FUS/TLS Is a Novel Mediator of Androgen-Dependent Cell-Cycle Progression and Prostate Cancer Growth


Abstract

Progression of prostate cancer is highly dependent upon the androgen receptor pathway, such that knowledge of androgen-regulated proteins is vital to understand and combat this disease. Using a proteomic screen, we found the RNA-binding protein FUS/TLS (Fused in Ewing's Sarcoma/Translocated in Liposarcoma) to be downregulated in response to androgen. FUS has recently been shown to be recruited by noncoding RNAs to the regulatory regions of target genes such as cyclin D1, in which it represses transcription by disrupting complex formation. Here we show that FUS has some characteristics of a putative tumor suppressor, as its overexpression promoted growth inhibition and apoptosis of prostate cancer cells, whereas its knockdown increased cell proliferation. This effect was reproducible in vivo, such that increasing FUS levels in tumor xenografts led to dramatic tumor regression. Furthermore, FUS promoted conditions that favored cell-cycle arrest by reducing the levels of proliferative factors such as cyclin D1 and Cdk6 and by increasing levels of the antiproliferative Cdk inhibitor p27. Immunohistochemical analysis revealed that FUS expression is inversely correlated with Gleason grade, demonstrating that patients with high levels of FUS survived longer and were less likely to have bone metastases, suggesting that loss of FUS expression may contribute to cancer progression. Taken together, our results address the question of how androgens regulate cell-cycle progression, by demonstrating that FUS is a key link between androgen receptor signaling and cell-cycle progression in prostate cancer. Cancer Res; 71(3); 914–24. ©2010 AACR.

Introduction

Prostate cancer is almost invariably dependent upon the androgen receptor (AR) pathway, which when activated stimulates cell proliferation. Several factors involved in cell-cycle progression are regulated in response to androgen — for example, cyclin D1, which is upregulated (1–3). Non–organ-confined prostate cancer is treated with analogues of luteinizing hormone releasing hormone (LHRH), which block androgen production, and/or antiandrogens, which bind to the AR and hold it in an inactive state. Although initially successful, these treatments consistently fail and the tumors progress to a more aggressive hormone-refractory stage for which few therapeutic options exist. Expression of the AR is maintained in this refractory stage and much evidence exists to suggest that the receptor is still driving growth (4). Downstream targets of the AR involved in cell growth are therefore important in terms of further characterising this disease and identifying new therapeutic targets.

FUS (Fused in Ewing’s Sarcoma), also known as TLS (Translocated in Liposarcoma), is a member of the TET family, along with Ewing’s Sarcoma (EWS) and TATA-binding protein–associated factor TAF15/TAF68 (5). These family members, which are structurally and functionally related, are defined by the presence of an N-terminal SYGQ-rich region, a C2/C2 zinc finger motif, an RNA-recognition motif and at least 1 RGG-repeat region (6). FUS was originally identified in human myxoid and round cell liposarcomas as an oncogenic fusion with the stress-induced DNA-binding transcription factor CHOP (CCAAT enhancer-binding homologous protein; ref. 7, 8). FUS is a multifunctional protein, being implicated in pre-mRNA splicing (9), chromosome stability (10), cell spreading (11), and transcription (12, 13). Recently, FUS has been...
shown to be directed to the regulatory regions of target genes by single stranded noncoding RNA (ncRNA) transcripts tethered to DNA; repressing transcription by binding to and inhibiting complexes bound to such elements (12). This suggests that ncRNAs, via recruitment of RNA-binding proteins such as FUS, can act cooperatively as selective ligands to regulate transcription.

Here we show that FUS is an AR target protein down-regulated in response to androgen. Overexpression of FUS significantly retards androgen-induced prostate cancer cell growth in vitro and in vivo, regulates the expression of several factors involved in cell-cycle progression (for example, cyclin D1), and induces G1 arrest and apoptosis. FUS therefore exhibits certain characteristics of a tumor suppressor. Immunohistochemistry performed upon human tissue arrays demonstrated that FUS expression is inversely correlated with prostate tumor grade, and that patients with high levels of FUS have longer survival rates and are less likely to have bone metastases and hence we surmise that loss of FUS expression is important in disease progression.

