA	11.353	6.526
9	T	CK1/CK1/CK1-D/CK1e	SATSNNSTVACI***	6.529	6.526
9	T	CMGC/CDK/CDK7/CDK7	SATSNNSTVACI***	0.667	0.406
4	T	CMGC/MAPK/p38/MAPK13	****LSATSNNSTVA	5	4.706
5	S	CMGC/MAPK/p38/MAPK13	***LSATSNNSTVAC	5.765	4.706
2	S	TKL/MLK/MLK/MLK1	*****LSATSNNST	3.333	2.6
8	S	TKL/MLK/MLK/MLK1	LSATSNNSTVACI**	3	2.6
8	S	TKL/MLK/MLK/MLK3	LSATSNNSTVACI**	3.75	3.6
2	S	TKL/STKR/STKR1/BMPR1B	*****LSATSNNST	5.667	5.158
4	T	TKL/STKR/STKR2/TGFbR2	****LSATSNNSTVA	1.75	1.344
Position: The position of the site that is predicted to be phosphorylated. Code: The residue that is predicted to be phosphorylated. Kinase: The regulatory kinase that is predicted to phosphorylate the site. Peptide: The predicted phosphopeptide with 7 amino acids upstream and 7 amino acids downstream around the modified residue. Score: The value calculated by GPS algorithm (http://gps.biocuckoo.org) to evaluate the potential of phosphorylation. The higher the value, the more potential the residue is phosphorylated. Cutoff: The cutoff value under the threshold. Different threshold means different precision, sensitivity and specificity.					

To further understand the molecular mechanisms by which FBXW2 regulates EGFR, a classic *in vivo* ubiquitylation assays was used for whether FBXW2 promotes EGFR ubiquitylation. We transfected the exogenous HA-tagged FBXW2 and/or His-Ub into PC3 cells and tried to pull down endogenous the ubiquitylated EGFR. The results showed that exogenously expressed FBXW2 significantly promoted the ubiquitylation of endogenous EGFR (Fig. 5A). We then determined whether FBXW2 can likewise promote

EGFR mutants' ubiquitination. The *in vivo* ubiquitylation assay demonstrated that FBXW2 could promote ubiquitylation of EGFR-WT and EGFR-MU1, but not EGFR-MU2 (Fig. 5B), further suggesting that EGFR-MU2 mutant played a decisive role in FBXW2-mediated EGFR ubiquitylation. Therefore, we strongly supported the notion that EGFR was a novel substrate for FBXW2 F-box protein E3 ligases, and FBXW2 bond to EGFR via its 1041/1042 and 1045/1046 binding motif, which promoted its ubiquitylation for subsequent degradation.

Fbxw2 Blocks Egf-induced Biological Effects

We further sought to determine whether FBXW2 has effect on EGF function. Treated EGF in PC3 and DU145 cells caused a significant stimulation in cell proliferation (Fig. 6A and Fig. S6A) and invasion (Fig. 6B and Fig. S6B), but this effect could be blocked by overexpression of FBXW2, suggesting that growth-stimulating effect of EGF is blocked by FBXW2-mediated downregulation of EGFR. We transfected FBXW2 and EGFR (WT versus mutant) alone or in combinations into PC3 and DU145 cells and then treated with EGF. Similarly, FBXW2 overexpression caused EGFR reduction to inhibit cell growth (Fig. 6C and Fig. S6D) and invasion (Fig. 6C and Fig. S6D), even co-transfection with EGFR-WT, but which was abrogated by simultaneous transfection of EGFR-MU2, suggesting that growth- and invasion- inhibiting effect of FBXW2 is mediated by targeted of EGFR via its 1041/1042 and 1045/1046 binding motif, as named EGFR-MU2. Collectively, these results strongly demonstrated FBXW2 had inhibition effect on EGF function via EGFR degradation.

