Novel gene C17orf37 in 17q12 amplicon promotes migration and invasion of prostate cancer cells

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Abstract

C17orf37/MGC14832, a novel gene located on human chromosome 17q12 in the ErbB-2 amplicon, is abundantly expressed in breast cancer. C17orf37 expression has been reported to positively correlate with grade and stage of cancer progression; however the functional significance of C17orf37 overexpression in cancer biology is not known. Here, we show that C17orf37 is highly expressed in prostate cancer cell lines and tumors, compared to minimal expression in normal prostate cells and tissues. Cellular localization studies by confocal and TIRF microscopy revealed predominant expression of C17orf37 in the cytosol with intense staining in the membrane of prostate cancer cells. RNA interference mediated downregulation of C17orf37 resulted in decreased migration and invasion of DU-145 prostate cancer cells, and suppressed the DNA binding activity of NF-κB transcription factor resulting in reduced expression of downstream target genes MMP-9, uPA and VEGF. Phosphorylation of PKB/Akt was also reduced upon C17orf37 downregulation, suggesting C17orf37 acts as a signaling molecule that increases invasive potential of prostate cancer cells by NF-κB mediated downstream target genes. Our data strongly suggest C17orf37 overexpression in prostate cancer functionally enhances migration and invasion of tumor cells, and is an important target for cancer therapy.

Introduction

Prostate cancer is the most common type of cancer diagnosed in American men and is the second leading cause of cancer related death in men (Pienta and Loberg, 2005). American cancer society estimates about 28,660 men will die of this disease in 2008. Most men

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diagnosed with prostate cancer can survive the primary localized tumor, but because of the metastasis related disease, mortality rates remain extremely high. Metastasis of prostate cancer is facilitated by migration and invasion of malignant tumor cells from localized neoplastic tumors to distant organs like bone by cleavage of extracellular matrix (ECM) (Arya *et al.*, 2006). Proteases that facilitate malignant cell migration and invasion by cleaving ECM proteins, such as matrix metalloproteinases (MMPs), cysteine proteases and serine proteases have been extensively studied (Sliva, 2004). Although the fundamental role of these proteases in prostate cancer cell migration, invasion and metastasis is clear, the underlying mechanism and regulation of these proteases to promote prostate cancer progression and invasion is vague.

Chromosome 17q12 -contains multiple genes that play important roles in different forms of cancer. Recent genetic studies have projected 17q12 loci genes as an important risk factor in prostate cancer progression in selected individuals (Eeles *et al.*, 2008; Sun *et al.*, 2008). ErbB-2 located in the same amplicon is known to play a significant role in prostate cancer progression and its expression correlates with poor hormone refractory prostate cancer in patients (Myers *et al.*, 1994). C17orf37 is a novel gene located in the same amplicon next to ErbB-2 in a tail to tail rearrangement, functional importance of which is presently unknown. C17orf37 has been reported to be frequently amplified with ErbB-2 in breast tumor cells, however independent activation of C17orf37 has been demonstrated in cell lines expressing low levels of ErbB-2 (Evans *et al.*, 2006). C17orf37 protein is abundantly expressed in breast tumor cells and clinical tissues, with reduced or limited expression in 38 different normal tissues (Evans *et al.*, 2006). C17orf37 expression positively correlates with grade and stage of breast cancer, and increased expression has been shown in patients with breast to liver and lungs metastasis (Evans *et al.*, 2006). This suggests C17orf37 protein may be an important mediator of cancer cell metastasis. However, to establish C17orf37 as a biomarker and therapeutic target for cancer treatment, it is utmost important to explore the functional importance of this novel protein.

We demonstrate C17orf37 expression is enhanced in prostate cancer cells and tissues specimens. We also observed the cellular localization of C17orf37 in prostate cells by confocal microscopy and finally by knockdown and overexpression studies, we demonstrate C17orf37 significantly contributes to the migration and invasion of prostate cancer cells. Our results clearly indicate C17orf37 as a critical cancer specific protein promoting tumor cell invasion by enhancing secretion of uPA, MMP-9 and VEGF through NF-κB pathway.

Results

Increased expression of C17orf37 in prostate cancer cells

To investigate C17orf37 expression in prostate cancer, we analyzed mRNA expression of *C17orf37* in a panel of prostate cancer cell lines including androgen-independent DU-145 and PC-3, and in a LNCaP cell line prostate cancer progression model consisting of androgen dependent LNCaP-R and androgen independent LNCaP-UR (Rothermund *et al.*, 2002) by q-RT-PCR (Figure 1a) and RT-PCR (Supplementary Figure 1). We find high levels of *C17orf37* mRNA expression in all the prostate cancer cell lines investigated, whereas expression in both the normal prostate epithelial cell lines HPV18 C-1 and PWR-1E

are relatively low (Figure 1a). To analyze the protein expression of C17orf37 in prostate carcinoma cells, we first determined the specificity of the anti-C17orf37 antibody (ORF37) using recombinant GST-C17orf37 protein. ORF37 antibody immunoreacts with both GST-C17orf37 (39kD) and GST-tag cleaved purified recombinant C17orf37 protein of 12kD size (Figure 1b). To verify the 12kD band is indeed C17orf37 protein, we analyzed the protein sequence by tandem mass spectrometry. As shown by high-resolution ESI-FTICR analysis (Supplementary Figure 2), the molecular weight of the intact recombinant protein was 11,918 Da and matched 54% amino acid sequence coverage of C17orf37 (Figure 1c).

To find any possible change in C17orf37 protein expression from androgen dependency to independency, we examined two additional prostate cancer cell lines LNCaP-RF, a fast growing androgen responsive LNCaP cell line (Rothermund *et al.*, 2002) and LNCaP/C4-2 (LNC4-2), a subline derived from parental LNCaP cells that acquired phenotypes of androgen independence (Thalmann *et al.*, 1994; Wu *et al.*, 1994). Among metastatic prostate cancer cell lines, DU-145 showed higher C17orf37 expression compared to PC-3 (Figure 1d), whereas *in vitro* LNCaP prostate cancer progression model - UR, RF and R along with LNCaP C4-2 showed similar level of protein expression (Figure 1d), suggesting C17orf37 expression persists both in androgen dependent and independent states of prostate cancer. As with our observations made at the mRNA level, normal prostate epithelial cell lines HPV18 C-1 and PWR-1E showed undetectable expression of C17orf37 protein (Figure 1d). These data indicated that expression of C17orf37 persists throughout prostate cancer disease progression, both in early stages of androgen dependency to late stages of androgen independency, with minimal expression in normal prostate cell lines.

C17orf37 is over-expressed in high grade neoplastic glands and stroma of prostate adenocarcinoma

To determine the expression of C17orf37 in normal and cancerous prostate tissue, we examined C17orf37 expression in archival formalin-fixed paraffin embedded prostate specimens by immunohistochemistry. The hematoxylin and eosin (H & E) stained specimens were classified into normal, benign prostatic hyperplasia (BPH), moderately differentiated prostate adenocarcinoma and poorly differentiated prostate adenocarcinoma (Banerjee *et al.*, 2003) (Figure 2a and 2d) by anatomic pathologists. Gleason scores of the prostate carcinoma tissues ranged from 6-10, thus no well differentiated prostate adenocarcinoma specimens (Gleason score 2-4) were available for our study. In both normal prostate glands (Figure 2b left) and transurethral resection of the prostate (TURP) BPH specimens (Figure 2b middle), C17orf37 staining was minimal in prostatic glands and stroma. Prostate intraepithelial neoplasm (PIN) showed moderately increased staining of C17orf37 (Figure 2c) compared to normal or BPH glands in the same section. However, in moderately differentiated specimens C17orf37 expression was found to be intense in the neoplastic glands and stromal cells surrounding the glands (Figure 2e left). Strong C17orf37 expression was found throughout the poorly differentiated prostate cancer sections with intense diffused stain in the stroma around the malignant cell mass and invasive edges (Figure 2e middle). C17orf37 expression was intense in the fused prostatic glands of poorly differentiated prostatic cancer (Figure 2e "inset" middle). The isotype controls (IgG) for each specimen did not show immunoreactivity (Figure 2b, 2e right). Taken together,

immunohistochemical detection of C17orf37 protein showed prevalent expression in the higher grades of prostate adenocarcinoma with densely stained malignant cells compared to either low or null expression in both normal and BPH specimens.