Materials and Methods

Cell culture

LNCaP cells (ATCC CRL-1740) were obtained in 2003 from American Type Culture Collection, where they are verified phenotypically and by short tandem repeat profiling, frozen in liquid nitrogen, and fresh aliquots defrosted for use every 4 to 6 months. Cell were grown in RPMI 1640 media as described previously (14) and their identity further verified at least every 1 to 2 months by testing for morphology (microscopic inspection), AR expression (immunoblotting), hormone sensitivity (reporter or PSA assay), and mycoplasma contamination (MycAlert; Lonza). The LNCaP-TR2 (15) and LNCaP-FUS lines were grown in RPMI 1640 media supplemented with 10% TET-free fetal calf serum (Clontech), in the presence of the relevant antibiotics for selection purposes. Seventy-two hours before exposure to ligand, media were replaced with phenol red-free RPMI, supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (Sigma-Aldrich) and 5% charcoal-stripped FBS (Labtech International).

2D-SDS PAGE

Four samples were prepared per experimental condition. Cells were incubated with ligand for 16 hours before lysis and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14). Gels were stained using Sypro-ruby (GE Healthcare) and proteins separated by 2D-SDS PAGE as previously described (14).

Generation of stable cells inducibly expressing FUS cell line

For insertion of FUS in to the pCDNA4-TO plasmid, FUS was amplified by PCR with the addition of BamHI and XhoI restriction sites (for 5′-GGA TCC ATG GCC TCA AAC GAT TAT ACC C-3′, rev 5′- CTC GAG TTA ATA CGG CCT CTC CCT GC-3′). Both the plasmid and PCR product were digested with BamHI and XhoI before ligation and subsequently verified by sequencing. The pCDNA4-TO-FUS plasmid was stably transfected in to the LNCaP-TR2 line as previously described (15, 17).

Depletion of FUS levels using siRNA

FUS levels were reduced in LNCaP cells, using a Dharmacoon On-Target siRNA pool (L-009497-00-0005; Thermo Scientific) as previously described (18). To calculate percentage knockdown, densitometry was performed using Image J (NIH). FUS levels were normalized to β-actin and expressed as a percentage of FUS levels following treatment with scrambled siRNA.

Real-time quantitative PCR

Cells were treated for the indicated times and RNA harvested using Qiashredders and RNaseasy kits (Qiagen Ltd.). Five hundred nano grams of RNA was reverse transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). Gene expression was quantified using quantitative real-time PCR on a Taqman 7900HT (Applied Biosystems; ref. 18).

Western blotting

Cells were lysed in RIPA buffer and protein concentration determined by DC protein assay (Bio-Rad). Fifteen micro grams of protein was separated on a 10% SDS polyacrylamide gel and electrophoretically transferred (Transblot; Bio-Rad) onto nitrocellulose membrane. Membranes were blocked for 30 minutes in PBS–0.5% Tween containing 5% nonfat milk powder followed by a 1 hour incubation with primary antibody against: FUS (4H11), cyclin E1 (HE-12), CDK2 (M2), and p27(C-19) were from Santa Cruz Biotechnology; β-actin (AC-15) and cyclin D1 (ab24249) were from Abcam; cleaved PARP (Aasp214) was from Cell Signaling Technology; retinoblastoma (554162) was from BD Biosciences; phosphospecific antibody for retinoblastoma Rb-pSer807/S811 was from Sigma-Aldrich (R6400; Sigma-Aldrich); cyclin A2 (E23-1) was a kind gift from Dr Gordon Peters (CRUK LRI). Membranes were washed 3 times with PBS–Tween and incubated for a further hour with the relevant secondary antibody (Dako). Three washes with PBS-T and 1 wash with PBS were performed before chemiluminescent detection using ECL-PLUS (GE Healthcare).

Cell-cycle analysis

Cells were washed with PBS, trypsinized, and pelleted (1,200 rpm, 5 minutes). After 2 washes with PBS, cells were fixed in 70% ethanol (overnight at 4°C). Cells were washed 3 times with PBS before incubation for 1 hour with 50 mg/mL propidium iodide and 50 mg/mL RNase A in PBS. FACS analysis was carried out using a Becton-Dickinson FACS Calibur machine using linear scale representation of forward and side scatter during flow analysis. A total of 10,000 events were measured per sample.