Discussion

The biological functions of FBXW2 in PCa remain unknown. Herein, we reported that FBXW2 acts as a novel tumor suppressor in PCa by promoting the ubiquitylation and degradation of the oncogenic EGFR pathway with following lines of supporting evidence: (1) FBXW2 is down-regulated in highly invasive PCa cells and high-grade PCa tissues; (2) Overexpression of FBXW2 significantly inhibits the cell growth and metastasis in PCa cells by inhibiting the EGFR downstream targets, such as p-AKT and p-STAT3, but while knockdown of FBXW2 caused accumulation of these proteins; (3) The tumor growth inhibition and metastasis suppression effects of FBXW2 is casually mediated by its targeted ubiquitylation and degradation of EGFR; (4) Overexpression of FBXW2, via decreasing the intratumoral protein level of EGFR, inhibits the tumor growth and osteolytic bone destruction in PCa. Hence, we have found that FBXW2 is a novel tumor suppressor in PCa by inhibiting the EGFR oncogenic pathway.

The F-box protein is a substrate recognition subunit of the SKP1-Cullin 1-F-box protein (SCF) E3 ligase complex, and plays a key role in multiple cellular processes through ubiquitylation and subsequent degradation of the target protein. F-box protein dysfunction plays an important role in the occurrence and development of PCa. The F-box protein SKP2 was first discovered as the E3 kinase of the p27[23]. Subsequent studied reported that high expression of SKP2 promotes cell proliferation, invasion and shortens the survival of PCa patients [24, 25]. FBXW2 is originally identified as a ubiquitin ligase for

polyubiquitination and degradation of GCM1, which suppresses placental cell migration and invasion [21, 26, 27]. Our recent study shows that FBXW2 is a tumor suppressor in lung cancer by promoting the ubiquitylation of SKP2 and beta-catenin for targeted [17, 18]. Here, consistent with our previous study in lung cancer, we found that FBXW2 is a tumor suppressor in PCa by inhibiting the cell growth and tumor metastasis.

Our study further identified that the tumor suppressing function of FBXW2 is through its ubiquitylation and degradation of EGFR and hence targeting EGFR in PCa will be a good therapeutic strategy. Abnormal activation of EGFR in PCa contributes to metastatic progression and poor prognosis of PCa[9]. EGFR reduces the tumor suppressor miR-1 and activates the oncogene TWIST1 to promote the progression of PCa and bone metastasis [10]. Cholesterol-rich Lipid Rafts in PCa cells promotes cell survival by activating the EGFR/PI3K/AKT pathway[22]. Furthermore, high expression of EGFR is specifically correlated with the high-grade stages and high risk for prostate-specific antigen recurrence [28]. Targeting EGFR alone[29] or combination with the conventional cytotoxic agents[30] or castration treatment[31] has shown optimistic tumor growth inhibition in *in vitro* cell lines and *in vivo* xenograft models of PCa. Even though, the single-agent trials with the EGFR inhibitors, including gefitinib[32] and erlotinib[33, 34] or panitumumab[35] haven't achieved any significant PSA response. These disappointing clinical trials may be explained by the well-known fact that EGFR inhibition in an unselected patient population is not a helpful approach. Indeed, inhibition of EGFR with cetuximab shows significantly improved PFS in PCa patients with overexpression of EGFR and persistent activity of PTEN [36]. Consistent with this finding, we speculated that it will be more beneficial to the patients if EGFR was targeted in a selected group of PCa patients with inactive FBXW2.

Our study also raised a question. It is well known that the biological functions of an E3 ligase might be mediated by different substrates. Except the newly-identified EGFR, the tumor growth and metastasis suppression effects of FBXW2 in PCa might probably be induced through the other potential substrates of FBXW2, like SKP2 [17]. We will continue to pursue whether both SKP2 and EGFR are simultaneously accumulated in FBXW2-low-expressed PCa and whether double targeting SKP2 and EGFR will be more effective than single agent of each.

Conclusions

Collectively, our study reveals that FBXW2 is a novel E3 ligase of oncogenic EGFR for targeted ubiquitination and degradation, and results in repressing STAT3 and AKT signaling, which inhibits PCa cell progression and metastasis (Fig. 7). These findings have motivated the development of a clinical trial to test whether targeting EGFR is effective in the selected patients with inactive FBXW2.