Subcellular localization of C17orf37 in prostate cancer cells

We investigated the intracellular localization of C17orf37 protein in prostate cells. In DU-145 and LNCaP cells, western immunoblot of cellular fractions (Figure 3a) and immunocytochemical detection by confocal microscopy (Figure 3b left - DU-145, Figure 3b right - LNCaP) showed endogenous expression of C17orf37 predominantly in the cytosol. Transient transfection of GFP-C17orf37 in DU-145 (Figure 3c left), LNCaP (Figure 3c middle) and HPV18C-1 (Figure 3c right) prostate cells also showed similar pattern of C17orf37 expression. Endogenous C17orf37 and GFP-C17orf37 localized primarily in the cytoplasm of the transfected cells, densely in the perinuclear area around the membrane (white arrows) and less in the nucleus (Figure 3b and 3c). Localization was similar in both C17orf37 positive prostate cancer cells DU-145 and LNCaP, and a null C17orf37 HPV18 C-1 (Figure 3c). To determine whether C17orf37 is a membrane bound protein, we performed total internal reflection fluorescence microcopy (TIRFM) to monitor membrane localization of C17orf37. In the TIRFM, the evanescent wave produced by the visible light at the cell/substratum interface penetrates only 100-200 nM with decaying intensity with the distance, allowing detection of protein molecules associated with the plasma membrane (Axelrod, 2001). DU-145 (Figure 4d left) and LNCaP (Figure 4d middle) cells labeled with ORF37 antibody tagged to Alexa flurophore-568 showed numerous dense spots of C17orf37 protein in the membrane region (Figure 4d). We used intracellular cytoskeletal protein tubulin as a control for our experiments, but TIRFM failed to image tubulin protein molecules on the membrane (Figure 4d right). These results indicated that C17orf37 is a cytosolic protein with predominant membrane localization in prostate cancer cells.

Expression of C17orf37 enhances the in vitro invasive and migratory potential of prostate cancer cells

Our results show expression of C17orf37 is higher in advanced prostate adenocarcinomas (Gleason grade: 6 and 9) compared to normal prostate tissues (Figure 2b and 2e). Metastases of malignant cells to distant tissues or organs are mediated by cellular migration, invasion and proteolytic activity that degrade tissue barrier (Sliva, 2004). To investigate the role of C17orf37 in prostate cancer cell migration, we studied the effect of C17orf37 downregulation on the migratory potential of DU-145 cells. Using gene specific siRNA at varying concentrations, we blocked the endogenous expression of C17orf37 in DU-145 cells (Figure 4a). An *in vitro* agarose gel bead assay was performed with C17orf37 knocked down DU-145 cells, to determine the dose dependent effect of siRNA-C17orf37 on DU-145 cell migration. As shown in Figure 4b, siRNA-C17orf37 treatment (Figure 4b, *III-V*) reduced the number of DU-145 cells that could migrate out of the agarose gel bead into the medium compared to wild type or control-siRNA treated (Figure 4b, *I-II*). siRNA-C17orf37 treatment at 100nM resulted in ∼10 fold decrease in migration of DU-145 cells (P <0.0001) compared to control wild type cells (Figure 4c). *In vitro* tumor invasion assay also showed reduced invasive ability of DU-145 cells treated with siRNA-C17orf37 (Figure 4d). To verify expression of C17orf37 has a dominant effect on tumor cells invasion, we assessed

invasiveness of DU-145 and PC-3 cells overexpressed with GFP-C17orf37. C17orf37 positive DU-145 prostate cancer cells showed ∼1.8 fold increase, where as PC-3, with low endogenous C17orf37 expression, showed ∼1.75 fold increase in invasion compared to respective controls (Figure 4e). Stable overexpression of GFP-C17orf37 construct in DU-145 cells (DU-GFP-C17orf37) dramatically increased the invasiveness ∼2.6 and ∼1.95 fold compared to parental DU-145 cells (Supplementary Figure 3), indicating overexpression of C17orf37 protein leads to increased invasive behavior in prostate cancer cells.

Downregulation of C17orf37 results in reduced expression of MMP-9, uPA and VEGF by lowering NF-κB DNA binding activity in DU-145 prostate cancer cells

Migration and invasion of malignant cells is facilitated by specific proteases like matrix metalloproteinases (such as MMP-9) and serine proteases (such as uPA) that degrade the ECM (Sliva, 2004). During prostate cancer progression these molecules are found to be predominantly up-regulated to facilitate migration and invasion (Helenius *et al.*, 2006; Li and Cozzi, 2007b; Lokeshwar, 1999; Wilson and Sinha, 1993; Wilson *et al.*, 2004). Prostate cancer cells also secrete high levels of growth factors like VEGF, which is an important mediator of angiogenesis, proliferation and migration (Hicklin and Ellis, 2005). To investigate whether C17orf37 protein has any effect on the metastasis related genes, we knocked down endogenous C17orf37 in DU-145 cells and analyzed expression of several genes that facilitate prostate cancer metastasis and promote ECM cleavage. We observed suppression of endogenous C17orf37, simultaneously reduced MMP-9, uPA and VEGF expression as measured by RT-PCR (Figure 5a) and western immunoblot (Figure 5b) compared to non-targeting siRNA (control-siRNA). To determine if C17orf37 expression increases MMP-9, uPA and VEGF protein in prostate cancer cells, we overexpressed C17orf37 in DU-145 cells by transient transfection of GFP-C17orf37 plasmid. As shown in Figure 5c, western immunoblot confirmed overexpression of C17orf37 increases endogenous MMP-9, uPA and VEGF protein compared to vector treated cells. Interestingly, silencing of C17orf37 in DU-145 prostate cancer cells resulted in reduced mRNA expression of both the isoforms of VEGF ($VEGF_{165}$ and $VEGF_{121}$) (Figure 5a). $VEGF_{121}$ isoform is rapidly secreted and freely diffuses into the tissues, where as $VEGF_{165}$ the potent isoform functionally active in most angiogenic states (Connolly and Rose, 1998; Woolard *et al.*, 2004) was found to be regulated by C17orf37 (Figure 5b,c). To quantitate the relative amount of secreted VEGF (both the isoforms) and MMP-9 we performed ELISA of conditioned media from DU-145 cells overexpressed or depleted C17orf37 protein. Our results show that secreted MMP-9 and VEGF protein is significantly altered due to C17orf37 expression compared to respective controls (Figure 5d).