Growth and caspase assays

LNCaP-FUS and the parental LNCaP-TR2 cells were seeded at 1,000 per well on a 96-well plate in ‘stripping media’ and left
for 24 hours. Cells were treated with and without mibolerone and with and without doxycycline for the indicated times. Changes in cell proliferation were quantified using WST1 assay (Roche), following the manufacturers instructions. Simultaneous plates were assayed for evidence of caspase 3/7 activity using Caspase-Glo assays (Promega) and activity normalized for cell proliferation.

**Chromatin immunoprecipitation**

LNCaP cells were grown to approximately 70% and serum starved for 72 hours. Cells were treated 0, 2, or 24 hours with 10 nmol/L mibolerone before cross-linking with formaldehyde (Sigma) for 10 minutes at RT. Chromatin immunoprecipitation (ChIP) was performed using the Millipore Chromatin Immunoprecipitation Kit (Millipore) following the manufacturer’s instructions, with the exception that a protein A/G sepharose mix was used. DNA was recovered by phenol–chloroform extraction and real-time quantitative PCR was used to quantify enrichment of regions of the CCND1 promoter. 

**Tissue microarray and immunohistochemistry**

Immunohistochemistry was performed using 3 tissue microarrays (TMA) of benign and malignant prostate biopsies derived from transrectal biopsy, transurethral resection, and radical prostatectomy as previously described (19). All materials were used in accordance with approval granted by the Northumberland, Tyne and Wear NHS Strategic Health Authority Research Ethics Committee (reference 2003/11/ The Freeman Hospital). The final study included 321 cancer biopsies and 69 benign biopsies. Antigen retrieval was achieved by immersion in 10 mmol/L citric acid buffer (pH 6.0), followed by microwaving for 15 minutes (at 1,000 W) in a pressure cooker. Sections were immunostained with a rabbit polyclonal antibody against FUS (Santa Cruz Biotechnology) on a DAKO autostainer using Vectastain ABC kits (Vector Labs), according to the manufacturer’s protocol. Sections known to stain positively were included in each batch, and negative controls were prepared by replacing the primary antibody with TBS buffer. FUS expression was scored blindly for epithelial nuclear intensity of staining and number of epithelial nuclei positive per field, in each biopsy core. Slides were scanned using a Scanscope GL scanner (Aperio) and analyzed using Spectrum software (Aperio). For statistical analysis, samples were split into low or high intensity/number of positive nuclei (low = 0, 1 and high = 2, 3).

**In vivo xenograft model**

About 2 × 10^6 LNCaP-FUS cells mixed with an equal volume of matrigel (BD Biosciences) were injected subcutaneously into the flanks of castrated male balb/c nude mice (Harlan Laboratories). Animals received bi-daily testosterone replacement injections until the tumors were established, following which the mice were split into experimental groups: ± doxycycline and ± testosterone. Tumors were measured using calipers and relative tumor volume (RTV) calculated as previously described (20). After sacrifice, tumors were resected and immunohistochemistry performed as previously described (20) using antibodies specific for phospho-histone H3 (Ser10; Millipore), active caspase 3 (AF835; R&D Systems), and cleaved PARP (Asp214; Cell Signaling Technology).

