## ABSTRACT

AN EXAMINATION OF THE FUNCTIONAL ROLE OF TMEFF2 IN PROSTATE CANCER AND THE TRANSLATIONAL REGULATORY MECHANISMS CONTROLLING ITS EXPRESSION

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September, 2012

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Prostate cancer is the most commonly diagnosed cancer in American men and accounts for approximately 11% of cancer-related deaths. Although promising treatment strategies have been developed and are currently being tested in clinical trials, standard practices in the treatment of prostate cancer remain inadequate. This is due in large part to the heterogeneous and multifocal nature of prostate tumors which severely complicates efforts to stratify patients and to identify therapeutic targets to effectively treat prostate cancer. A better understanding of the molecular mechanisms that drive prostate cancer initiation and progression to advanced stages is needed in order to design effective diagnostic and treatment strategies.

The transmembrane protein with EGF-like and two follistatin domains 2 (TMEFF2) is selectively expressed in the adult brain and the prostate and is overexpressed in prostate cancer,

suggesting a potential role in the establishment and/or progression of the disease. Previous reports on TMEFF2 function have revealed a complex biology with seemingly diverse cellular effects, and its role in prostate cancer has remained unclear. The studies presented here examine the biological function of TMEFF2 in prostate cancer in order to evaluate its potential as a molecular target for prostate cancer or as a diagnostic/prognostic biomarker. Data obtained using prostate cancer cell lines pointed to a role for TMEFF2 as a tumor suppressor as its overexpression resulted in a potent inhibition of anchorage-independent growth, reduced proliferation rates, the promotion of apoptosis, and a decrease in invasion. The tumor suppressor function of TMEFF2 was further demonstrated through the inhibition of subcutaneous tumor development in a TRAMP-C2 allograft model.

Evidence that TMEFF2 expression can be upregulated by androgen stimulation in a post-transcriptional fashion suggests a potential mechanism by which its expression can be modulated in prostate cancer. We therefore investigated the post-transcriptional regulatory pathway controlling the expression of TMEFF2 in prostate cancer cells and its connection with androgen signaling. The presence of conserved upstream open reading frames (uORFs) in the 5' leader region of its mRNA transcript prompted us to investigate the possibility that androgen signaling stimulates TMEFF2 translation through these regulatory sequences. Our results show that TMEFF2 translation is inhibited by its uORFs under normal conditions; however, the uORFs mediate a translational increase in TMEFF2 expression in response to androgen stimulation through the phosphorylation of the initiation factor eIF2α. This effect is dependent on a functional androgen receptor (AR). During the course of prostate tumorigenesis, the selective translational increase in uORF-containing transcripts by androgen signaling may represent a mechanism by which certain transcripts are selectively regulated to influence tumor progression.

As a tool to study role of TMEFF2 in prostate tumorigenesis *in vivo* and to evaluate its potential as a biomarker, we generated and initiated the characterization of a novel transgenic mouse model with TMEFF2 expression exclusively in the prostate epithelium. Ultimately this model will be used to study the function of TMEFF2 in the development/function of the prostate gland and in prostate cancer.

# An Examination of the Functional Role of TMEFF2 in Prostate Cancer and the Translational Regulatory Mechanisms Controlling its Expression

A Dissertation presented to:

The Faculty of the Department of Biochemistry and Molecular Biology

East Carolina University

In Partial Fulfillment

Of the Requirements of the Degree

Doctor of Philosophy in Biochemistry and Molecular Biology

By

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September, 2012

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## DEDICATION

I would like to dedicate this work to my wife for making this research possible and for her support, to my children for inspiring and motivating me, and to my parents for many years of academic guidance and support.

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## ABBREVIATIONS

| | |
|---|---|
| 4EBP1 | eIF4E binding protein 1 |
| AA | Amino acid |
| ADAM | A disintegrin and metalloproteinase |
| ADT | Androgen deprivation therapy |
| AR | Androgen receptor |
| ATF4 | Activating transcription factor 4 |
| BACT | Beta-actin |
| BIC | Bicalutamide |
| CDK | Cyclin-dependent kinase |
| cDNA | Complementary DNA |
| CLT | Clotrimazole |
| CMV | Cytomegalovirus |
| CRPC | Castration-resistant prostate cancer |
| CSS | Charcoal-stripped serum |
| DHT | Dihydrotestosterone |
| DMEM | Dulbecco’s modified eagle’s medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DOX | Doxycycline |
| eEF | Eukaryotic translation elongation factor |
| eIF | Eukaryotic translation initiation factor |

| | |
|---|---|
| EGF | Epidermal growth factor |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| GCN2 | General control nonderepressible kinase 2 |
| GLuc | Gaussia luciferase |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HIS | Histidine |
| HRI | Heme-regulated eIF2α kinase |
| HRP | Horseradish peroxidase |
| IL-1 | Interleukin-1 |
| KSF | Keratinocyte serum-free medium |
| MEF | Murine embryonic fibroblasts |
| mRNA | Messenger RNA |
| mTOR | Mammalian target of rapamycin |
| MTT | (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| OD | Optical density |
| PB | Probasin |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PERK | Pancreatic endoplasmic reticulum kinase |
| PIN | Prostatic intraepithelial neoplasia |

| | |
|---|---|
| PKR | Protein Kinase R |
| PSA | Prostate-specific antigen |
| PTEN | Phosphatase and tensin homolog |
| PVDF | Polyvinylidene difluoride |
| qRT-PCR | Quantitative reverse transcription-PCR |
| RIPA | Radioimmunoprecipitation assay buffer |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RPMI | Roswell park memorial institute medium |
| SARDH | Sarcosine dehydrogenase |
| SD | Standard deviation |
| SEAP | Secreted alkaline phosphatase |
| SHH | Sonic hedgehog |
| siRNA | Small interfering RNA |
| TA | Transactivator |
| TBS-T | Tris buffered saline- Tween-20 |
| TE | Tris-EDTA |
| TG | Thapsigargin |
| TGF-β | Transforming growth factor-β |
| TMEFF | Transmembrane protein with EGF-like and two follistatin domains |
| TNF-α | Tumor necrosis factor-α |
| TRAMP | Transgenic adenocarcinoma of the mouse prostate |
| uORF | Upstream open reading frame |

| | |
|---|---|
| UTR | Untranslated region |
| WT | Wild-type |

# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

## The hallmarks of cancer

Tumorigenesis is a multistep process driven by a succession of genomic alterations that confer a phenotype of uncontrolled growth and survival. The specific genetic or epigenetic alterations that drive tumor initiation and progression can vary greatly in distinct forms of cancer. However, a common set of phenotypic traits or "hallmarks" have been identified which are common to most cancer cells and that are critical for malignant progression. These traits include increased proliferation and invasion capabilities, resistance to cell death, evasion of growth suppressing signals, angiogenesis, and replicative immortality (Fig. 1)(44). Each of these traits plays a distinct role in the disease process; however, they are often controlled by overlapping signaling pathways and multiple traits can cooperate to promote tumor progression.

The acquisition of the hallmarks of cancer during tumor progression is driven by certain characteristics of both the cancer cells themselves and of the cells that make up the tumor microenvironment. Genomic instability is one of the drivers of almost all cancer cells (60) and serves a critical role in tumor progression by promoting the selection of traits that provide the cancer a growth or survival advantage. The onset of genomic instability in cancer cells can occur through a variety of mechanisms that are often dependent on the type of cancer (60) and generally involves the loss of function of DNA repair or checkpoint genes (e.g. p53). Additionally, inflammatory cells in the tumor microenvironment accelerate the accumulation of genetic aberrations though the release of reactive oxygen species. In solid tumors, many cell types that make up the tumor microenvironment, including inflammatory cells and other stromal cells, are capable of directly encouraging certain hallmark traits of cancer cells by supplying

growth/survival factors and extracellular matrix-modifying enzymes (44). A complex interaction or "crosstalk" between the stromal compartment and cancer cells has been demonstrated to play a pivotal role in the establishment or progression of many types of cancer, including breast, prostate, pancreatic, and lung cancers (20, 29, 98).

Stromal-epithelial crosstalk also plays an important role in organ/tissue development, regulating processes like differentiation, proliferation, survival, and branching of the epithelium. However, it is now evident that many of the molecular pathways involved in the stromal-epithelial crosstalk during development are critical drivers of tumorigenesis. For instance, the stromal secretion of matrix-degrading enzymes (e.g. MMP-9) that mediate branching morphogenesis during development serves a crucial role in the invasion of cancer cells into the stromal compartment (94). Additionally, two stromal-derived signaling molecules with established roles in prostatic development, Notch1 and Shh (sonic hedgehog) are now implicated in prostate cancer metastasis (7, 69). In fact, the re-activation of early vertebrate developmental pathways including Notch, Shh, BMP, and Wnt, is a common observation in the progression of most cancers (7). The activation of these pathways has been demonstrated to drive a variety of hallmark tumorigenic traits, and the mechanisms by which these developmental pathways instigate tumor progression largely depend on the type of cancer.

The identification of these hallmark traits of cancer cells and the features that promote them has provided an avenue to therapeutically target the specific pathways that drive tumor growth and survival. Several drugs have been developed that target one or more of these tumorigenic traits and have shown efficacy in clinical trials. However, as previously mentioned, these traits are often regulated by multiple signaling pathways which are capable of compensating for one another in the event that one is targeted therapeutically. Additionally,

tumor cells rarely depend on just one of these hallmark capabilities, and highly advanced cancers often display several if not all of these hallmarks. Inhibiting specific tumorigenic traits has therefore proven to be a promising but complex strategy to treat cancer and requires further work in identifying biomarkers and the molecular pathways that drive the tumorigenic phenotype.