MMP-9, *uPA* and *VEGF* genes are transcriptionally up-regulated by NF-κB, which is constitutively active in prostate cancer cells (Suh and Rabson, 2004). We performed EMSA to evaluate the DNA binding activity of NF-κB in C17orf37 silenced DU-145 cells. Our results indicate increasing concentration of C17orf37 specific siRNA effectively inhibited NF-κB DNA binding activity in a dose-dependent manner (Figure 6a). These results show that C17orf37 increases migration and invasion in prostate cancer by NF-κB mediated genes MMP-9, uPA and VEGF. In prostate cancer cells, constitutive activation of NF-κB is

mediated by upstream protein kinase B (PKB/Akt) (Fresno Vara *et al.*, 2004), we performed western immunoblot to detect the levels of phosphorylated Akt (p-Akt) and total Akt. p-Akt is the active form of the protein that cascades the intracellular signaling and our results show blocking of C17orf37 by siRNA dramatically reduced p-Akt level in DU-145 cells (Figure 6b). C17orf37 specific siRNA (100nM) treatment abolished 80% phosphorylation of Akt in DU-145 cells (Supplementary Figure 4) where as the total Akt expression was constant when normalized to loading control GAPDH. We also observed reduction in the phosphorylated form of ERK1/2 compared to total ERK1/2 (Figure 6b), however the extent of reduction was not significant when compared to Akt activation (Supplementary Figure 4). To validate our observation, we measured p-Akt/Akt level and NF-κB DNA binding activity in DU-145 cells stably overexpressing GFP-C17orf37 (DU-GFP-C17), and as expected phosphorylation of Akt and NF-κB activity was significantly higher in C17orf37 overexpressed cells compared to vector control cells (DU-GFP) (Figure 6c). These results demonstrate that C17orf37 mediates prostate cancer cell migration and invasion through NF-κB downstream target genes MMP-9, UPA and VEGF.

Discussion

C17orf37 is a novel gene located on human chromosome 17q12, in the 'hot spot locus of cancer' which contains multiple genes that have been shown to be involved in the progression of cancer. C17orf37 gene is 505 nucleotides from ErbB-2 oncogene, which has been demonstrated as an important factor for development of hormone refractory prostate cancer (Berger *et al.*, 2006). Although C17orf37 overexpression in breast cancer has been linked with genomic amplification of Her-2/neu locus, recent report has confirmed overexpression of C17orf37 in Her-2/neu negative breast cancer patients and breast tumor cell lines, suggesting independent transcriptional control mechanisms for C17orf37 (Evans *et al.*, 2006). In the present study, we show that C17orf37 expression is consistently higher in both androgen dependent and independent prostate cancer cell lines and clinical prostate cancer tissues examined, compared to either low or null expression in normal and BPH prostate cells and tissues (Figure 1, 2). In clinical prostate cancer specimens, higher Gleason scored tumors showed increased C17orf37 expression compared to lower scores, suggesting C17orf37 expression may increase with grade and stage of cancer. However, the small number of established prostate cancer cell lines does not show significant changes in C17orf37. A larger study with more patient population will be necessary to establish C17orf37 as a biomarker for prostate cancer progression. Our data provide the first evidence that C17orf37 expression positively correlates with the migratory and invasive potential of metastatic prostate cancer cells and thereby can be regarded as a potential biomarker for the disease progression. This is the first report delineating the role, functional significance and a proposed mechanism through which this novel gene C17orf37 governs prostate cancer progression.

C17orf37 gene encodes a protein (Accession no. NP_115715) of 115 amino acids with a molecular weight of ∼12 KD (Fig. 1*C* and Supplementary Fig. S2) that has no sequence similarity with any known genes or proteins (Evans *et al.*, 2006; Kauraniemi and Kallioniemi, 2006). By immunoconfocal microscopy, TIRFM and cellular fractionation studies we show that C17orf37 is predominantly a cytosolic protein, densely located in the

cell membrane (Figure 3). In migrating prostate cancer cell, C17orf37 protein primarily localizes to the leading edge of cell (Supplementary Figure 5). Careful analysis of the C17orf37 protein sequence (Figure 1c) revealed a "CaaX" prenylation motif comprising of last four amino acids "CVIL" at the C-terminal end. "CaaX" group of proteins are either prenylated or geranylgeranylated by enzymes farnesyltransferase (FTase) and geranylgeranyl transferase type I (GGTase-I), respectively. If "X" is leucine, protein is preferentially modified by GGTase-I enzyme (Fu and Casey, 1999), suggesting C17orf37 translocation to the membrane may be mediated by GGTase-I. Pre-PS (prenylation prediction suite) predicted C17orf37 to be prenylated preferentially by the enzyme GGTase-I with a P-value=0.00049 (Maurer-Stroh and Eisenhaber, 2005) and this idea was also supported by a recent report (Evans *et al.*, 2006).

"CaaX"-type prenylated proteins are primarily located at the cytoplasmic face of cellular membranes and in cancer cells these proteins are found to have significant role in oncogenic transformation, cytoskeletal organization, cellular proliferation, migration, invasion and metastasis (Kelly *et al.*, 2006; Virtanen *et al.*, 2002; Winter-Vann and Casey, 2005). By RNAi mediated C17orf37 gene silencing and also by overexpressing C17orf37 in prostate cancer cells, we successfully established that C17orf37 regulates migration and invasion (Figure 4). Even in patients with metastasis of breast cancer to liver and lungs, abundant expression of C17orf37 protein has been reported (Evans *et al.*, 2006). Migration and invasion of cancer cells are the hallmark of malignant neoplastic proliferation beyond the vascular boundaries facilitated by ECM cleavage by proteolytic enzymes. siRNA mediated knockdown of C17orf37 in DU-145 cells, reduced mRNA and protein expression of important proteolytic enzymes MMP-9 and uPA, and potent angiogenic molecule VEGF (Fig.5), suggesting C17orf37 may act as an important upstream signaling molecule enhancing the transcription of these genes. uPA binding to its receptor uPA-R at the cell surface converts inactive plasminogen to active plasmin, and there by cleaves ECM proteins inducing migration and invasion of malignant cells (Li and Cozzi, 2007). MMP-9 specifically cleaves ECM proteins like gelatin, collagen I and IV, vitronectin and fibronectin resulting in metastatic dissemination of malignant cells (Price *et al.*, 1997). In prostate cancer development, angiogenic growth factor VEGF plays key role in promoting tumor growth and metastatic progression of the disease. MMP-9, uPA and VEGF have been reported to be overexpressed in invasive prostate cancer (Wilson and Sinha, 1993) and are positively correlated with poor prognosis (Sheng, 2001). We show here that by modulating C17orf37 expression in DU-145 metastatic prostate cancer cells, expression of these key molecules is significantly reduced, there by inhibiting prostate cancer cell migration and invasion.

In invasive prostate cancer cells, increased expression of *MMP-9*, *uPA* and *VEGF* genes are regulated by transcriptional activation of NF-κB (Huang *et al.*, 2001). Activation of NF-κB is due to increased activity of upstream kinase IKK complex which greatly reduces the half-life of the inhibitory IkBα, there by inducing nuclear localization of NF-κB (Suh and Rabson, 2004). Knockdown of C17orf37 by gene specific siRNA simultaneously reduced the DNA binding activity of NF-κB, indicating C17orf37 mediated signaling may modulate MMP-9, uPA and VEGF genes through NF-κB pathway. This also justifies C17orf37 as an

upstream signaling molecule of NF-κB. PI3K-Akt and MAPK are the two upstream signaling pathways that have been shown to be responsible for constitutive activation of IKK complex and NF-κB (Suh and Rabson, 2004). In prostate cancer cells, PI3K-Akt works like a signaling hub mainly as a downstream effector of tyrosine kinase growth factor receptors. Modulation of C17orf37 dramatically altered the phosphorylation of Akt at Ser-473 (Figure 6) which indicates C17orf37 may act as an inducer of Akt phosphorylation. The ratio of phosphorylated ERK1/2 to the total ERK1/2 also decreased, albeit minimally, when compared to the control-siRNA treated DU-145 cells. This decrease in ERK1/2 phosphorylation may be due to the reduced expression of uPA protein upon C17orf37 silencing. In prostate cancer cells, uPA binds to its cell surface receptor uPA-R there by activating ERK1/2 signaling (Jo *et al.*, 2005). Several studies have shown that knockdown of uPA abolishes the ERK1/2 phosphorylation (Aguirre Ghiso *et al.*, 1999), indicating decrease in ERK1/2 phosphorylation in C17orf37 knockdown cells may be due to the downstream target molecule uPA.