**Results**

**FUS is downregulated by androgen treatment**

To identify targets regulated by the androgen receptor, a 2D-proteomic screen was performed on the AR positive LNCaP prostate cancer cell line, which is dependent on androgen for growth, treated with mibolerone (a synthetic androgen) or vehicle for 16 hours. Proteins were separated using 2D SDS-PAGE, stained with Sypro-Ruby and spots found to have a significant change in density in response to androgen excised and identified using mass spectrometry. A spot found to be downregulated in response to androgen, running around 75 kDa and pl 9.4, was identified as FUS (Fig. 1A). To confirm androgen regulation of FUS expression, the LNCaP line was treated with androgen for 0 to 72 hours and immunoblotting performed (Fig. 1B). FUS expression was found to be reduced by more than 90% after 72 hours of stimulation with androgen. This regulation appears to be at least partly at the RNA level, as qRT-PCR demonstrated a significant decrease in FUS over a 72-hour time course (56% reduction at 72 hours; Fig. 1C). As a control, expression of the known androgen regulated gene prostate specific antigen (PSA) was measured and increased transcription in response to androgen was confirmed. C-jun has been previously shown to regulate FUS degradation (21), and hence we investigated whether this posttranscriptional regulation was also important in the androgen induced downregulation of FUS. In accordance with this hypothesis, and in agreement with the work of Perrotti and colleagues (21), we found that c-jun expression was androgen-dependent, with upregulation of c-jun protein evident within 8 hours of androgen treatment (Supplementary Fig. 1A), preceding the decrease in FUS. However, reducing c-jun levels by siRNA or treating cells with the protease inhibitor lactacystin did not affect the androgen-induced downregulation of FUS (Supplementary Fig. 1B and 1C). We therefore surmise that the androgen-dependent regulation of FUS is independent of c-jun and proteasomal degradation and is instead predominantly regulated at the transcriptional level.

**FUS represses LNCaP growth**

Because androgen treatment results in both prostate cell proliferation and a reduction in FUS expression, we tested the hypothesis that FUS is a suppressor of growth. To investigate this, we created a stable cell line to allow doxycycline-inducible overexpression of FUS. Addition of doxycycline led to an increase in FUS expression, with maximal expression at 10 nmol/L and within 24 hours (Supplementary Fig. 2). Light microscopy revealed that this exogenous FUS expression results in cell rounding within 4 days and a marked reduction
in cell number by day 6 (Fig. 2A, top). To quantify this, growth assays were performed over the same timecourse. In the absence of ligand, little proliferation was evident, whereas addition of mibolerone resulted in a 6-fold increase in proliferation after 8 days (Fig. 2A, bottom). Exogenous FUS expression in the presence of ligand resulted in a decrease in cell number, with fewer cells present after 8 days than were seeded. To ensure that these effects were not an artifact of the...
doxycycline treatment, growth assays were performed on the parental cells (LNCaP-TR2), which were unaffected by doxycycline treatment, demonstrating that the inhibition of androgen-stimulated growth is as a result of FUS overexpression (Fig. 2A). To investigate the effect of FUS upon cell-cycle progression, cells were propidium iodide (PI) stained and analyzed using FACS. In agreement with previous studies (for example, 2, 14), in the absence of ligand LNCaP cells

Figure 2. FUS blocks androgen-dependent prostate cell growth. A, the LNCaP-FUS line was treated with and without doxycycline (DOX) and with mibolerone (MIB) and images taken with a phase contrast microscope at the indicated times (bars, 100 µm). The effect of FUS overexpression upon androgen-induced cell proliferation was quantified using WST1 assays. WST1 assays were also performed upon the parental line, LNCaP-TR2. B, at the indicated time point, cells were fixed, stained with PI, and cell-cycle was analyzed by FACS. Data were analyzed using FlowJo v8.8.6 (TreeStar). C, the 4 days FACS data were reanalyzed to exclude the sub-G1 pool. D, LNCaP cells were transfected with scrambled siRNA or siRNA to target FUS and incubated for 3 days before treatment with different doses of ligand. Cells were left for 3 or 6 days following treatment with mibolerone (MIB) before WST1 growth assays were performed. t test *, P < 0.05; **, P < 0.005; ***, P < 0.0005.
were found to arrest in G1 phase (Fig. 2B and C). Addition of ligand resulted in cells progressing through to S/G2/M. In the presence of mibolerone, exogenous FUS resulted in a large increase sub-G1 peak (from 1.9% of cells to 4.3% at 4 days and from 2.7% to 31% at 8 days; Supplementary Table 1 and Fig. 4A). Analysis of the FACs data with the sub-G1 population removed (to avoid skewing the data) revealed that exogenous FUS expression (addition of doxycycline) blocks the action of androgen, resulting in an increase in cells in G1 and reducing the percentage of cells progressing to S and G2/M (Fig. 2C). FACS analysis of the parental LNCaP-TR2 showed no change in cell-cycle profile in response to doxycycline (Fig. 2C and Supplementary Fig. 2), confirming that these differences are as a result of increased FUS levels.