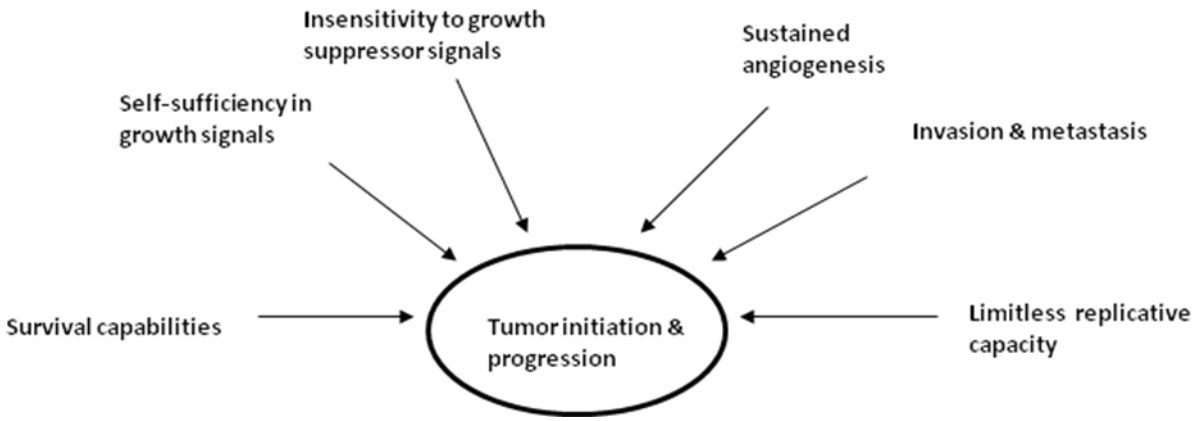


**Figure 1. The hallmark traits of cancer cells.** (Adapted from Hanahan and Weinberg, 2011 (44))

**Prostate cancer background and pathology**

Prostate cancer is the second most deadly cancer for men in the United States with approximately 30,000 deaths attributable to the disease every year (1). If detected early and confined to the prostate capsule, these cancers can be effectively treated by radical prostatectomy and radiation therapy. Although this treatment has a high success rate, patients with advanced prostate cancer do not have a treatment strategy that will improve their long-term survival. The current gold standard for treating advanced prostate cancer is androgen withdrawal, which entails surgical or chemical castration and the administration of androgen antagonists. Androgen withdrawal is effective in causing the cancer to regress; however, it inevitably returns with an aggressive phenotype that is characterized by deregulated androgen signaling (Castration-resistant prostate cancer, CRPC), and for which there is no cure (73, 92). It is therefore imperative to gain a better understanding of the mechanisms that lead to the establishment and progression of this disease in order to design effective treatments and identify novel biomarkers for its diagnosis and prognosis.

An insufficient understanding of the molecular pathways driving prostate tumorigenesis has hindered the molecular targeting and personalized therapies which have gained traction in many other cancers. This shortage of information on the molecular basis of prostate cancer initiation is likely due several reasons. First, the heterogeneous and multifocal nature of prostate cancer makes it difficult to obtain homogeneous samples for analysis, and to tease out the pathways that are critical for its survival. Additionally, most clinical samples are derived from highly advanced cancers that are characterized by a plethora of mutations and other genomic alterations, making it difficult to determine the genetic cause of progression to each stage. Furthermore, understanding the disease process is further complicated by the transition from

androgen-dependent to castration-resistant prostate cancer that occurs during disease progression, which is accompanied by or driven by alterations in several molecular pathways that allow for androgen-independent growth and survival.

Few "signature" genes have been implicated as hereditary factors involved in the pathogenesis of prostate cancer. The vast majority of prostate cancer cases are sporadic and do not involve hereditary factors (57). However, a few gene alterations have been identified in families with hereditary prostate cancer that are believed to drive disease initiation and/or progression (reviewed in (57)). For instance, Ewing et al. recently identified a mutation in HoxB13, a member of a key pathway in prostatic development that is associated with a significantly increased risk of hereditary prostate cancer and early disease onset (31). Overexpression of HoxB13 has been observed in CRPC was shown to provide a growth advantage to prostate cancer cells in the presence of low androgen levels (54).

Genetic and epigenetic studies have identified numerous sporadic chromosomal aberrations that are frequently detected in prostate cancer specimens and are associated with certain stages of progression. Although the specific roles of many of the altered genes in driving the disease are not fully understood, *in vitro* functional studies and transgenic mouse models have demonstrated that modulations in their expression can be driving forces of prostate tumorigenesis rather than secondary effects of tumor progression. The proceeding sections will briefly review the stages of prostate tumorigenesis and reference some of the most common genetic/chromosomal alterations that have been associated with certain stages of progression.

The human prostate is divided into the peripheral, central, and transition zones. The majority of prostate tumors develop in the peripheral and central zones in small glandular acini which empty secretions into the urethra. Each acini is comprised of secretory luminal cells

which, in healthy prostate tissue, are surrounded by a thin layer of basal and neuroendocrine cells (Figure 2; normal epithelium). The tissue surrounding the acini is a fibromuscular stroma that plays a critical role in prostate development, epithelial cell differentiation, and in prostate tumorigenesis (20). Prostate cancer first appears in the epithelium of the prostate as a hyperplastic lesion of cells, a state known as prostatic intraepithelial neoplasia (PIN). These lesions are a result of unchecked proliferation and/or a reduction in rates of apoptosis in a subset of the epithelial cells that line the glandular lumina; however, at this stage the cells are confined by the basal cell layer and the lesion does not extend into the stroma. The loss of chromosome region 8p12-21 is associated with this early stage of cancer and several studies point to a role for the *NKX3.1* homeobox tumor suppressor gene, located in this region, as a cause for driving PIN formation (11, 12). Transgenic mouse models that target *NKX3.1* for inactivation display PIN formation that resembles human PIN features, however it is not enough to lead to invasive cancer (11). In addition to *NKX3.1*, the *MYC* oncogene is frequently upregulated in both PIN and prostate cancer tissue and has been suggested to play a role in the initiation of PIN (42). The chromosomal region which contains the *MYC* gene, 8q24, is amplified in several cancer types including prostate cancer (51, 56), and its oncogenic function is well established *in vitro* and *in vivo*. In support of its role in prostate cancer development, Myc overexpression in a transgenic mouse model leads to the development of PIN and adeno-carcinoma (28, 50).

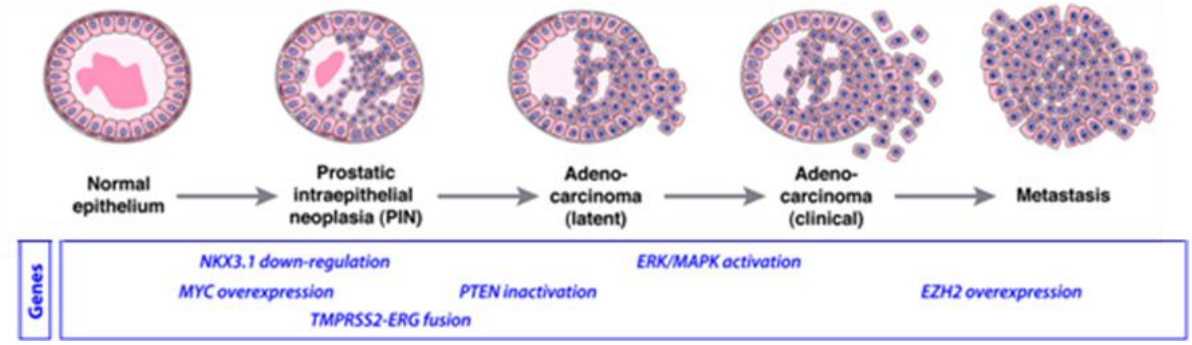


**Figure 2: General stages of prostate cancer progression.** Progression through each stage is associated with gene alterations (chromosomal aberrations, mutations, epigenetic modifications) that result in the loss of tumor suppressor genes or the activation of oncogenes. The deregulated genes/pathways have been associated with certain stages of disease progression based on expression profiling studies from prostate cancer specimens derived from different stages of progression.

*This figure was originally published in Genes & Development. Shen, M. and C Abate-Shen. Molecular genetics of prostate cancer: new prospects for old challenges**.** *Genes & Dev*. 2010; 24:1967-2000. © Cold Spring Harbor Laboratory Press (90)

Following the establishment of PIN lesions, further genetic aberrations lead to disease progression to prostate adeno-carcinoma, when neoplastic cells begin to obstruct the glandular lumina and invade the stromal compartment. These cells are no longer constrained by the basement membrane which in early stages of tumorigenesis acts as a mechanical barrier to prevent malignant cells from invading surrounding tissue. Cases of prostate adeno-carcinoma are described as latent if there are no symptoms for the patient or evidence of spreading to other tissues; and in many cases these will remain latent. However, they can also progress to clinical adeno-carcinoma as cells invade the tissue surrounding the prostate such as the seminal vesicles and/or bladder, often coinciding with the onset of symptoms. Some genetic aberrations have been identified in a large fraction of postate adeno-carcinoma specimens that are believed to be involved in the progression of the disease after the initiation of PIN. For example, region 10q-23.1 is frequently lost during prostate cancer progression at some point after cancer initiation, and this genetic alteration is rarely found in PIN lesions (88). This locus harbors the phosphatase and tensin homologue (PTEN) tumor suppressor. PTEN dephosphorylates and inactivates phosphatidylinositol (3,4,5) triphosphate (PIP-3) and reduces signaling through AKT/PKB, a pathway that strongly promotes cellular growth, metabolism, proliferation, and survival (15). Decreased PTEN expression or function is frequently observed in prostate cancer and several other cancers including glioblastoma, breast, and endometrial cancers (86). Its tumor suppressor function in prostate cancer has been demonstrated in a PTEN homozygous null mouse model which targeted both alleles of the gene for inactivation exclusively in the prostate. These mice develop prostate tumors which rapidly progress through the entire continuum of carcinogenesis,

from low grade PIN to metastasis (105). Another common chromosomal aberration that has been implicated in driving progression past PIN is the TMPRSS2-ERG gene fusion. This fusion results in the androgen-regulated activation of ERG, a member of the ETS transcription factor family. The functional consequences of ERG activation are not fully understood, however it is believed to inhibit epithelial cell differentiation programs and cooperate with other oncogenic events to promote disease progression after the initiation step (90).