Thus, based on our data we propose a mechanistic model for C17orf37 mediated signaling in prostate cancer invasion and signaling (Figure 6d). In response to extracellular stimuli, growth receptors (tyrosine kinase) present on the prostate cancer cells cascade down the signal activating PI3K. This results in the activation of AKT which translocates to the membrane where it is phosphorylated at Ser-473 (Chan *et al.*, 1999). Prenylated protein C17orf37 also remains localized to the cytosolic face of the membrane (may be as an adapter protein) and in turn controls the downstream signaling of activated p-AKT. Whether this effect is direct or mediated through other adapter proteins that physically interact with C17orf37 on the membrane needs further investigation. Activated AKT phosphorylates IKK complex which then results in the translocation of NF-κB to the nucleus by reducing the half life of IκBα (Suh and Rabson, 2004). In the nucleus NF-κB acts as transcription factor resulting in the enhanced expression of downstream target genes MMP-9, uPA and VEGF. These molecules are then secreted by prostate cancer cells and there by increases the invasiveness of prostate cancer cells (Sliva, 2004).

In summary, we find that C17orf37 is highly expressed in prostate cancer cells and tissues, compared to minimal expression in normal prostate cells. C17orf37 is predominantly expressed as a membrane bound cytosolic protein and actively regulates prostate cancer cell migration and invasion by upregulating the expression of MMP-9, uPA and VEGF. Additionally, downregulation of C17orf37 by specific siRNA reduced the DNA binding activity of NF-κB, substantiating the fact that C17orf37 expression contributes to the development of invasive prostate cancer through NF-κB mediated downstream target genes. Interestingly, AKT activity was reduced due to C17orf37 gene silencing suggesting membrane bound C17orf37 may be a potential regulator of AKT phosphorylation. We believe our results are the first report to deduce the functional importance of so called 'hypothetical protein' C17orf37 that may be considered as a potential therapeutic target for cancer therapy.

Materials and methods

Cell lines and culture conditions

Details describing cell culture and reagents are provided in the supplementary information.

Cloning, expression and purification of recombinant C17orf37

Information about the plasmids constructed for the study and details about the protein expression and purification is included in supplementary information.

Transfection procedures and siRNA mediated gene silencing of C17orf37

Stable and transient transfection of DU-145, LNCaP and HPV18 C-1 cells were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with plasmid DNA (GFP vector or GFP-C17orf37). For C17orf37 knockdown experiments, human C17orf37 (NM_032339) smart pool siRNA (siRNA-C17orf37) and human non-targeting scrambled siRNA duplex (Control-siRNA) were purchased from Dharmacon (Lafayette, CO) and transfected in DU-145 cells according to manufacturer's instructions. Details provided in supplementary information.

Isolation of total RNA and quantitative reverse transcription-PCR

Information about the RNA extraction and q-RT-PCR is included in supplementary information.

Cell fractionation and immunoblotting

Whole cell protein extracts were prepared from prostate cell lines as described previously (Das *et al.*, 2007). Cytosolic and nuclear fractions of DU-145 and LNCaP cells were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) according to manufacturer's instructions. Bacterial cell lysates were prepared using B-PER extraction kit (Pierce, Rockford, IL). Western immunoblot was performed using a 15% gel for C17orf37 protein analysis and 10% for other proteins, as described previously (Das *et al.*, 2007). List of antibodies purchased for the study are included in the supplementary.

Mass spectrometry and liquid chromatography–tandem mass spectrometry

Mass spectrometry performed using FTICR instrument (LTQ-FT, Thermo Fisher, San Jose, CA) as described in supplementary information.

Confocal microscopy, immunofluorescence and total internal reflection fluorescence microscopy

DU-145 and LNCaP cells were grown on glass cover slips and treated with C17orf37 antibody (Zymed, Carlsbad, CA). For confocal images, cells were visualized on a Zeiss LSM 410 confocal microscope and images were captured using LSM 4 software (Carl Zeiss Microimaging). For, total internal reflection fluorescence microscopy (TIRFM), cells were grown on coverslips and then fixed by 2% paraformaldehyde. Unpermeabilized cells were then washed with PBS and treated with C17orf37 or tubulin antibody (Calbiochem) followed by Alexa 568 conjugated secondary antibody. Coverslips were then mounted on

specialized cover glass #1 (Corning) of 22×50 mm size. For TIRF images, cells were visualized on an Olympus I×71 microscope with commercial TIRF attachment as described previously (Burghardt *et al.*, 2006) by 60× oil immersion objective.

Immunohistochemistry

Frozen prostate tumors from patients were provided by the UNMC/Eppley Cancer Center Tumor Bank (Omaha, NE). Two anatomic pathologists independently graded the Hematoxylin & Eosin (H & E) stained sections and Gleason scores for these tissues varied between 6 and 10. Immunohistochemistry was performed as described previously (Das *et al.*, 2007). C17orf37 antibody (Zymed, Carlsbad, CA) and rabbit IgG (Sigma, St. Louis, MO) were used for the immunohistochemistry study. Representative images of the clinical prostate sections were captured and analyzed by the pathologist.

Cell migration assays

Forty eight hours post-transfection with siRNA-C17orf37 or control-siRNA, 1×10^6 number of DU-145 cells were mixed with low melting agarose and growth medium to form a semi-solid gel and migration assay performed as described before (Das *et al.*, 2007). The cells were allowed to migrate out of the semi-solid beads for 36 hours and then visualized on an Olympus Microscope (Carl Zeiss). Representative images of the cells were captured and number of cells that migrate out of the beads were counted from three independent experiments. Fold change of migration was determined by a ratio of migrated cells in experimental group to the control group.

In vitro tumor invasion assay

Tumor invasion assay was performed using BD Biocoat Tumor Invasion System (BD Biosciences, Bedford, MA) according to manufacturer's instructions. Details about the procedure are included in the supplementary information.

Reverse Transcription-PCR (RT-PCR)

One-step RT-PCR (Invitrogen, Carlsbad, CA) was performed using 1μg of total RNA as described before (Das *et al.*, 2007). Custom primers were synthesized as mentioned in Supplementary Table. 1.

ELISA assay for MMP-9 and VEGF

Elisa assay performed from the conditioned media as described in Supplementary Materials and Methods section.

Electrophoretic mobility shift assay (EMSA)

NF-κB DNA binding activity was determined by gel shift assay in C17orf37 knockdown and overexpressed cells using NF-κB specific EMSA gel-shift assay kit (Panomics Inc., Redwood, CA).

Statistical analyses

Results were expressed as mean and statistical analysis performed using GraphPad Prism 4.02 software. One sample t-test was performed and *P* <0.05 was considered to be significant.