To establish the role of FUS in androgen-induced growth we performed the reciprocal experiment, reducing FUS expression using transiently transfected siRNA. LNCaP cells were transfected with siRNA, successful knockdown was confirmed at the levels of RNA (93%) and protein (74%; Supplementary Fig. 4), and growth analyzed at days 3 and 6 in response to different concentrations of mibolerone (Fig. 2D). After 3 days, reduction of FUS expression resulted in a significant increase in growth at the highest concentration (10 nmol/L) of ligand. After 6 days this growth-promoting effect was significant at both 1 nmol/L and 10 nmol/L mibolerone.

**FUS regulates the expression of factors involved in cell-cycle progression**

Previously it has been shown that FUS is a negative regulator of cyclin D1 expression in RAW264.7 cells (12). We were therefore interested to see whether increasing FUS expression in the LNCaP line altered the expression of cell-cycle regulators, either directly or as cyclin D1 is also androgen-regulated; refs. 22–24 perhaps via preventing androgen-induced changes, which could potentially explain G1 accumulation and growth inhibition. Western blotting of lysates from the LNCaP-FUS line demonstrated that increasing FUS levels altered the expression levels of several factors involved in G1 progression (Fig. 3A). Specifically, cyclin D1 and CDK6 levels were decreased in response to FUS overexpression, whereas the level of the kinase inhibitor p27 was increased. Little change was observed in levels of the other cell-cycle regulators investigated and levels of the AR were also found to remain unchanged. This indicates that the effects of FUS on growth are at least in part due to it promoting G1 arrest, possibly via regulation of cyclin D1, CDK6 and p27.

Wang and colleagues have previously demonstrated that FUS binds, via noncoding RNA, to the regulatory regions of cyclin D1 and blocks transcription (12). In agreement with their study, the regulation of cyclin D1 by FUS in these prostate cancer cells appears to be at the transcriptional level, as overexpression or knockdown of FUS respectively reduces or enhances androgen-induced cyclin D1 expression at the RNA level (Fig. 3B). Furthermore, we performed ChIP on the CCND1 promoter to analyze FUS recruitment to 2 regions demonstrated by Wang and colleagues to express ncRNA (regions A and D) and 1 negative region that has been shown not to express ncRNA (region C; ref. 12; Fig. 3D). FUS was found to bind to regions A and D but not C, and binding was only evident in the absence of androgen, supporting our hypothesis that, in prostate cancer cells, FUS regulates cyclin D1 expression via recruitment to the CCND1 promoter and this is modulated by androgen treatment.

**FUS induces apoptosis**

We have shown that increasing FUS expression in cells cycling in response to androgen results in an increase in the sub-G1 population, which suggests an increase in apoptosis (Fig. 4A and Supplementary Table 1). To confirm whether FUS can influence rates of apoptosis, caspase 3/7 activity was measured (Fig. 4B). Exogenous expression of FUS resulted in an increase in caspase 3/7 activity of 2.9-fold at 4 days and 34-fold at 8 days in the mibolerone-treated cells. No such doxycycline-induced increase in caspase activity was evident for the parental LNCaP-TR2 upon androgen treatment. We also investigated the downstream apoptotic marker of PARP cleavage. Western blotting demonstrated a ligand- and FUS overexpression-dependent increase in cleaved PARP (Fig. 4C), which was evident after 4 days treatment. Hence, it appears that increasing FUS expression results in an increase in cell death due to activation of apoptotic pathways.