The invasion of prostate cancer cells into tissues surrounding the prostate puts the cells into a position to metastasize and colonize distant sites. If cancer cells are able to detach from the original tumor and enter blood and lymph vessels, they may colonize distant site(s) and form metastatic foci, representing the terminal stage of cancer progression. In human prostate cancer the primary site of metastasis is the bone, but it can also spread to the lung and lymph nodes. The progression of localized prostate cancer to metastasis is a multi-step process that requires dramatic changes in the phenotype of the cancer cells, as well as the cross-talk between the cells and the surrounding environment, to be able to survive and colonize foreign tissue. The activation of oncogenes that drive a metastatic phenotype and/or the loss of tumor suppressor genes that protect against metastasis provide the cells the ability to spread to other tissues and survive to form secondary tumors. Although an abundance of genes have been described as having roles in the process of metastasis, some of the most common gene alterations detected in metastatic prostate cancer that are thought to play a causative role in late-stage progression are mentioned below. Late-stage prostate cancers commonly show the loss of a region of chromosome 13q, which contains the Retinoblastoma (*RB*) tumor suppressor gene (61). The protein encoded by this gene is well known to have a negative influence on tumorigenesis by antagonizing transcription factors of the E2F family, and its loss leads to increased expression of

cell cycle genes and increased AR activity (89). Additionally, mutations in the tumor suppressor p53 and allelic loss at the p53 locus, 17p, have been detected primarily in late stage prostate cancers, although the frequency of these aberrations has varied greatly in the literature (18, 88). The p53 tumor suppressor function has been implicated in the progression to CRPC and metastasis (76); and although its effects are pleiotropic, it primarily antagonizes cell proliferation and survival in tumor cells (40). In support of a role for Rb and p53 loss of function in prostate cancer, a conditional Rb and p53 compound knockout mouse model displays invasive and metastatic carcinoma at a rapid pace, indicating that the loss of both genes may have a synergistic effect that leads to a highly malignant phenotype in some cases (110). The lysine methyltransferase *EZH2* gene may also have a role in late-stage disease progression as it is overexpressed in a significant fraction of castration-resistant, metastatic prostate cancers but not in early-stage tumors (87, 102). Furthermore, gene silencing is a common feature of advance prostate cancer, and EZH2 has been proposed to be involved in this process. I*n vivo* studies support a role for EZH2 in promoting metastasis (97), however its mechanism of action as an oncogene has remained unknown for many years. Recently, Min et al demonstrated that EZH2 epigenetically silences key metastasis suppressor genes, leading to simultaneous Ras and NF-κB activation and metastasis (70). These genes are strong candidates that may have an influential role in driving prostate cancer progression to later stages, and targeting their expression/activation may significantly impair the metastatic progression of prostate cancer.

Altogether, these observations support a need for multiple "hits" or gene alterations (mutations, deletions, epigenetic modifications) to drive prostate cancer progression. The aforementioned genetic aberrations are associated with certain stages of the disease based primarily on cytogenetic or mutational analyses of prostate cancer specimens and on functional

studies of the affected genes in prostate tumorigenesis. However, these genes represent only a small fraction of the genes that are altered in prostate cancer and that have been implicated in driving the disease process. Newly identified genes with potential roles in prostate cancer are continuing to emerge, along with novel pathways that deregulate the expression of those genes through a variety of mechanisms.

**The role of androgens and the AR in prostate cancer establishment and progression**

Although deciphering the molecular pathways that drive prostate carcinogenesis has proven to be complicated, it is indisputably clear that androgens and the androgen AR play a major role in nearly all aspects of prostate biology, from prostate development to the establishment and progression of prostate cancer. Androgens and the AR have been primary targets for the treatment of prostate cancer since the 1940's, and although the strategies to target androgen signaling have changed, it remains a major focus of prostate cancer research and treatment strategies.

The primary androgen, testosterone, is synthesized almost exclusively in the testes, although a small amount can be produced in the adrenal glands. Once testosterone enters the cells of the prostate it can be converted to dihydrotestosterone (DHT), a more potent form of the androgen. The biological effects of androgens on prostate tissue are principally mediated though the AR, a member of the nuclear receptor transcription factor family that is activated upon androgen binding. Basal levels of testosterone and DHT are required for cell growth and survival in normal prostate tissue. AR signaling in both the stromal and epithelial compartments promotes its proliferative homeostasis, epithelial cell differentiation, and secretory and metabolic processes (8). The influence of androgens on these cellular processes is principally mediated

through AR transcriptional activation of androgen-regulated target genes, and its actions can be heavily influenced by a number or coregulators as well as crosstalk with other pathways that can modulate its activity (83).

In addition to its classical role as a transcriptional activator, new roles are surfacing for androgens and the AR that are independent of its transcriptional effects or "non-genomic". Non-genomic androgen signaling can influence a wide variety of cellular processes, mostly mediated through rapid changes in [$Ca^{2+}$] levels or second messenger signaling through several pathways including MAPK, PKA, and PKC (33). The effects of this type of androgen signaling are relatively rapid and can be dependent on or independent of the AR. The physiological effects of non-genomic androgen actions are currently not well understood and it is not known if they are linked with AR transcriptional effects or if they function independently.

The influence of the AR in prostate cancer is critical as prostate cancer cells are dependent on androgen signaling to grow and survive. Therefore, blocking androgen action is currently the gold standard treatment for tumors that cannot be effectively removed by prostatectomy and radiation. Androgen Deprivation Therapy (ADT) is defined by a combination of approaches to eliminate the actions of androgens and the AR, usually through castration and the administration of AR antagonists. Through ADT, androgen regulated genes are suppressed from the molecular network of prostate cancer cells, leading to massive apoptosis and prostate cancer regression. However, despite continued attempts to block it, AR signaling returns along with the cancer in an aggressive and lethal form of CRPC. Several mechanisms have been proposed to account for the return of AR signaling, including *AR* gene amplification, increased AR ligand sensitivity, ligand-independent AR activation, and increased androgen synthesis (Fig.

3). Studies in castration-resistant mouse models have demonstrated that these cancers remain dependent on AR signaling throughout prostate tumorigenesis (8).

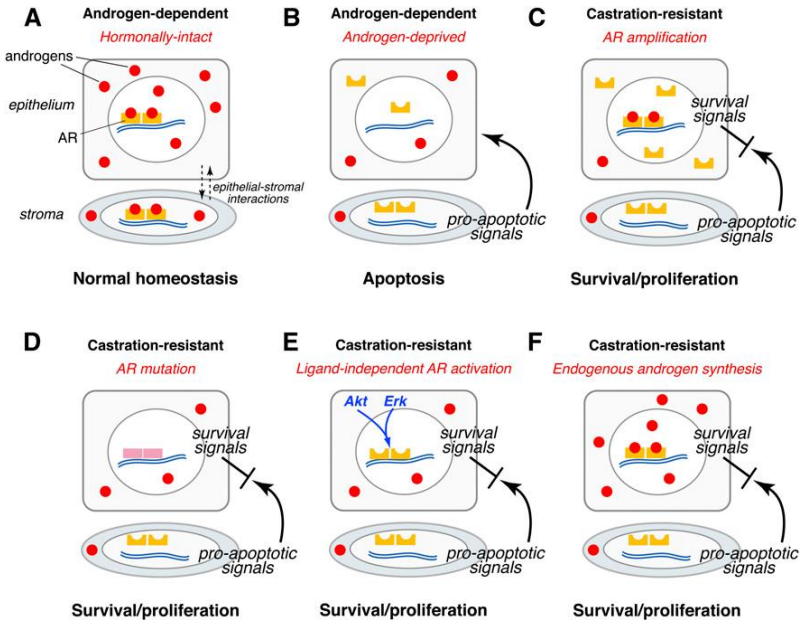


**Figure 3. Role of androgen signaling in prostate cancer.** **(A)** Androgens and the AR are critical for the function and homeostasis of the prostate gland. **(B)** Removal of androgens from androgen-dependent prostate cancers triggers massive apoptosis and disease regression. *(C-F)* Mechanisms proposed to contribute to the development of castration-resistant prostate cancer include **(C)** amplification of the *AR* gene, **(D)** AR mutations that increase its activity, **(E)** activation of oncogenic pathways that lead to the induction of androgen-regulated gene expression, and **(F)** increased androgen synthesis.

*This figure was originally published in Genes & Development. Shen, M. and C Abate-Shen. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes & Dev*. 2010; 24:1967-2000. © Cold Spring Harbor Laboratory Press (90)

The role that AR signaling plays in promoting the establishment and/or progression of prostate cancer has been extensively studied; however, the expansive influence of the AR on a wide variety of cellular processes makes it difficult to tease out its specific roles in driving the disease process. As mentioned, AR signaling activity is critical for the growth and survival of prostate cancer cells and has therefore conventionally been ascribed a tumorigenic role in the disease. The identification of an abundance of androgen-regulated genes that promote proliferation and survival support its pro-tumorigenic role, including cyclin-dependent kinases (CDK's), anti-apoptotic genes, and genes involved in epithelial cell differentiation and secretory function (8, 68). However, this conventional concept of the AR as tumorigenic was challenged recently by the discovery that AR signaling can suppress prostate epithelial cell growth and induce differentiation (78, 107). It has been demonstrated that the AR acquires oncogenic traits in advanced prostate cancer cells that promote the survival and growth of the cancer cells (101). These results made clear that the role of the AR is complex and much remains unknown regarding its function in prostate cancer. Through both the classical transcriptional network activated by the AR and through rapid, post-transcriptional or "non-genomic" effects, androgen signaling capabilities are vast and new roles for the AR continue to emerge.

**The *TMEFF2* gene and its potential role in prostate carcinogenesis**

TMEFF2 is an evolutionarily conserved, type I transmembrane protein expressed exclusively in the adult brain and prostate tissue under normal conditions, and is frequently upregulated in prostate cancer specimens relative to benign tissue (39, 99). It has been proposed

that TMEFF2 may play an influential part in the establishment and/or progression of prostate cancer; however the role of TMEFF2 in the disease process has remained unclear.