Supplementary Material

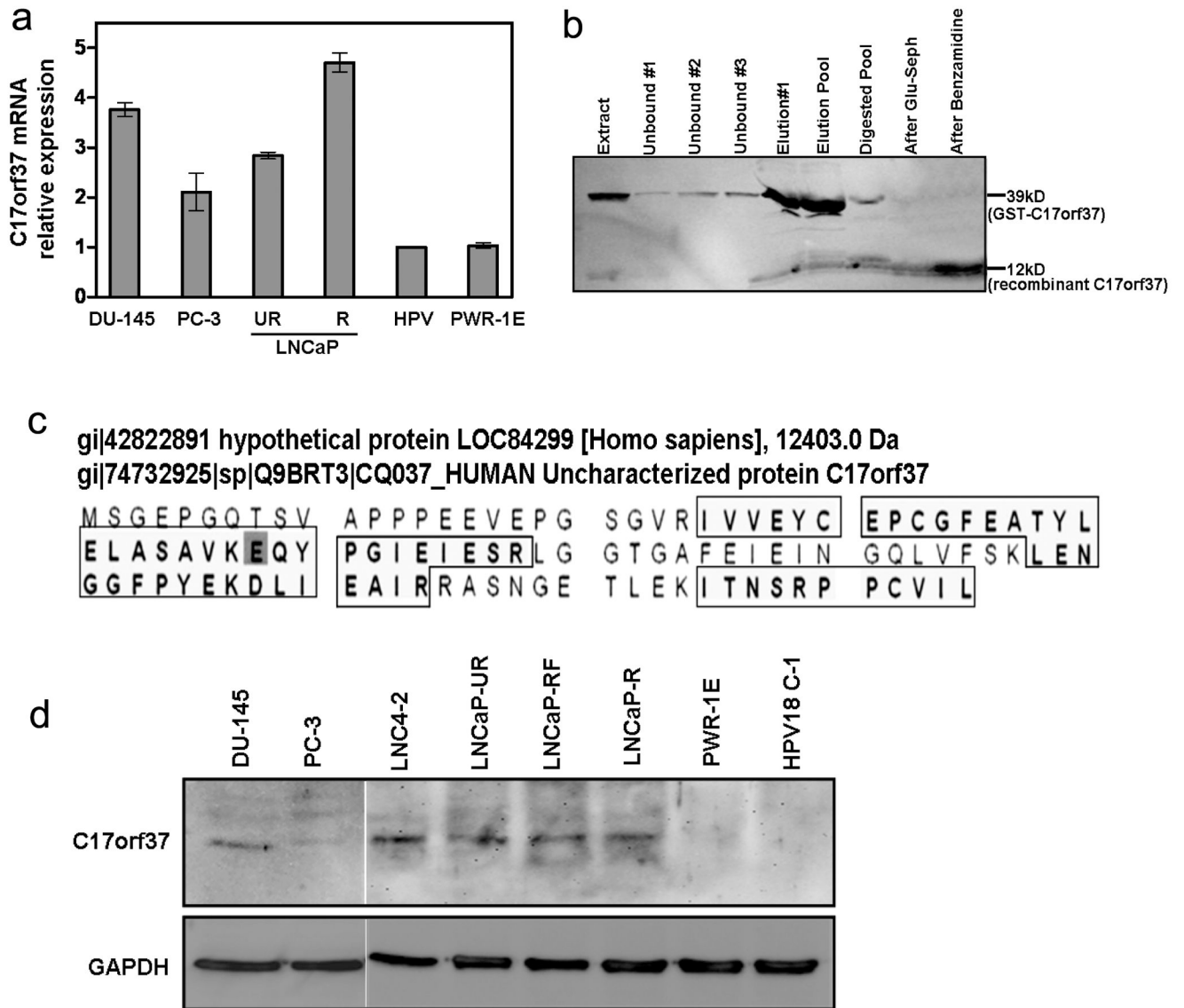Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
C17orf37 mRNA and protein expression in human prostate cancer cells and tissues. (a) *C17orf37* mRNA expression was analyzed by real time PCR in prostate cancer cell lines DU-145, PC-3, LNCaP-UR and LNCaP-R, and normal prostate epithelial cell line HPV18 C-1 and prostate derived cell line PWR-1E. Housekeeping gene β-actin was used as an internal control. (b) Western immunoblot showing the expression of GST-C17orf37 protein that was purified and thrombin cleaved to generate recombinant C17orf37 protein. (c) Summary of LC/ESI-MS/MS analysis for the predicted C17orf37 after tryptic digestion (4 unique peptides and 54% sequence coverage, 62 out of 115 amino acid residues shown; the box indicates the amino acid residues covered and grey highlight denotes conversion of E to <E upon proteolytic digestion). (d) C17orf37 protein was analyzed by western immunoblot in a panel of prostate cancer cell lines including - DU-145, PC-3 and LNC4-2 (androgen independent), LNCaP progression model- LNCaP-UR, LNCaP-RF and LNCaP-R (from
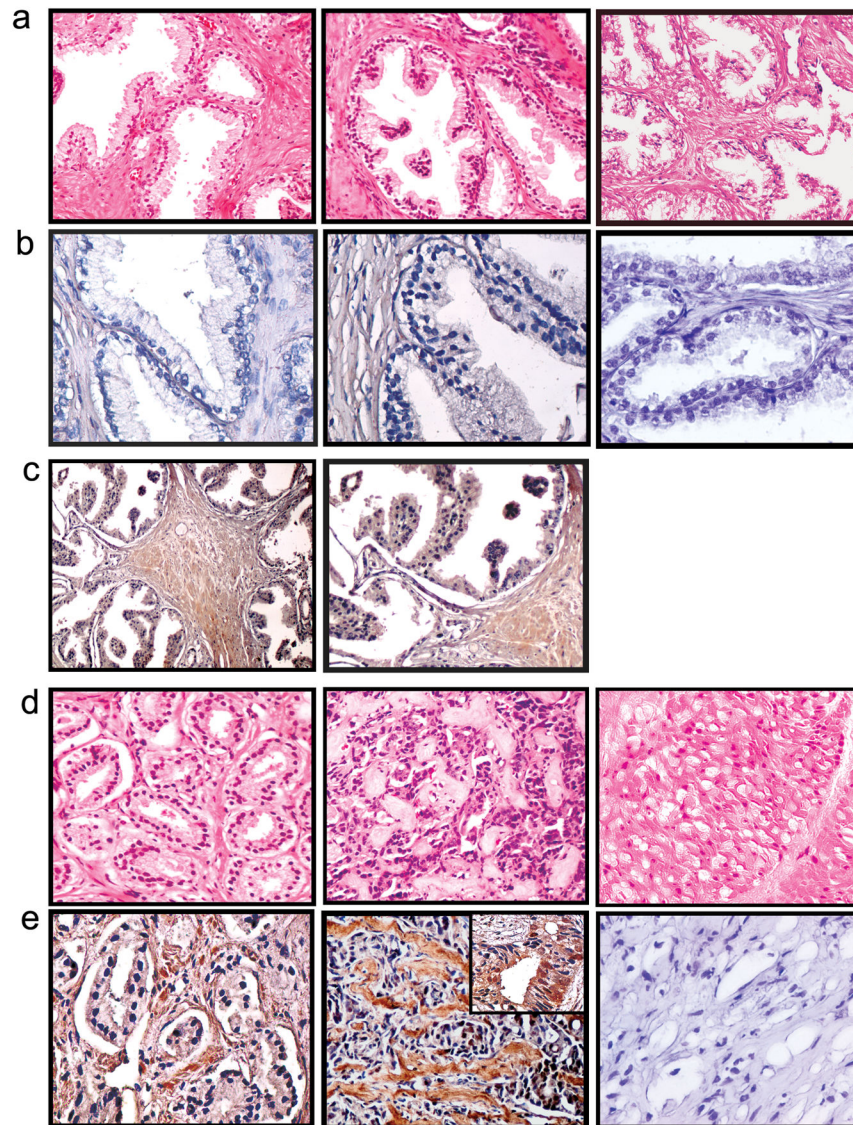
androgen independent to dependent); and, normal prostate epithelial cell line HPV18 C-1 and PWR-1E. Housekeeping gene GAPDH served as loading control. Expression of C17orf37 protein was higher in prostate cancer cells compared to normal prostate cells.