**FUS blocks tumor growth in vivo**

Having demonstrated FUS to be a repressor of androgen-dependent proliferation in culture, we went on to investigate the role of FUS in prostate tumor progression in vivo. The LNCaP-FUS line was subcutaneously injected into both flanks of castrated male nude BALB/c mice. Animals were given bi-daily injections of testosterone until tumors had reached an average size of approximately 250 mm³, upon which (day 0) animals were split into experimental groups of ± testosterone and ± doxycycline (Fig. 5A). In the absence of testosterone tumors did not increase in size during the course of the experiment, in fact some regression was seen, whereas testosterone promoted a significant increase in growth (t test, 7 days P < 0.05). Addition of doxycycline to testosterone-treated mice led to a significant reduction in tumor volume compared with testosterone alone (P < 0.05 at 7 days), with tumor volumes falling to sizes comparable to those in animals receiving no testosterone.

To test whether the effects of FUS overexpression are reversible, mice that were treated with testosterone and doxycycline were monitored for an extended period (Fig. 5B). At day 13 doxycycline was withdrawn and the tumors were found to grow, expanding up to an average of 1.5 times original tumor size at day 29 (P < 0.0005). Doxycycline was reintroduced at day 30 and the RTV again regressed, this time in a dramatic fashion to approximately 50% of maximum size at day 37. Following animal sacrifice, immunohistochemistry was performed upon tumor sections to investigate the expression of markers of proliferation and apoptosis. The number of cells expressing the mitotic marker phospho-histone H3 was found to be significantly decreased following exogenous FUS expression, whereas markers of apoptosis (active caspase 3 and cleaved PARP)
were significantly upregulated (Fig. 5C and Supplementary Fig. 4).

**FUS expression is inversely correlated with Gleason grade, survival, and bone metastasis**

To determine whether alterations in FUS expression are associated with prostate cancer progression, immunohistochemistry was performed on prostate cancer tissue microarrays (examples of staining in Supplementary Fig. 5). Sections were scored for primary Gleason grade and epithelial cells scored for the number of cells positive for nuclear FUS staining per field (Fig. 6A) and the intensity of nuclear staining (Fig. 6B). An inverse correlation of FUS expression with Gleason grade, a determinant of aggression of prostate cancer by histology, was found using both scoring methods for all grades except for benign prostatic hyperplasia (BPH) versus...
primary Gleason grade 3. Analysis of patient survival and data on the presence of bone metastases found no significant correlation with the number of cells positive for FUS. FUS nuclear intensity, however, showed significant correlation with the presence of bone metastases at the time of biopsy (data available for 77 patients with confirmed absence of bone metastases and 37 with confirmed bone metastases), with patients with high levels of FUS significantly less likely to present with bone metastases (Mann–Whitney, 2-tailed \( P \) value = 0.0325). Furthermore, a significant difference in patient survival was observed. Patients with high FUS expression show significantly longer survival than patients with low FUS expression, with mean survival increasing from 70.8 to 91.8 months and median from 57 to 109.2 months in the high expressers versus the low expressers (Fig. 6C and Table 1).

Discussion

Prostate cancer growth is almost always dependent upon the AR pathway and therefore identification of downstream targets critical for growth is important for the further characterization of this disease. In an attempt to identify novel androgen-regulated targets, we performed a proteomic screen on the LNCaP prostate cancer cell line following stimulation with androgen. One of the proteins found to be significantly regulated was the RNA-binding protein FUS. Addition of androgen was found to result in a decrease in FUS expression at the RNA and protein level. Perrotti and colleagues have shown that FUS is regulated at the protein level by c-jun (21), which targets the protein to the proteasome. Velasco and colleagues reported regulation of c-jun by androgen (25), and in support of this we saw upregulation of c-jun protein within 8 hours of androgen treatment (Supplementary Fig. 1A). Because c-jun upregulation precedes the observed decrease in FUS levels, we hypothesized that androgens may induce FUS degradation via increasing c-jun. However, knockdown of c-jun or treatment with proteasomal inhibitors did not reduce the androgen-dependent downregulation of FUS. We therefore conclude that the regulation of FUS in response to androgen is predominantly at the transcriptional level.