TMEFF2 is a member of the relatively new TMEFF (transmembrane protein with EGF-like and two follistatin domains) protein family. TMEFF2 and the only other member of the TMEFF family, TMEFF1, contain similar structural features and may therefore serve similar biological functions. Both TMEFF proteins contain an EGF-like domain similar to those previously described in the EGF-like protein family (100). Another common feature of these proteins is the presence of two follistatin modules toward their N-termini. Follistatin domains in generally are known to bind and generally antagonize members of the TGF-β family such as activin and inhibin (106). Additionally, the C-terminal domains of these proteins contain a putative G-protein-activating motif, suggesting a potential role in second messenger signaling. The extracellular portion of the TMEFF2 protein can be cleaved by the metalloproteinases ADAM 10/17, releasing from the membrane a soluble form of the protein that contains the EGF-like domain and two follistatin modules (5)(Fig. 4). Due to the high degree of sequence similarity between the TMEFF proteins, it is likely that TMEFF1 can also be cleaved to release an ectodomain; however its cleavage has not been confirmed experimentally. These structural features reflect the potential of these the TMEFF proteins to influence a variety of cellular process, conceivably acting as a membrane-bound receptor, co-receptor, or a ligand precursor.

Investigations into the biological function of TMEFF1 have been limited; however, insights into its function and mechanim(s) of action may provide clues into the role of TMEFF2 in prostate tumorigenesis. The brain appears to be the primary site of function for TMEFF1 as it is predominantly expressed in the brain and to a lesser extent in the heart, placenta, and skeletal muscle (36). A role for TMEFF1 in cancer was proposed when it was shown to be

downregulated in 96% of 54 brain tumor samples relative to normal brain tissue (36). Furthermore, overexpressing TMEFF1 in the glioblastoma cell line U118 had a growth inhibitory effect on the cells (36), consistent with a role as a tumor suppressor. TMEFF1 expression has also been detected in early mouse embryos and it may play a role in vertebrate development (26). Supporting this possibility, studies in Xenopus embryos demonstrated that TMEFF1 is able to block nodal signaling, and that this inhibition required both the follistatins motifs or the EGF-like domain (13). Nodal has a crucial role in embryonic development, but like many developmental pathways, has also been shown to be re-activated in cancer and to promote prostate cancer cell growth (58). Modulating TGF-β/nodal action may therefore represent a mechanism by which TMEFF1 functions as a tumor suppressor.

Previous studies into the function of TMEFF2 in tumorigenesis have demonstrated a complex biology for the protein and its role in prostate cancer has remained unclear. Functional studies to date from our lab and others are suggestive of a potential dual role in tumorigenesis. Overexpression of full-length TMEFF2 inhibited cell growth and proliferation in PC3 and DU145 prostate cancer cells (35), and inhibited colon cancer cell growth and survival (27). Additionally, Lin et al. recently reported that TMEFF2 can bind to Platelet Derived Growth Factor (PDGF) and inhibit PDGF-stimulated fibroblast proliferation (66). Its ability to modulate PDGF signaling could be an important clue to the function of TMEFF2 in prostate cancer as aberrant PDGF signaling has been associated with prostate tumorigenesis (55), stimulating cell growth and promoting metastasis through the activation of the AKT/PKB pathway (24). Contrary to results with the full-length protein, overexpression of the TMEFF2 ectodomain has been shown to promote growth in HEK293T cells through ERK activation (5) as well as survival effects in some neurons (47). The pro-tumorigenic effects of the ectodomain region are

consistent with the observation that its overexpression in MKN28 gastric cancer cells stimulated ErbB4 phosphorylation, a member of the EGF receptor protein family (99). These results indicate that TMEFF2 is capable exerting opposing functions in cancer, both tumor suppressive and oncogenic, and its role in tumorigenesis may therefore be dependent on the tissue type, stage of the disease, or other environmental factors that can augment its function.

Like TMEFF1, TMEFF2 may play a role in development as its expression has been detected in the middle to late stages of embryogenesis (99). However, the functional role of TMEFF2 in development is currently not known. The presence of the follistatin domains presents the possibility that TMEFF2 may modulate the TGF-β/nodal signaling pathway during development as was demonstrated for TMEFF1; however, no studies have confirmed this function for TMEFF2. In light of the re-activation of developmental signaling pathways in the progression of prostate tumorigenesis (7, 15, 82), deciphering its role in development may provide important insights into its function in prostate cancer.


**Figure 4: Structural domains of the TMEFF2 protein.** SP=signal peptide, FS=follistatin, EGF=epidermal growth factor-like domain, TM=transmembrane region. ▼ represents the putative metalloproteinase cleavage site, which releases the TMEFF2 ectodomain. A potential G protein-activating motif is located near its C-terminus facing the cytoplasm. (Adapted from Horie et al., 2000 (47))

As previously mentioned, TMEFF2 is frequently overexpressed in prostate cancer tissue relative to benign prostate tissue. However, the mechanism(s) that lead to the upregulation of TMEFF2 in prostate cancer are not well understood. Although multiple studies have demonstrated that TMEFF2 is transcriptionally regulated by androgens (35, 71), the addition of testosterone to an androgen-deprived prostate cancer xenograft model stimulated a post-transcriptional increase in TMEFF2 expression (71). The post-transcriptional regulatory connection between androgen signaling and TMEFF2 expression was not investigated further, and it is not known if the increase in TMEFF2 was the result of increased translation, the stability of the TMEFF2 protein or mRNA, or decreased proteolysis. It is possible that TMEFF2 is regulated by androgens at both the transcriptional and post-transcriptional levels, and that the regulatory pathways controlling its expression may be altered during tumor development or progression. An evaluation of the post-transcriptional regulatory mechanism(s) controlling TMEFF2 expression could lead to a broadened understanding of the role of androgen signaling in prostate tumorigenesis and potentially provide novel targets to block androgen actions.

**Post-transcriptional control in prostate cancer**

The deregulated expression of oncogenes and tumor suppressors at the post-transcriptional level has been well-documented in several cancers and can occur through a variety of mechanisms including changes in mRNA stability, alterations in RNA-binding protein activity/specificity, and alterations in translation initiation efficiency (6). In recent years, the expression of an abundance of genes with suspected roles in the pathogenesis of prostate cancer have been shown to be regulated by the AR through post-transcriptional mechanisms (77, 108),

indicating that the critical influence of AR signaling on prostate tumorigenesis goes beyond the transcriptional stimulation of AR target gene expression.

The overexpression or increased activity of several translation initiation factors in prostate cancer suggests that translational control may play a pivotal role in the establishment and/or progression of the disease. As the rate-limiting step in for the translation of most transcripts, components of the translation initiation machinery are most often deregulated in tumorigenesis such as the eIF4F cap-binding complex, the 43S pre-initiation complex, and eIF3proteins (91, 95). For instance, the translation initiation factors eIF3h and eIF4E are frequently upregulated in advanced prostate cancer tissue, and the expression of each of these proteins is associated with an aggressive disease prognosis (21). The increased activation of eIF4E has also been demonstrated in prostate cancer through either its direct phosphorylation or through the phosphorylation and inhibition of 4EBP1 (an eIF4E inhibitory binding protein) (91). In fact, the translational regulator mTOR was recently shown to have a critical role driving prostate cancer cell invasion and metastasis by modulating 4EBP1 phosphorylation, increasing eIF4E availability and promoting the translation of a subset of pro-invasion genes (48). It has also been reported that the activity of mTOR is modulated by DHT treatment in LNCaP prostate cancer cells (108), suggesting that androgen signaling can promote prostate tumor progression by modulating the activity of a major translational regulator. A potential role for the translation initiation factor eIF2 in prostate cancer has also been proposed in light of the observation that the tumor suppressor function of PTEN requires the phosphorylation of eIF2α by PKR (74). Like eIF4E, eIF2 is one of the main regulatory targets of translation initiation, and therefore may play an important role in cancer establishment or progression. It is primarily regulated by the phosphorylation of its α subunit which leads to a global decrease in translation; however, some

transcripts can be selectively upregulated under these conditions (e.g. genes involved in cell survival under conditions of severe stress) (72). At this time little is known concerning the role of eIF2α in tumorigenesis. Studies in mouse models and various cancer cell lines have produced conflicting results regarding the role of eIF2α phosphorylation in cancer (21), in some cases it has demonstrated tumor suppressive effects and in other models it has been shown to promote tumorigenesis. These highly contradictory results have may reflect a function for eIF2α phosphorylation that is dependent on the tissue type or on the stage of tumorigenesis.

## CHAPTER 2: EXPERIMENTAL PROCEDURES

**Cell culture-** All cell lines were obtained from the American Type Culture Collection unless stated otherwise. LNCaP and 22RV1 cells were maintained in RPMI 1640 (Gibco) supplemented with either 10% FBS (Gemini Bio-products) or 10% charcoal-stripped serum (Atlantic Biologicals) for hormone starvation. PC3 cells were obtained from Dr. D. Terrian (Department of Anatomy and Cell Biology, East Carolina University) and were also maintained in RPMI 1640. Dihydrotestosterone, bicalutamide, and actinomycin D were purchased from Sigma-Aldrich. RWPE1 cells were maintained in Keratinocyte Serum Free medium (Gibco) supplemented with the provided bovine pituitary extract and recombinant epidermal growth factor following the manufacturer's recommendations. HEK293T cells were maintained in DMEM (Gibco) supplemented with 10% FBS. TRAMP-C2 cells were a gift from Dr. L. Yang (Department of Internal Medicine, East Carolina University) and maintained as previously described (34). A/A and wt/wt MEF cells were provided by Dr. R. Kaufman (Burnham Institute, La Jolla, CA) and were grown in DMEM with 10% FBS. All cell lines were incubated at 37ºC with 5% $CO_2$.