Figure 2.
Expression of C17orf37 in human normal, benign prostatic hyperplasia (BPH) and prostate adenocarcinoma tissue specimens. Representative images of normal prostate gland (a, b - left), BPH (a, b -middle), prostate intraepithelial neoplasm (c), moderately differentiated (Gleason Score 6) (d, e - left) and poorly differentiated (Gleason Score 9) (d, e -middle) prostate carcinoma. Sections were stained with H & E (a, d) and ORF37 antibody (b, c, e). (b) Left, C17orf37 expression was found to be minimal in both normal prostate glands (*n=6*), (b) middle, in BPH prostate specimens (n=6), and (b) right, negative in IgG control. (c) C17orf37 staining in PIN glands show presence of protein in early stages of cancer progression. (e) Left, Moderately differentiated prostate adenocarcinoma tissue sections show increased expression of C17orf37 in both neoplastic glands and prostatic stroma (*n=15*), (e) middle panel, Poorly differentiated prostate adenocarcinoma tissue sections showed fusion of neoplastic glands with intense diffused C17orf37 immunostain *(n=12)*,
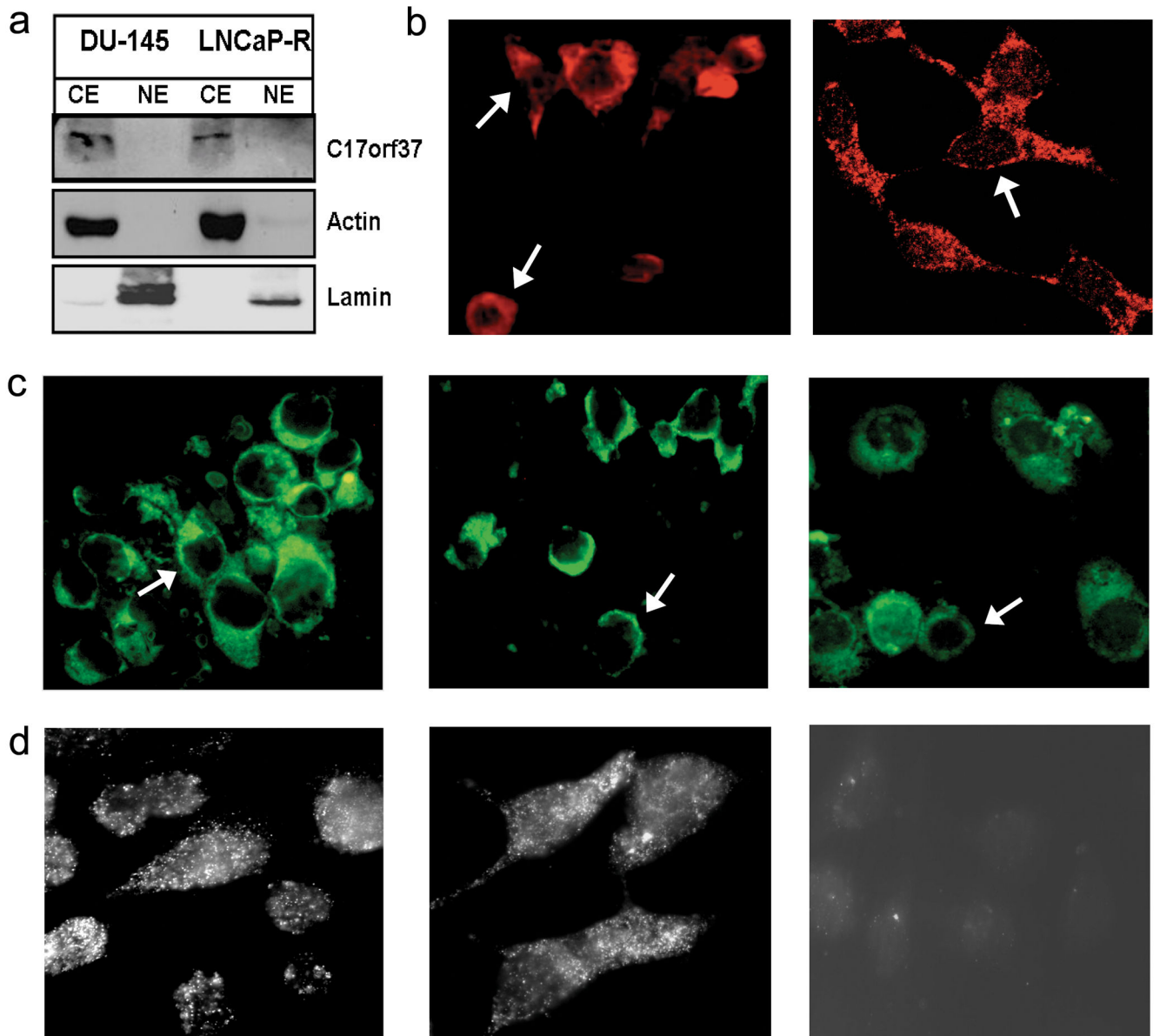
Inset shows C17orf37 expression in the prostatic glands in poorly differentiated carcinoma, and (e) right panel, shows negative staining in IgG control. All images are *100×* of original magnification except (c) left, which is *40×* of original magnification.
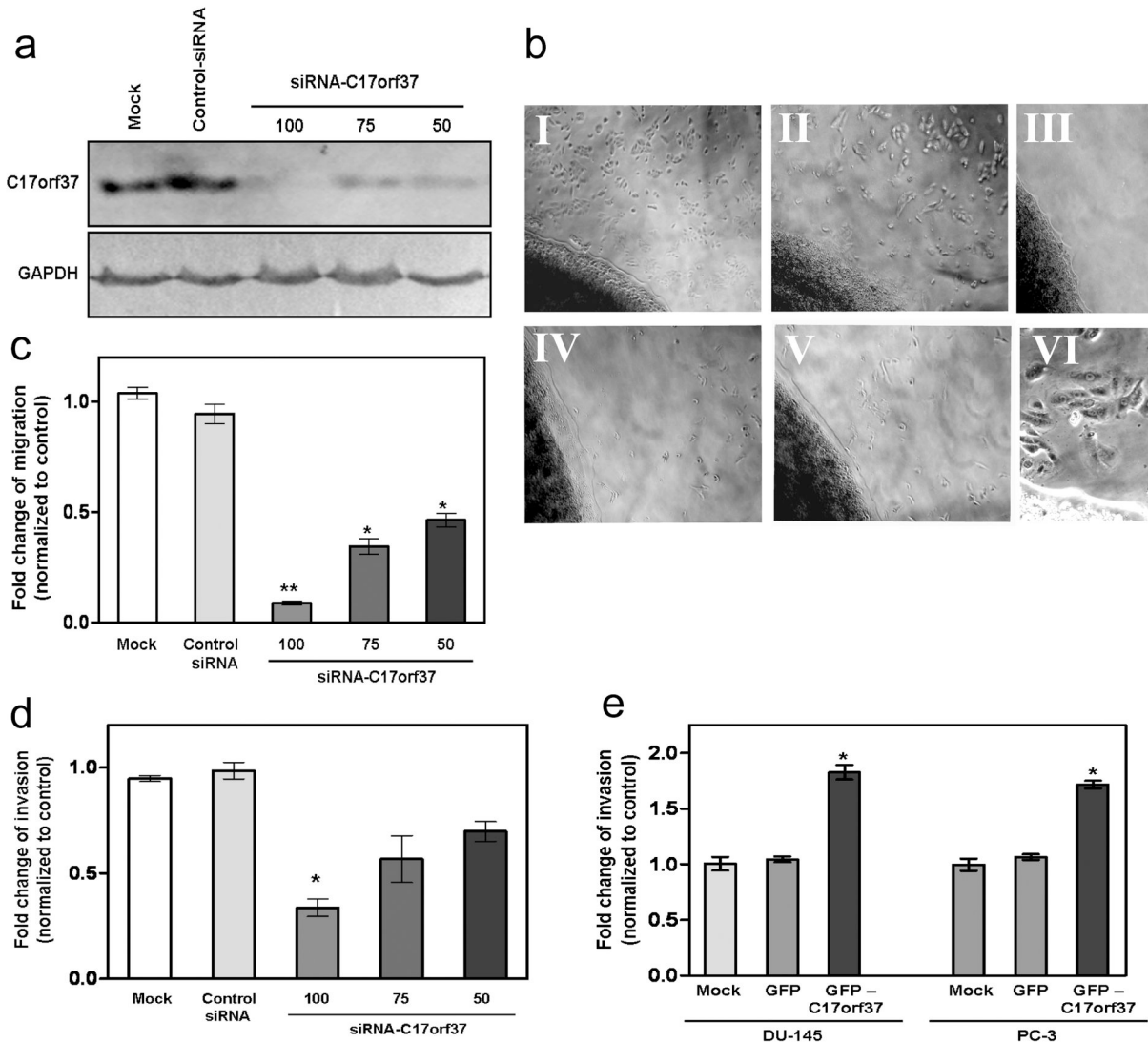
Figure 3.
Subcellular localization of C17orf37 in human prostate cancer cell lines. (a) Subcellular fractionation followed by western immunoblot showing endogenous expression of C17orf37 in the cytosolic fractions of DU-145 and LNCaP-R prostate cancer cells. The purity of the cytosolic (CE) and nuclear extracts (NE) were determined using cytosolic marker protein actin and nuclear protein lamin. (b) Immunofluorescent staining of endogenous C17orf37 expression in DU-145 (middle) and LNCaP-R (right) prostate cancer cells. Confocal images show intense C17orf37 staining in the cytosolic compartment with dense spots in the membrane. Original magnification, ×40. (c) GFP-C17orf37 construct was transiently transfected in DU-145 (left), LNCaP (middle) and HPV18 C-1 cells (right). The cells were then fixed and imaged by confocal microscopy. GFP-C17orf37 was visualized as green