Abbreviations

PCa

Prostate cancer

PSA
Prostate specific antigen
AR
Androgen receptor
CRPC
Castration-resistant prostate cancer
TIC
Tumor-initiating cells
CTC
Circulating tumor cells
c-Cbl
Casitas Blineage lymphoma
SCF
SKP1-Cullin1- F-box protein
GCM1
Glial cell missing homolog 1
SKP2
S phase kinase-associated protein 2
FBS
Fetal bovine serum
CCK-8
Cell counting Kit-8
OD
Optical density
eGFP
Enhanced green fluorescence protein
IB
Immunoblotting
IP
Immunoprecipitation
CHX
Cycloheximide
Ni-NTA
Nickel-nitrilotriacetic acid
IHC
Immunohistochemistry
HRP
Horseradish peroxidase
M-PCa
Metastatic prostate cancer

SCF
SKP1-Cullin 1-F-box protein.

Declarations

Ethics approval and consent to participate

The present study was approved by the Hospital's Protection of Human Subjects Committee. All animal experiments were performed in accordance with protocols approved by Laboratory Animal Welfare and Ethics Committee of Third Military Medical University of China.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

Author Contributions

W.H.Z. and J.X. did the conception and design of the research. T.T.C. and T.Z. performed the experiments. T.T.C. interpreted the results of the experiments. W.F.Z., B.L. and T.Z. prepared the figures. W.H.F. and X.L. drafted the manuscript. W.H.Z. and J.X. edited and revised the manuscript. T.Z., T.T.C., B.L., W.F.Z., X.L., W.H.F., W.H.Z. and J.X. approved the final version of the manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

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Figures

Figure 1

Figure 1

FBXW2 is lowly expressed in highly-progressive PCa and augmented FBXW2 inhibits cancer progression and metastasis. A mRNA levels and protein levels of FBXW2 were significantly decreased in M-PCa tissue group. 9 clinical samples and divided them into three groups: prostatic hyperplasia (Nor), Non-metastatic prostate cancer (PCa, Gleason score ≤ 7 , no metastasis) and metastatic prostate cancer (M-PCa, Gleason score > 7 , metastasis.). B FBXW2 protein expressed in PCa cell lines. Cell lysates from four PCa cell lines

were subjected to IB. C-E Overexpression of FBXW2 in PC3 cells inhibited cell proliferation (C), migration (D) and invasion (E), while knockdown of FBXW2 (si-FBXW2-#1014) in 22RV1 cells showed the opposite effect. Cell viability was detected with Cell Counting Kit-8 (CCK-8) assay. The cell migration ability and invasion ability were determined using the wound-healing and transwell assays, respectively. F Effects of FBXW2 overexpression or FBXW2 knockdown (si-FBXW2-#1014) on the expression of cell progression-related proteins in PCa cells. GAPDH levels served as the control for equal loading. Data were presented as means $\pm$ SD. ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

Figure 2

Figure 2

Loss of FBXW2 induces tumor development and promotes osteolytic bone destruction in PCa cancer. A-D Overexpression of FBXW2 inhibited PCa tumor formation in vivo. PC3 cells stably expressing Vector and HA-FBXW2 (1×10^6 cells) were inoculated s.c. in both flanks of nude mice. The tumor growth was monitored twice a week for up to 30 days and growth curve plotted (D). The tumor growth was monitored and growth curve was plotted (B) and tumors were harvested (C) and photographed. E FBXW2 attenuated osteolytic bone tumor tumorigenesis in vivo. 2.5×10^6 PC3 stable cells (Vector and HA-FBXW2) were resuspended in 100 μ L PBS, and 10 μ L of cell solution was slowly injected NOD/SCID mice. Representative radiographical images of osteolytic bone tumor in the indicated tibia of the mice (left). Bars, 4mm. Representative H&E-stained sections of the indicated tibia of the mice (right). Bars, 500 μ m and 50 μ m. The sum of bone metastasis scores in the tibia of the Vector (n = 10) or HA-FBXW2 (n = 10) mice groups. Data were presented as means $\pm$ SD. ***p < 0.001, compared with the control group.