**Generation of TMEFF2-overexpressing cell lines- 1)** HEK293T cells stably overexpressing TMEFF2 were developed by transfecting an expression construct containing the full-length human TMEFF2 cDNA inserted into the pSecTag2A vector (Invitrogen) followed by antibiotic selection. The construct was transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. **2)** Inducible cell lines were developed using the Tet-on Tetracyclin Inducible Expression System (Clontech) following the

manufacturer's instructions. Briefly, an initial transduction introduces a constitutively expressed transactivator protein (rtTA) that is activated upon binding doxycyline. Following antibiotic selection and expansion of resistant clones, a second transduction introduces a construct containing full-length TMEFF2 cDNA downstream from a promoter that is specifically recognized by the transactivator from the previous transduction. TMEFF2 expression was induced by incubation of the cells with 250 ng/ml doxycycline for 48 hrs.

**Proliferation assay-** Cells were seeded at 3,000-5,000 cell/well in 96-well plates. After incubation for the indicated times, MTT reagent (Sigma) was added at a concentration of 0.5 mg/ml in phenol red-free RPMI containing 1% FBS. Following a 3.5 hr incubation at 37ºC, 200 µl DMSO (Sigma) was added to each well, incubated for 15 min with rocking, and the OD measured at 562 nm.

**Apoptosis assay-** To induce apoptosis, 30,000 cells/well were plated into 6-well plates and treated with 2 or 3 µM staurosporine (Sigma) for 24 hrs. Cells were harvested and washed with 1X PBS, and resuspended in 100 µl binding buffer [100 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM $CaCl_2$]. To stain the cells, 1 µl Annexin V-FITC reagent (BioVision) and 1 µl of a 5 µg/ml propidium iodide solution (Invitrogen) were added to the cell suspension and incubated for 10 min at 4˚C. The cell suspensions were spun down and resuspended in 400 µl binding buffer in 12 x 75 mm polystyrene tubes (Becton Dickinson). Cells were then analyzed by flow cytometry (FACScan, Becton Dickinson). Technical assistance was provided by Mitch Harris.

**Anchorage-independent growth**- 2,500-3,500 cells were suspended in 0.35% agarose in DMEM containing 5% FBS. This suspension was overlaid onto a solidified layer of 0.4% agarose in a 60 mm plate. Fresh DMEM was maintained on top plates during a 14-21 day incubation, at which time the cells were stained with 0.005% crystal violet, gently washed with 1X PBS, photographed, and counted.

**Invasion assays-** Cell invasion was assayed using Boyden chambers containing a layer of matrigel (BD Biocoat; Becton Dickinson) and using NIH 3T3 conditioned medium as a chemoattractant. To analyze sarcosine induced invasion, TMEFF2 inducible and control RWPE1 cells were grown in the presence of 50 μM sarcosine or alanine as a control. 48 hrs later, doxycycline was added to induce TMEFF2 expression and the cells were incubated for an additional 48 hrs. In total, the cells were grown 96 hrs in the presence of sarcosine or alanine and 48 hrs in the presence of doxycycline (250 ng/ml) to induce TMEFF2 expression before they were added to the Boyden chambers. Following a 36-48 hr incubation to allow for invasion, the number of invading cells at the bottom side of the matrigel chamber and the number of non-invading cells at the top of the matrigel were determined using a MTT assay and the percentage of the invading cells calculated from the total. Alternatively, following 48-hr incubation, cells adhering to the bottom of the membrane were fixed with 70% ethanol and stained with 0.1% crystal violet, photographed and counted in several random fields of view.

**Subcutaneous TRAMP-C2 Injections**- TRAMP-C2 cells were incubated for 48 hrs in medium containing 250 ng/ml doxycycline to induce TMEFF2 expression prior to the injections. Following this incubation period, cells were harvested and washed once with cold PBS. Mice

were anaesthetized by brief inhalation of isoflurane and hind flanks were shaven using clippers. 2.5 x $10^6$ TMEFF2 inducible and control TRAMP-C2 cells suspended in 0.2 ml PBS were then subcutaneously injected into the flank of 7-8 wk. old C57BL/6 male mice using a 26 gauge needle. Mice were continuously fed chow containing 200 mg/kg doxycycline (BioServe) to induce TMEFF2 expression during the incubation period. Mice were sacrificed and tumors were then excised. Technical assistance was provided by Greg Tipton.

**TMEFF2 Fusion Constructs and Reporter Asssays-** PCR mutagenesis was used to mutate the start codons of the uORFs in the TMEFF2 5' UTR from AUG to GUG (see Table 1). The wild-type and mutant 5' UTRs were inserted upstream of the Gaussia luciferase gene in the pCMV-GLuc vector (New England Biolabs). For uORF analyses, cells were grown to 70-90% confluency in 6-well plates and transfected with 1.5 µg of each construct per well and the same amount of the pSeap-Control Vector II (BD Biosciences) using Lipofectamine 2000 (Invitrogen) and following the manufacturer's protocol. Cells and supernatants were collected 24 hrs post-transfection and Gaussia luciferase levels were determined from the supernatants using the BioLux kit (New England Biolabs). Seap (secreted alkaline phosphatase) levels were measured from the supernatants using the Great Escape Seap Chemiluminescence Kit 2.0 (Clontech Laboratories) for normalization. Luminescence was measured for the Luciferase and Seap assays using a $20/20^n$ luminometer (Turner Biosystems). mRNA was extracted from the cells to measure Gaussia luciferase transcript levels for normalization as described under the qRT-PCR procedures.

For DHT-stimulated reporter assays, cells were hormone-starved for 48 hrs in phenol red-free medium containing 10% charcoal-stripped serum (CSS) prior to stimulation with DHT.

Twenty-four hrs after hormone removal the cells were transfected using Fugene HD transfection reagent (Promega) following the manufacturer's recommendations. Briefly, 10 µg of each construct and 10 µg of pSeap-Control Vector II were diluted in serum-free RPMI along with 30 µl Fugene HD reagent for a total volume of 500 µl. 100 µl of this transfection mix was added per well of a 6-well plate. The following day, DHT or ethanol vehicle were added to fresh CSS-RPMI and the cells were incubated for another 48 hrs prior to harvesting cells and supernatants.

**qRT-PCR** - RNA was isolated from the cells using the RNAqueous kit (Ambion) following the supplied protocol. cDNA was then synthesized using the iScript kit (BioRad Laboratories) using 0.25 µg of RNA as the template. Message levels were measured using IQ SYBR Green Supermix and the IQ5 Real-Time PCR Detection System (BioRad Laboratories). mRNA levels were normalized to β-actin using the IQ5 Optical System Software (BioRad Laboratories).

**Androgen Receptor knockdown**- AR expression was reduced in 22RV1 cells using the ON-TARGET plus SMART pool for human AR (Thermo Scientific). Five µl of silencing RNAs and 7.5 µl of DharmaFECT Transfection Reagent (Thermo Scientific) were each separately diluted in 300 µl serum-free, phenol red-free RPMI. After 5 min incubation, the solutions were combined and incubated for another 20 min at room temperature, then added to an 85% confluent cell monolayer in a T-25 flask containing 2.4 ml of complete, phenol red-free RPMI. siRNA-treated cells were then incubated for 48 hrs prior to treatments with DHT or vehicle.

**Immunoblotting-** Cell lysates were prepared with RadioImmunoPrecipitation Assay (RIPA) buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] supplemented with 0.1 mM β-glycerophosphate and 0.5 mM sodium orthovanadate and protease inhibitor cocktail (Sigma). Twenty µg of lysates were separated on Mini-protean TGX gels (BioRad) and transferred to PVDF membranes. These were then blocked for 30 min in 5% non-fat dry milk diluted in 1X Tris-buffered saline + 0.1% Tween-20 (TBS-T) and incubated with the primary antibody overnight at 4˚C. Incubations with a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) were for 1 hr at room temperature. Detection was carried out using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 min. In some cases, blots were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Scientific) following the manufacturer's recommendations. Antibodies against TMEFF2, PSA, and eIF2α-P (Ser51) were from Abcam, eIF2α and CREB2/ATF4 antibodies were purchased from Santa Cruz Biotechnology, and the AR antibody was from Cell Signaling Technology.

**Polysome Analysis-** Cell monolayers were scraped with lysis buffer [100 mmol/L KCl, 10 mmol/L HEPES (pH 7.4), 0.5% NP40, 5 mmol/L $MgCl_2$, 100 µg/ml cycloheximide], and incubated on ice for 10 mins, followed by a 5 min spin at 10,000 rpm at 4°C to pellet cellular debris. Equal protein concentrations of cytoplasmic extracts (1.8 mg) were then overlaid onto a linear sucrose gradient [15–45% (w/v) 10 mmol/L HEPES (pH 7.4), 100 mmol/L KCl, 5 mmol/L $MgCl_2$] and centrifuged at 35,000 rpm for 2 hrs at 4°C in an SW41-Ti rotor without the brake. Using an ISCO UA-6 fractionator, fractions were collected with continuous UV monitoring at 254 nm. Sucrose gradient fractionations were performed by Dr. V. Chappell (Department of

Anatomy and Cell Biology, East Carolina University). RNA was isolated from fractions using Trizol reagent (Ambion). Twenty-five µg of RNA was then used for cDNA synthesis using the iScript kit (BioRad Laboratories). A sample of 0.1 µg of each cDNA preparation was used to amplify *TMEFF2*, *ATF4*, and *β-actin* by PCR using the Platinum Taq HiFi DNA Polymerase system (Invitrogen). PCR products were then visualized on a 1% agarose gel and band intensity was analyzed using the public domain NIH Image J program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

**PB-TMEFF2 mouse generation and identification** – The PB-TMEFF2 transgenic expression construct (see Ch. 5 for plasmid construction) was amplified with the PureLink HiPure Plamid Maxiprep Kit (Invitrogen) following the manufacturers recommendations and resuspended in TE buffer supplied with the kit. The DNA was then transferred to the University of North Carolina at Chapel Hill Animal Models Core Facility where pronuclear injections were performed as previously described (49). Tail snips of the mice produced were provided at weaning and the presence of the transgenic DNA was detected by PCR using the Terra PCR Direct Kit (Clontech Laboratories) following the supplied protocol and primers specific for the transgene (See figure 14(A) and Table 1).