fluorescent signals mostly in the cytosolic area predominantly in the membrane. Original magnification, ×*40*. (d) DU-145 (left and right) and LNCaP-R cells (middle panel) were grown in coverslips, fixed, unpermeabilized and treated with anti-C17orf37 (left and middle) or anti-tubulin (right) antibody. Coverslips were mounted on special TIRF coverslips as mentioned in the methods section. Slides were visualized by TIRFM. Spots as seen in the images depict the membrane localization of C17orf37 in prostate cancer cells. Original magnification, ×*60* oil immersion. White arrows indicate membrane localization of C17orf37 in prostate cancer and non-cancerous cells.
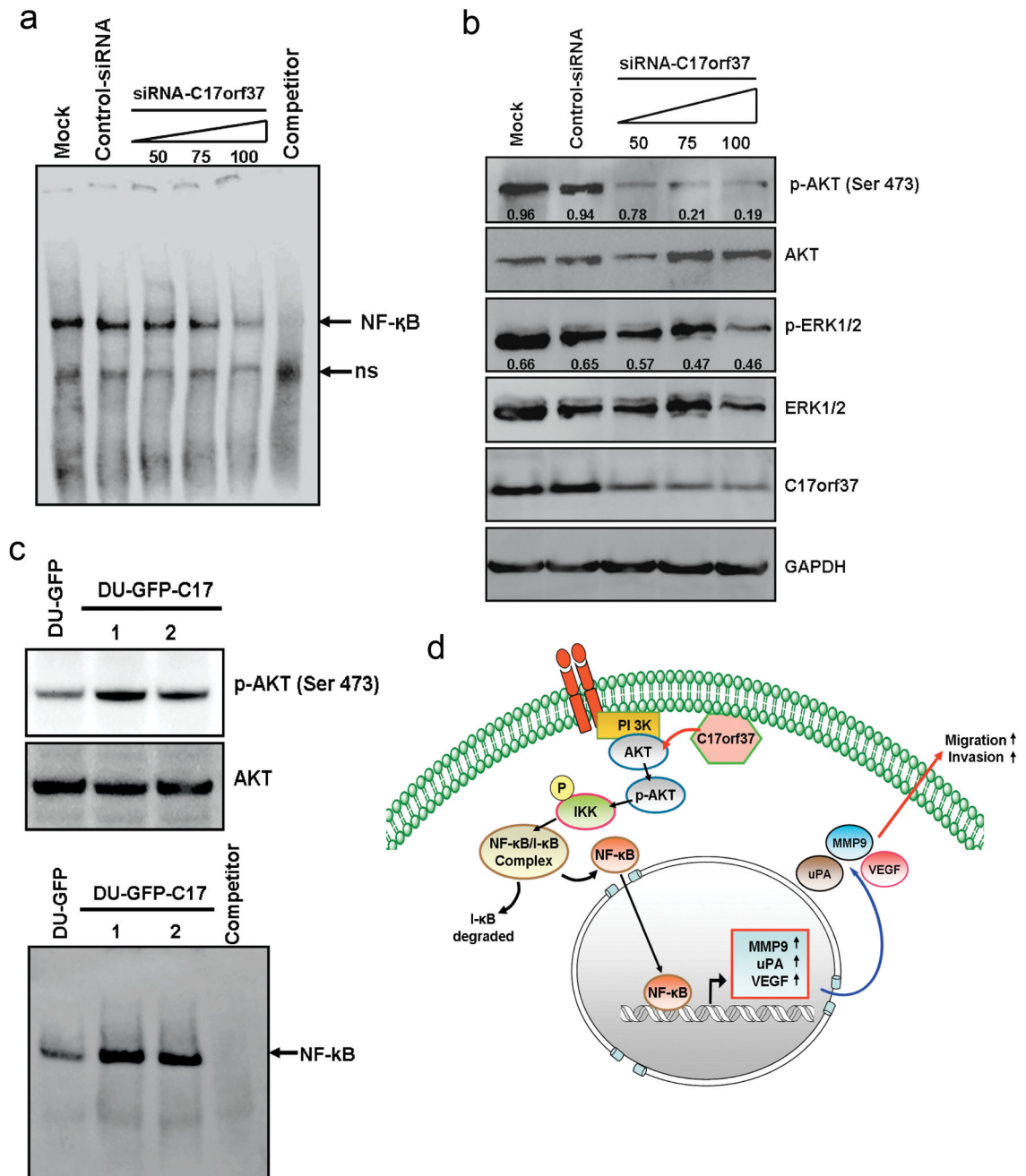
Figure 4.
C17orf37 expression regulates migration and invasion of prostate cancer cells. (a) DU-145 cells were transfected with transfection media alone (mock); non-targeting scrambled siRNA (control-siRNA); and 100, 75 and 50 nM doses of C17orf37 specific siRNA. Following 48 hours of transfection, C17orf37 protein knockdown was confirmed by Western immunoblot using ORF37 antibody and compared to GAPDH. Blot is represented image of 4 independent experiments. (b) 48 hours of post-transfection DU-145 cells were mixed in low melting agarose and growth medium, and plated on fibronectin coated plates to form a semisolid gel bead. Representative pictures taken after 36 hours show the number of DU-145 cells that migrate out of the gel beads treated with, *I* - transfection medium only (mock), *II* - nontargeting siRNA (control-siRNA), *III* - 100nM of C17orf37 specific siRNA, *IV* -75 nM of C17orf37 siRNA and *V* - 50nM of C17orf37 specific siRNA. Figure *C-VI* shows the normal morphology of the DU-145 cells that migrate out of the agarose gel beads. (c) Graphical representation of fold change in DU-145 prostate cancer cell migration