Because FUS levels are decreased by growth-promoting androgen treatment we hypothesized that FUS may be a repressor of prostate cancer growth. In stably transfected LNCaP cells, we found exogenous FUS expression significantly inhibits cell growth, causes G1 arrest and promotes apoptosis. The AR is known to regulate factors important in cell-cycle progression and appears to be particularly important in G1/S progression because androgen depletion results in G1 arrest (24). In agreement with this we also found LNCaP cells to arrest in G1 following removal of androgen, whereas addition of androgen resulted in an increase in the number of cells progressing to S and G2/M. Overexpression of FUS, however, blocked the effects of androgen, leading to G1 arrest and also an increase in the sub-G1 population. Increased caspase 3/7 activity and an increase in the levels of PARP cleavage confirmed that this sub-G1 population contained apoptotic cells. Hence, FUS appears to promote apoptosis in prostate cancer cells.

Analysis of cell-cycle regulators revealed that manipulation of FUS levels is associated with altered expression of several factors important in G1/S transition, specifically cyclin D1, CDK6, and p27. It is known that cyclin D1 and p27 are androgen targets, and that an increase in cyclin D1

Figure 4. Exogenous FUS expression promotes apoptosis. LNCaP-FUS cells were treated with and without DOX, and with and without MIB. A, cells were fixed, PI-stained, and FACS analysis used to quantitate the % number of cells in sub-G1. B, caspase 3/7 activity was analyzed using the Caspase 3/7-Glo assay (Promega) and activity normalized to account for changes in cell proliferation. Graph shows fold change in DOX-treated over vehicle-treated cells for each point. C, cells were harvested, lysed, and proteins separated by SDS PAGE. Proteins were visualized using immunoblotting as indicated.
and a decrease in p27 promote G1 transition (22-24). Our observed reduction in the expression of this cyclin and the increase in p27 following exogenous expression of FUS suggests that FUS induces G1 arrest and thus affects androgen-dependent proliferation, at least in part, via modulation of these factors. Recently, FUS was demonstrated to be directly recruited to the regulatory regions of various points on the 5' upstream region. This recruitment leads to interference with transcriptional complex formation hence decreased expression of cyclin D1 (12). The regulation of cyclin D1 in response to FUS overexpression or knockdown was found to be at the RNA level (Fig. 3B). Furthermore, ChIP revealed FUS binding to ncRNA-expressing regions of the CCND1 promoter in the absence of androgen, which was abrogated by androgen treatment. This data therefore fits with the mechanism of regulation proposed by Wang and colleagues (12) and suggests that androgen withdrawal-mediated repression of cyclin D1 expression is via alterations in recruitment of FUS to the CCND1 promoter. It thus appears that cyclin D1 is a target of both androgens (22-24) and FUS (12; and data herein). We have shown that FUS levels are regulated by androgens, and others have shown that cyclin D1 itself is a corepressor of the androgen receptor (26). It is therefore possible that complex functional interactions between FUS, the androgen receptor, and cyclin D1 that merit further investigation. Notwithstanding this, our data demonstrate that manipulation of FUS levels influences the levels of a number of key cell-cycle regulatory proteins, indicating that FUS may be a critical link between androgen signaling and cell-cycle progression.

Our data from both in vitro and in vivo systems demonstrate that FUS has characteristics suggestive of a putative tumor suppressor. FUS expression in prostate tumor samples was inversely correlated with Gleason grade and analysis of patient data demonstrated that those with high expression levels of FUS had longer survival rates and were less likely to have bone metastases (the primary cause of morbidity in prostate cancer patients), suggesting that loss of expression may be important in disease progression. Our study in xenograft models suggests that this correlation is not merely circumstantial, as not only did increasing FUS levels result in decreased tumour growth, but this effect was also reversible because removing the exogenous expression increased tumor growth while reexpressing it halved the tumour volume within a week.

From the work presented here, we suggest that androgen signaling downregulates FUS and that FUS subsequently regulates factors important in cell-cycle progression. This, combined with the finding that FUS expression is reduced in advanced stages of prostate cancer, suggests that loss of FUS may enhance androgen signaling and promote prostate cell growth. Furthermore, the demonstration that...
Table 1. FUS expression is correlated with patient survival

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NOTE: The intensity of FUS staining was correlated with patient data and median and mean survival times calculated.

*Estimation is limited to the largest survival time if it is censored.
overexpression of FUS in vivo reduces tumor growth suggests that methods to manipulate FUS expression could be useful for the treatment of prostate cancer.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

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