**Statistical Analysis-** Data are expressed as mean ±SD. Differences were analyzed using paired, two-tailed t-tests. P values $\leq 0.05$ (*) or $\leq 0.01$ (**) were considered significant.

**Table 1. Primers used for experimental procedures.**

| | | |
|---|---|---|
| **Primers used for plasmid construction or qRT-PCR detection** | | |
| | **Construct name** | **Primer sequence** ***underlined sequences represent the uORF start codons** |
| **1** | pTM1234-GLuc (wt) | 5'-TAGGATCCCTCCACCCTGCCTCCTCG<br>5'-TAACTAGTTCGTGCAACTCTGCAGCAG |
| **2** | pTMX234-GLuc | 5'-GCTGCTGCCACAAGGAGGGAGC<br>5'-GCTCCCTCCTTGTGGCAGCAGC |
| **3** | pTM1X34-GLuc | 5'-GAGTTTCAGCAACACCCAGGGACT<br>5'-AGTCCCTGGGTGTTGCTGAAACTC |
| **4** | pTM12X4-GLuc | 5'-CCCGCGCACGATGTCGAGAG<br>5'-CTCTCGAGATCGTGCGCGGG |
| **5** | pTM123X-GLuc | 5'-GCTACTGAGCACCCCGCGGAC<br>5'-GTCCGCGGGGTGCTCAGTAGC |
| **6** | *TMEFF2* | 5'-TCTTGCAGGTGTGATGCTGG<br>5'-GCTCCCTTTAGATTAACCTCG |
| **7** | *β-actin* | 5'-GGACTTCGAGCAAGAGATGG<br>5'-AGCACTGTGTTGGCGTACAG |
| **8** | *ATF4* | 5'-TCAAACCTCATGGGTTCTCC<br>5'-GTGTCATCCAACGTGGTCAG |
| **9** | Gaussia luciferase | 5'-GGAGGTGCTCAAAGAGATGG<br>5'-TTGAACCCAGGAATCTCAGG |
| **Primers used for genotyping transgenic mice by PCR** | | |
| **10** | PB-TMEFF2 | 5'-CAGGGCACTACAGTTCGACA<br>5'-CAAATGTGGTATGGCTGATTATG |

# CHAPTER 3

## TMEFF2 FUNCTIONS AS A TUMOR SUPPRESSOR IN PROSTATE CANCER CELLS

***Adapted from Xiaofei Chen, Ryan Overcash, Thomas Green, Donald Hoffman , Adam Asch and Maria J. Ruiz-Echevarría. (2011) J Biol Chem 286(18), 16091–16100 (**(17)**)**

## Introduction

TMEFF2 is an evolutionarily conserved type I transmembrane protein expressed in the embryo (99) and selectively in the adult brain and prostate (35, 39). It is expressed in several regions of the brain, and overexpression of the TMEFF2 ectodomain has been demonstrated to promote survival in primary neurons (47). A critical role for this protein in tumorigenesis is suggested by the fact that it is upregulated in a significant fraction of primary and metastatic prostate tumors (35, 39, 71). It has been suggested that TMEFF2 may function as a tumor suppressor because ectopic expression of full-length TMEFF2 demonstrates anti-growth effects *in vitro* and suppresses tumor growth in nude mouse xenografts (27, 35). Consistent with a tumor suppressor function, TMEFF2 has been shown to be hypermethylated in a number of cancer types (41, 62) and the TMEFF2 promoter is repressed by c-Myc (37).

The present study expands our understanding of the role of TMEFF2 in tumorigenesis. For this purpose, we overexpressed TMEFF2 in HEK293T cells and RWPE1 prostate epithelial cells and assessed the effects on specific traits that are hallmarks of a cancerous phenotype. These traits include elevated proliferation rates, invasion, survival, and anchorage-independent growth capabilities. We show that the ectopic expression of full-length TMEFF2 results in monolayer and anchorage-independent growth inhibition in HEK293T cells. Additionally, although TMEFF2 overexpression alone did not induce apoptosis, it resulted in a marked increase in apoptotic cells after the chemical induction of apoptosis with staurosporine, demonstrating that it can sensitize cells to undergo the apoptotic program. To evaluate the effects of TMEFF2 overexpression on prostate cell invasion we incubated RWPE1 cells with the

metabolite sarcosine, a proposed prostate cancer biomarker that promotes cell invasion (93), and demonstrate that TMEFF2 overexpression blocks RWPE1 cell invasion. No significant impairment of proliferation was observed as a result of TMEFF2 overexpression in these cells. These results collectively indicate that TMEFF2 functions as a tumor suppressor.

In order to test the tumor suppressor activity of TMEFF2 *in vivo*, we generated TRAMP-C2 murine prostate cancer cells that are inducible for TMEFF2 expression and subcutaneously injected the cells into syngeneic male mice. The induction of TMEFF2 expression significantly inhibited the ability of the TRAMP-C2 cells to form tumors relative to cells that did not express TMEFF2.

## Results

### Increased expression of TMEFF2 inhibits cell growth in HEK293T cells

To investigate the function of TMEFF2 in tumorigenesis, we first determined whether ectopic expression of this protein could affect cellular proliferation. HEK293T cells stably expressing untagged (TMEFF2-wt) or c-Myc-His-tagged (TMEFF2-Myc-His) TMEFF2 proteins, along with control cells transfected with empty vector or untransfected cells, were generated for this purpose. Overexpression of either untagged (Fig. 5*A*) or C-terminal c-Myc-His-tagged TMEFF2 (not shown) in HEK293T cells decreased cell numbers by 20–30% with respect to the untransfected cells or the cells transfected with the empty vector. The presence of the C-terminal c-Myc-His tag did not change the effect of TMEFF2 on cell growth. Therefore, subsequent experiments were done using the c-Myc-His-tagged form of the protein.

To further characterize the nature of TMEFF2 overexpression on cell tumorigenicity, FACS analysis was used to investigate the effect of TMEFF2 on apoptosis. HEK293T cells stably transfected with TMEFF2-Myc-His or with the empty vector as a control were induced to undergo apoptosis with staurosporine, a protein kinase inhibitor that triggers both caspase-dependent and caspase-independent apoptotic pathways (10). Overexpression of TMEFF2 on its own had no effect on the number of apoptotic cells in HEK293T cells. However, it increased the sensitivity of the cells to staurosporine-induced apoptosis when compared with empty vector transfected cells (Fig. 5, *B* and *C*). The observed effects of TMEFF2 overexpression on proliferation rates are consistent with a role as a tumor suppressor.

Most normal mammalian cells require adhesion to the extracellular matrix to grow and survive; however, in the course of tumor progression, cancer cells often lose this requirement and

acquire anchorage-independent growth capabilities. This feature is particularly important for the spread of tumor cells outside of the primary tumor and in the metastatic dissemination of cancer cells. To investigate the tumor suppressor potential of TMEFF2, we assessed its ability to promote anchorage-independent growth using a soft agar growth assay. HEK293T cells stably expressing TMEFF2-Myc-His formed ~5-fold fewer colonies, which were of smaller size than cells carrying the empty vector (Fig. 5, *D* and *E*). Thus, TMEFF2 suppresses the formation and the growth of HEK293T colonies in soft agar. Overexpression of TMEFF2 had no effect on the migration or invasion ability of HEK293T cells as measured using Boyden chambers (not shown).

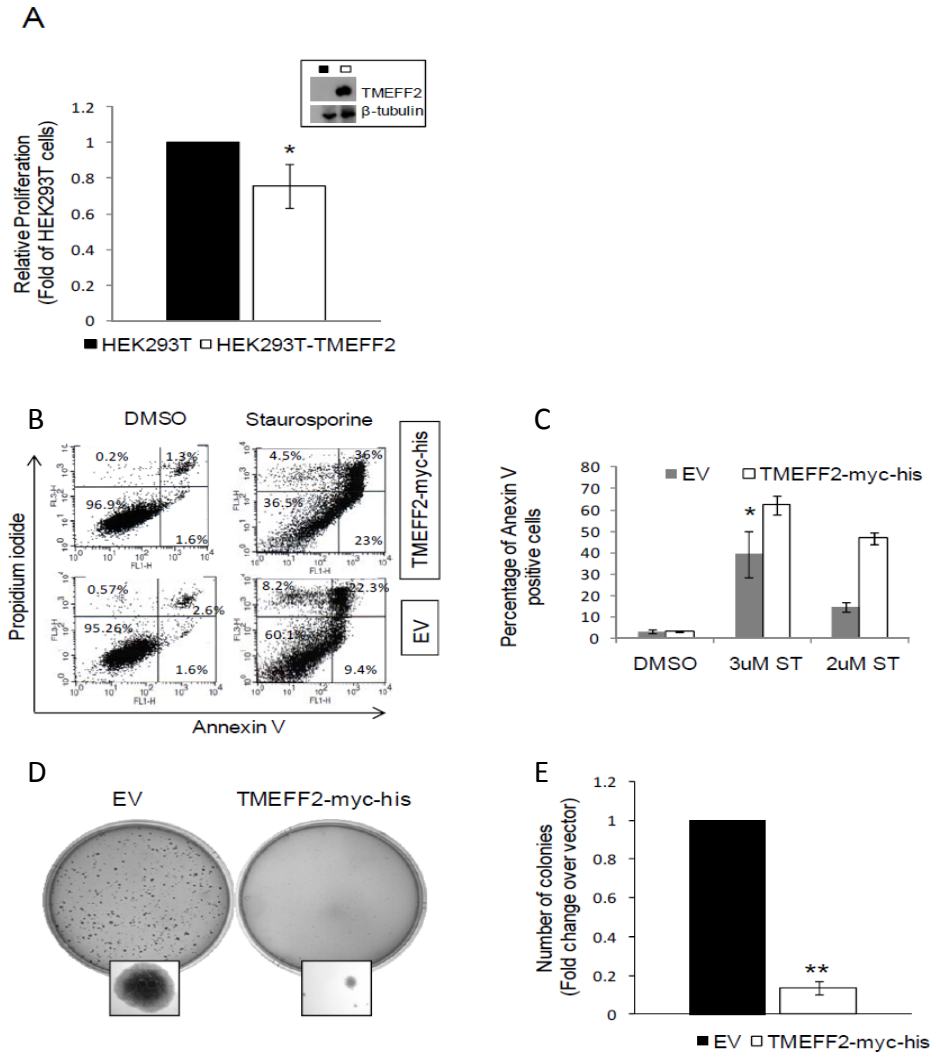


**FIGURE 5. TMEFF2 inhibits proliferation and anchorage-independent growth and sensitizes cells to apoptosis.** **(A)** *S*table expression of TMEFF2 decreases proliferation of HEK293T cells, **(B and C)** sensitizes the cell to an apoptotic stimulus, and **(D and E)** inhibits anchorage-independent growth. Overexpression of TMEFF2 was confirmed by western blot analysis (A insets). The effect of TMEFF2 on growth **(A)** was determined using an MTT assay after 96 h of growth. The $A_{562}$ at 96 h was normalized first to the value obtained at zero time (to correct for plating variability) and then to the value obtained for the parental cell line (HEK293T; A). The effect of TMEFF2 on apoptosis in HEK293T cells **(B and C)** was