compared to control (untreated) wild type cells. (d) DU-145 cells transfected with Mock, non-targeting control siRNA, and 100nM, 75nM and 50nM siRNA-C17orf37 were incubated for 48 hours. After the transfection phase, DU-145 cells were seeded onto Matrigel coated BD Biocoat Tumor Invasion system and allowed to migrate towards 10% serum for 24 hours. Fold change of invasion was calculated as described in materials and methods section. (e) DU-145 and PC-3 cells were transiently transfected with transfection media alone (mock), EGFP-C1 vector only (GFP) and EGFP-C17orf37 plasmid (GFP-C17orf37). Twenty four hours later, cells were seeded onto matrigel coated tumor invasion chambers and allowed to migrate toward serum for 24 hours at 37°C. Fold change of invasion and migration was calculated as described in materials and methods section. *Columns* (c, d and e) are mean of three independent experiments; *bars*, SD. **, $P < 0.001$ and *, $P < 0.05$, relative to mock treatment of cells; statistical analysis included Student's *t* test for calculating significant differences within groups.
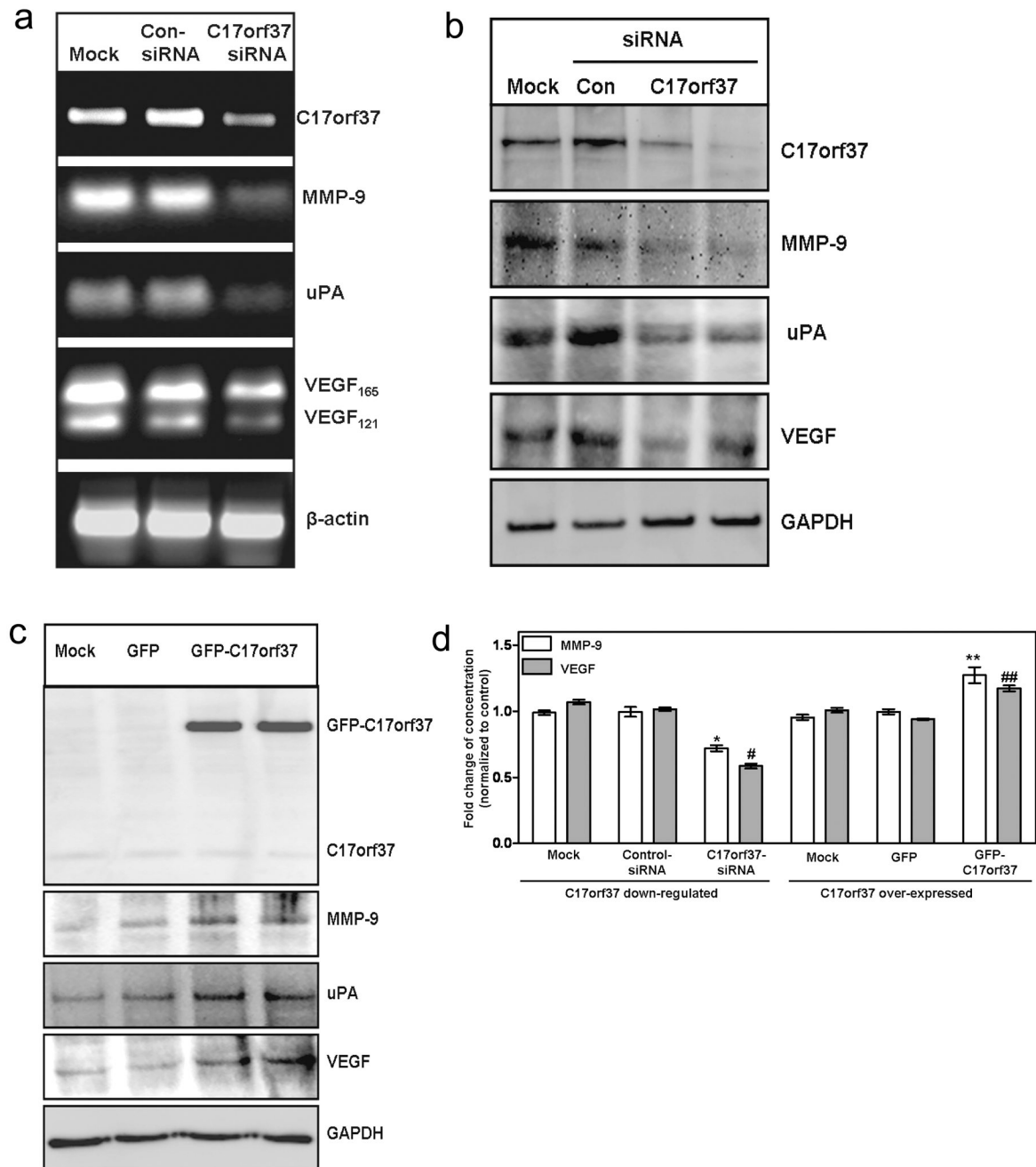
Figure 5.
siRNA mediated silencing of *C17orf37* results in the downregulation of *MMP-9*, *uPA* and *VEGF* expression. DU-145 cells were transiently transfected with transfection media-Dharmafect alone (mock), non-targeting siRNA (control siRNA) and SMART pool siRNA specific to C17orf37 (100nM). (a) Seventy hours later total RNA was isolated and the mRNA expression of MMP-9, uPA and VEGF was analyzed by RT-PCR. House keeping gene β-actin served as internal control. (b) Ninetysix hours of post transfection total protein was isolated and 30 μg of protein was used to perform western immunoblot. Expression of

MMP-9, uPA and VEGF protein were analyzed in mock, control-siRNA and C17orf37 specific siRNA - 100nM (in duplicate) treated DU-145 prostate cancer cells. (c) C17orf37 was over-expressed by transiently transfecting DU-145 cells with GFP-C17orf37 plasmid. Lipofectamine treated (mock) and GFP vector transfected were used as controls. Twnetyfour hours later total protein was isolated and 15 μg of protein was used to perform western immunoblot. Figures show expression of MMP-9, uPA and VEGF in mock-treated, GFP-vector only and GFP-C17orf37 treated DU-145 cells (in duplicate). All western blot images are representative of 4 independent experiments. (d) Elisa assay of conditioned media from DU-145 cells treated as in (b) and (c) were used to estimate MMP-9 and VEGF. Fold change of concentration was determined by a ratio of quantitative values of MMP-9 (ng/mL/10^5 cells) and VEGF (pg/mL/10^5 cells) in experimental group to the control group. *Columns* mean of four independent experiments; *bars*, SD. *, $P < 0.005$ relative to mock (Dharmafect) treated (MMP-9); #, $P < 0.005$ relative to mock treated (VEGF); **, $P < 0.05$ relative to mock (lipofectamine) treated (MMP-9) and ##, $P < 0.005$ relative to mock treated (VEGF); statistical analysis included Student's *t* test for calculating significant differences within groups.

Figure 6.
Knockdown of C17orf37 by siRNA reduces the NF-κB DNA binding activity via suppressing the AKT activation. (a) EMSA was done by incubating 5μg of nuclear protein extracts from DU-145 cells treated with non-targeting siRNA (control siRNA) and 50, 75 and 100nM siRNA specific to C17orf37. DU-145 prostate cancer cells without any treatment (untreated) and in presence of competitive oligonucleotides to the probe (competitor) were used as controls. The non significant band (NS) present in all the lanes did not have any effect even in presence of competitor. (b) DU-145 prostate cancer cells

were transiently transfected with non-targeting siRNA (Control-siRNA), and increasing concentration of C17orf37 specific siRNA at 50nM, 75nM and 100nM dose for 48 hours. The expression of C17orf37, phospho-AKT (Ser 473), total AKT, phospho-ERK1/2 and total ERK1/2 were detected by western immunoblotting. Housekeeping gene GAPDH was used as loading control to normalize the total AKT and ERK1/2 protein levels, and then used to determine the ratio of the phosphorylated/total AKT and ERK, respectively. Numbers indicate the normalized expression ratio expressed as mean of three independent experiments. (c) Western immunoblotting showing phospho-AKT and total AKT (top panel), and EMSA showing NF-κB DNA binding activity (bottom panel) in polyclonal populations overexpressing either DU-GFP (DU-145 stably expressing GFP vector) or DU-GFP-C17 (DU-145 stably expressing GFP-C17orf37) in two independent pools #1 and #2. Western blot and EMSA images are representative of three independent experiments. (d) Proposed working model for C17orf37 signaling in prostate cancer cells.