Figure 3

Figure 3

FBXW2 is inversely correlated with EGFR, and regulates EGFR protein level. A Inverse correlation at the protein levels of EGFR versus FBXW2 in PCa cell lines. B Expression of FBXW2 and EGFR in PCa tissues. PCa tissue microarrays were stained with FBXW2 and EGFR, and then photographed (Scale bars, 100μm). Association analysis of FBXW2 and EGFR in PCa tissues. Data were analyzed by using SPSS software ($P < 0.001$, Pearson's test). C Protein levels of EGFR and its downstream pathways were markedly

decreased by FBXW2 overexpression, while FBXW2 depletion caused them accumulation. D The levels of endogenously expressed EGFR and its downstream pathways were reduced upon FBXW2 transfection, in a dose-dependent manner. E EGFR half-life is shortened by FBXW2, but not by FBXW2-ΔF. FLAG-EGFR was transfected into 293T cells, along with the vector control or plasmid expressing HA-FBXW2 or HA-FBXW2-ΔF. Cells were switched to fresh medium (10% FBS) containing cycloheximide (CHX) 48h post transfection for indicated time periods and harvested for IB. F FBXW2 RNAi silencing extended protein half-life of EGFR. FLAG-EGFR was transfected into 22RV1 cells, along with the control RNAi, or si-FBXW2-#1014. Cells were cultured in fresh medium (10% FBS) containing CHX 48h post transfection for indicated time periods before being harvested for IB. GAPDH levels served as the control for equal loading.

Figure 4

Figure 4

FBXW2 binding to its phosphorylated binding motif on EGFR and promotes EGFR degradation. A FBXW2 could bind to endogenous EGFR: Cell lysates from PC3 cells were pulled down with anti-FBXW2 Abs, followed by IB with indicated Abs. B Two possible binding sites on EGFR, MU1 (446/447, 452) and MU2 (1041/1042, 1045/1046). C Loss of FBXW2-EGFR binding in phosphorylated binding site mutant: EGFR or its mutants at the phosphorylated binding site was co-transfected with HA-FBXW2, followed by IP with

HA Abs and IB. D FBXW2 reduced basal level of EGFR-WT and EGFR-MU1 by in a dose manner, but not EGFR-MU2. E FBXW2 shortened the protein half-life of EGFR-WT and EGFR-MU1, not EGFR-MU2. Stable 293T cells were incubated with CHX for indicated time periods and harvested for IB. F CK1 kinase mediated EGFR phosphorylation at FBXW2 binding motif. PC3 cells were transfected HA-FBXW2, and treated with or without CK1 inhibitor IC-261 (10mM), and harvested for IB. GAPDH levels served as the control for equal loading.

Figure 5

FBXW2 promotes EGFR ubiquitylation via its binding motif (TSNNST) on EGFR for subsequent degradation. A FBXW2 promoted ubiquitylation of EGFR in vivo: PC3 cells were transfected with indicated plasmids, lysed under denatured condition at 6M guanidinium solution, followed by Ni-beads pull-down. B FBXW2 promoted ubiquitylation of EGFR-WT and EGFR-MU1, not EGFR-MU2 in vivo: 293T cells were transfected with indicated plasmids, lysed under denatured condition at 6M guanidinium solution, followed by Ni-beads pull-down. GAPDH levels served as the control for equal loading.

Figure 6

FBXW2 weakened EGF function. A-B Transfection of FBXW2 significantly abrogated proliferation (A) and invasion ability (B) caused by EGF. Transfected the vector control or plasmid expressing HA-FBXW2 for 48h in PC3 cells were treated with and without EGF. The cell ability and invasion ability were detected by CCK-8 assay and transwell assays, respectively. C-D Transfected FBXW2 alone or co-transfection with EGFR-WT suppressed cell proliferation (C) and invasion (D), but was abrogated by simultaneous transfection of EGFR-MU2. Transfected HA-FBXW2 alone or in combinations with FLAG-EGFR (WT versus MU2) into PC3 cells and then treated with EGF. The cell ability and invasion ability were detected by CCK-8 assay and transwell assays, respectively. Data were presented as means $\pm$ SD. **p < 0.01, ***p < 0.001, compared with the control group.

Figure 7

Figure 7

Schematic model for FBXW2-induced EGFR degradation inhibiting progression and metastasis of PCa cell. During tumorigenesis, FBXW2, as a tumor suppressor, promotes ubiquitylation and degradation of EGFR, leading to repression of AKT and STAT3, and accumulation of p21 and p27, eventually to control proliferation and metastasis of cancer cells.

Supplementary Files

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