determined in the presence of staurosporine or the vehicle, as a control, by analyzing the number of annexin V-positive cells and comparing it with the numbers obtained when expressing the empty vector. *B* and *C*, a representative image of the flow cytometry analysis **(B)** and percentage of apoptotic cells **(C)**. *D* and *E*, a representative image showing anchorage-independent growth **(*D*)** and the number of colonies formed by HEK293T cells stably expressing TMEFF2-Myc-His or the empty vector as a control **(E)** after 14 days of growth. Data shown are mean ± S.D. of at least three independent experiments with multiple replicates. Several clones were tested to rule out that the effects are due to the insertion site. *, $p < 0.05$, and **, $p < 0.01$.

*This research was originally published in the Journal of Biological Chemistry. Chen, X., Overcash, R., Green, T., Hoffman, D., Asch, A., MJ Ruiz-Echevarria. The tumor suppressor activity of the transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) correlates with its ability to modulate sarcosine levels. *J Biol Chemistry*. 2011; 286:16091-16100. 

**TMEFF2 Inhibits Sarcosine-induced Cell Invasion of Prostate Epithelial Cells**

Because the expression of TMEFF2 is mainly restricted to brain and prostate, we sought to analyze the effect of TMEFF2 overexpression in prostate cells. We selected RWPE1 cells, derived from non-neoplastic human prostatic epithelial cells (9), which express very low levels of endogenous TMEFF2 as demonstrated by quantitative RT-PCR (not shown). Full-length

TMEFF2 was introduced into the RWPE1 cells by retroviral gene transfer to generate an RWPE1 cell line that inducibly expresses TMEFF2 with the addition of doxycycline to the growth medium (RWPE1-TMEFF2i). Control cells were transduced with the transactivator construct only (RWPE1-tet). High levels of TMEFF2 expression in the RWPE1-TMEFF2i cell line upon the addition of doxycycline was demonstrated (Fig. 6*A*). To test whether TMEFF2 affects the growth rate of RWPE1 cells, RWPE1-TMEFF2i cells were grown in the absence (no TMEFF2 expression) and presence (TMEFF2 expression) of doxycycline, and the effect of TMEFF2 on the growth rate was determined. No significant effect of TMEFF2 on the growth rate of RWPE1 cells was observed when compared with the RWPE1-tet cells (Fig. 6*B*).

The invasion of prostate cancer cells across the basement membrane and eventually to extra-prostatic tissue is a critical tumorigenic program leading the way to metastasis. We therefore tested the effects of TMEFF2 overexpression on the invasive capability of RWPE1 cells. As mentioned, sarcosine is a proposed marker for prostate cancer progression and the addition of sarcosine to RWPE1 cells increases the invasive capability of the cells (93). We therefore tested whether TMEFF2 can reverse sarcosine-induced invasion. Briefly, RWPE1-TMEFF2i cells were grown in the presence of sarcosine to induce invasion and doxycycline to induce TMEFF2 expression. Alanine was used as a control for sarcosine-induced invasion. The invasive potential was then analyzed using Boyden chambers containing a thin layer of matrigel to simulate a basement membrane. The effect of TMEFF2 was investigated by comparing the invasion of RWPE1-TMEFF2i cells with the invasive ability of the control cell line, RWPE1-tet, both in the presence of doxycycline. As expected, the addition of sarcosine resulted in an increase in the invasion of the RWPE1-tet cells (Fig. 6C) when compared with cells grown in the presence of alanine. Overexpressing TMEFF2 in these cells reduced cell invasion both in the

control (alanine-treated) cells and to a greater extent in the cells treated with sarcosine (Fig. 6*C*). These results suggest that TMEFF2 can block the intrinsic and the sarcosine-induced invasive potential of RWPE1 cells. It is worth noting that although in HEK293T cells TMEFF2 negatively affects cell growth but has no effect on migration or invasion (data not shown), it has no effect on cell growth in RWPE cells while it substantially reduces invasion, indicative of the cell line-specific effect of TMEFF2.

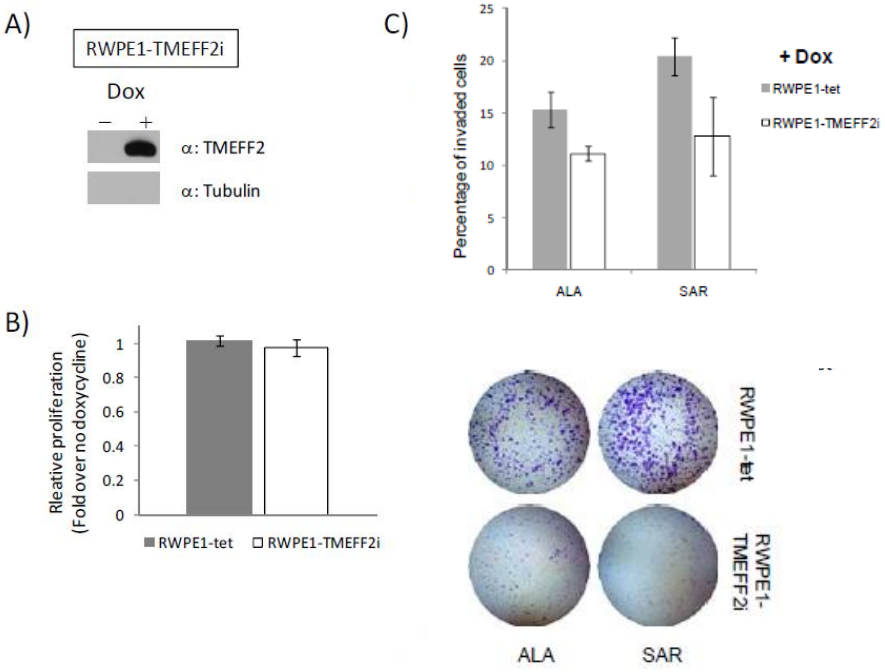


**Figure 6. TMEFF2 inhibits invasion in RWPE cells**. **(A)** Western blot demonstrating the induction of TMEFF2 expression in response to doxycycline (*Dox*, 250 ng/ml) in the RWPE1-TMEFF2i cell line. β-Tubulin was used as loading control. **(*B*)** The effect of TMEFF2 overexpression on the growth of RWPE1 cells was determined using an MTT assay after 96 h of growth. The *A*560 at 96 h was normalized first to the value obtained at zero time (to correct for plating variability) and then to the value obtained for same cells grown in the absence of doxycycline (no TMEFF2 expression). **(C)** The effect of TMEFF2 overexpression on the invasion ability of RWPE1 cells was determined using a MTT-based modified Boyden chamber assay. RWPE1-TMEFF2i or RWPE1-tet cells were grown for 96 hours in the presence of 50 μM

alanine or sarcosine and for 48 hours in the presence of doxycycline (250 ng/ml) and then added to the Boyden chambers and allowed to invade for 48 hrs. The number of invading cells at the bottom side of the matrigel chamber and the number of non-invading cells at the top of the matrigel were determined using a MTT assay and the percentage of the invading cells calculated from the total. Invasive cells from a random experimental repeat were visualized by fixing the cells adhering to the bottom of the membrane with 70% ethanol and staining with 0.1% crystal violet. Cells were then photographed (bottom).

*This research was originally published in the Journal of Biological Chemistry. Chen, X., Overcash, R., Green, T., Hoffman, D., Asch, A., MJ Ruiz-Echevarria. The tumor suppressor activity of the transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) correlates with its ability to modulate sarcosine levels. *J Biol Chemistry*. 2011; 286:16091-16100. 

**TMEFF2 inhibits TRAMP-C2 tumor growth *in vivo***

The tumor suppressor activity of TMEFF2 was next evaluated *in vivo* using a TRAMP-C2 allograft model. The TRAMP-C2 cell line was derived from a prostate tumor in a transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. These mice develop prostate tumors as a result of the prostate-specific expression of the SV40 T antigen in C57BL/6 mice. The TRAMP-C2 cells have an epithelial origin and are tumorigenic when injected into syngeneic mice (34). As described for the inducible RWPE1 cells, we developed TRAMP-C2 cells that are inducible for TMEFF2 expression with the addition of doxycycline. To establish subcutaneous

TRAMP-C2 tumors, cells were incubated in doxycycline for 48 hrs, collected, and 2.5 X $10^6$ inducible or non-inducible (vector-only) cells were injected into the flank of 7-8 wk. old male C57BL/6 mice and monitored for tumor growth. Our results demonstrate that overexpression of TMEFF2 significantly inhibited TRAMP-C2 tumor development, with only 12.5% of mice that developed tumors when injected with inducible TRAMP-C2 cells while 62.5% of mice injected with vector-only cells developed tumors (Fig. 7). It is important to note that we were unable to detect TMEFF2 protein expression in any of the tumors that developed following the injection of inducible TRAMP-C2 cells despite being continuously supplied doxycycline to maintain TMEFF2 expression. It is not clear why the cells in these tumors did not express TMEFF2; however, it is possible that some TRAMP-C2 cells with low or no TMEFF2 inducibility had a growth or survival advantage and outgrew the cells expressing TMEFF2.

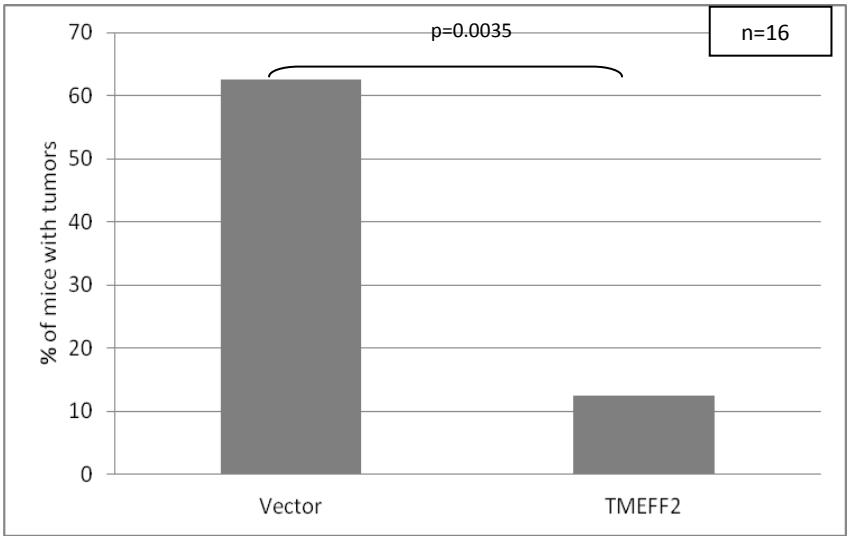


**Figure 7. TMEFF2 inhibits TRAMP-C2 tumor growth *in vivo*.** Bars represent the percentage of mice that developed subcutaneous tumors out of 16 mice injected with TRAMP-C2 cells per condition. Mice were injected with TRAMP-C2 cells that are inducible for TMEFF2 expression or cells carrying the vector only, and continuously fed a diet containing 200 mg/kg doxycycline to maintain TMEFF2 expression.

## Discussion

The upregulation of TMEFF2 expression in primary and metastatic prostate cancers relative to benign prostate tissue suggests a role for the gene in the establishment and/or progression of prostate cancer. To date, however, functional studies have not established a clear role for TMEFF2 in prostate tumorigenesis as many of these studies have produced conflicting results. Part of the discrepancy from previous reports may be due to the evaluation of TMEFF2 function in cells from several different cancer types or tissues, which has demonstrated cell-type-specific effects of its function. Furthermore, the structural features of the TMEFF2 protein give it the potential to influence a variety of pathways or cellular processes. For example, the follistatin domains of TMEFF2 are generally known to bind and inactivate members of the TGF-β signaling pathway; however, the effects of TGF-β signaling can be diverse and its role in cancer is dependent on the stage or specific genetic aberration(s) driving the cancer, sometimes tumor suppressive and sometimes oncogenic (2). The presence of a putative G-protein activating motif at the C-terminus of TMEFF2 suggests a potential role in second messenger signaling through G-proteins, a class of membrane proteins with diverse cellular effects that has been implicated in driving several tumorigenic processes including hormone-independence, inflammation, and metaststasis (25). Additionally, the cleavage and release of TMEFF2 from the membrane by ADAM proteins may be a critical regulated step in influencing the biological role of TMEFF2 as the regulated cleavage or "shedding" of ectodomains has been documented to play an important role cancer progression. Ectodomain shedding is critical for the activation of many growth factors in cancer and in some cases can produce proteins with functions that oppose the membrane-bound form (84). Altogether, the functional studies to date suggest a

complex biological function for TMEFF2 that may be largely dependent on its environment or other cell-type-dependent factors.

Here we evaluate the functional role of TMEFF2 in tumorigenesis and assess its influence on several phenotypic traits that are hallmarks of cancer cells. We show that the full-length TMEFF2 functions as a tumor suppressor capable of inhibiting cell proliferation and invasion, antagonizing survival, and severely blocking anchorage-independent growth. Consistent with previous observations, our results show some differences in the specific tumor suppressive effects of TMEFF2 in different cell lines. For instance, ectopic TMEFF2 expression reduced proliferation rates in HEK293T cells but had no effect on proliferation in RWPE-1 cells. Similarly, while TMEFF2 overexpression strongly inhibited RWPE-1 cell invasion, it had no effect on HEK293T cell invasion. Despite the cell-type-specific effects, TMEFF2 clearly demonstrated tumor suppressor activity in each of the cell lines examined. Confirming its tumor suppressor function *in vivo*, the subcutaneous development of TRAMP-C2 tumors was significantly inhibited as a result of TMEFF2 overexpression.

The functional assays presented here provide insight into the potential effects of TMEFF2 tumor suppression in prostate tumorigenesis. Results from the phenotypic assays demonstrate that TMEFF2 is capable of influencing multiple hallmark tumorigenic traits, and therefore has the potential to influence prostate cancer progression at multiple levels/stages. Interestingly, some of the most profound effects of TMEFF2 overexpression from the *in vitro* analysis of TMEFF2 function were the inhibition of cell invasion and a substantial inhibition of anchorage-independent growth. These features are critical components of the metastatic spread of cancer cells, and the inhibition of these traits by TMEFF2 suggests a possible role in the suppression of metastasis. However, in contrast to most of the current established “metastasis suppressor

genes", TMEFF2 is also capable of inhibiting cell proliferation and can therefore effect tumor growth in the earlier stages as well. Additionally, our results show that TMEFF2 overexpression has a negative effect on cell survival in the presence of an external apoptotic stimulus but does not induce apoptosis on its own. Therefore, in the stressful microenvironment of a tumor or in a cell attempting to colonize a foreign site, the sensitization of apoptotic signaling pathways may also represent an important mechanism by which TMEFF2 functions. The propensity of TMEFF2 to inhibit the development of tumors in the TRAMP-C2 allograft model as opposed to influencing tumor size may reflect its ability to antagonize tumor cell colonization/survival in a foreign environment.

Although our results clearly demonstrate a tumor suppressor function, we cannot deduce from these results that TMEFF2 always functions to suppress tumor growth within the environment of a tumor, where conditions surrounding the cancer cells are inundated with mitogens, inflammatory cytokines, and overactive oncogenic signaling pathways (23). As previously mentioned, the ectopic expression of the TMEFF2 ectodomain has been shown to exert pro-growth effects (5, 17), and its cleavage is stimulated by pro-inflammatory cytokines including IL-1β and TNF-α (65). It is therefore conceivable that TMEFF2 can exert either a tumor suppressive or tumor-promoting role in cancer that is dependent on signals from the tumor environment. A complete understanding of the role of TMEFF2 in prostate tumorigenesis will require an evaluation of the effects of its overexpression in a mammalian model of prostate cancer.

# CHAPTER 4

# ANDROGEN SIGNALING PROMOTES TRANSLATION OF TMEFF2 IN PROSTATE CANCER CELLS VIA THE PHOSPHORYLATION OF THE α SUBUNIT OF THE TRANSLATION INITIATION FACTOR 2 (eIF2α)

## Introduction

Androgens signaling through the AR play an essential role in normal prostate development and contribute to the progression of prostate cancer. Binding of androgens to the AR promotes a conformational change that ultimately leads to its translocation to the nucleus and regulation of transcription of a specific set of androgen-responsive genes. Clinical and experimental evidence suggest that prostate cancer progression occurs through alteration of the normal androgen signaling, reducing the specificity or the amount of AR ligand required for proliferation and survival (8). Importantly, recent results indicate that the function of the AR is specific to the disease stage, triggering a different gene expression program in androgen-dependent as compared to androgen-independent prostate cancer (104). While the role of the AR signaling axis in transcriptional regulation is well documented, very little is known regarding its role in translation initiation proposed in early studies (63, 64).

As previously mentioned, TMEFF2 is expressed in the embryo (99) and selectively in the adult brain and prostate (3, 35, 39). A role for TMEFF2 in prostate cancer was suggested by studies indicating that TMEFF2 expression is altered in a significant fraction of primary and metastatic prostate tumors (3, 35, 39, 71). In addition, we recently demonstrated that TMEFF2 interacts with sarcosine dehydrogenase (SARDH), the enzyme responsible for conversion of sarcosine to glycine (17). Importantly, sarcosine was identified as a marker for prostate cancer progression in a large-scale screen of metabolites from human prostate samples (93). Increased plasma and urine sarcosine levels distinguished prostate cancer from benign prostate tissue, and were further elevated in metastatic cancer. In addition, sarcosine metabolism and the enzymes involved in it (i.e. SARDH) were shown to act as regulators of cell invasion and metastasis (93).

Therefore, the interaction of TMEFF2 with SARDH further suggests a role for TMEFF2 in prostate cancer progression. In fact, we have also established that full-length TMEFF2 functions as a tumor suppressor and that this role correlates, at least in part, with its ability to interact with SARDH and modulate the cellular levels of sarcosine (17). In this study we report that translation of TMEFF2 is regulated by androgens, and this effect requires a functional AR. Results using xenograft models and prostate cancer cell lines established that TMEFF2 expression changes in response to androgens and/or the androgen-dependent or -independent condition of the cells (35, 71). As demonstrated by Gery et al., (35) these changes are in part due to transcriptional activation of *TMEFF2* in response to androgens. However, increased TMEFF2 protein levels in the absence of a corresponding increase in mRNA levels have been observed after addition of androgens to castrated animals carrying CWR22 xenografts, suggesting that TMEFF2 may also be post-transcriptionally regulated (71).

The *TMEFF2* mRNA has several potential upstream open reading frames (uORFs) in its leader region, and sequence analysis suggests that they are well conserved among mammals. Although only present in 5-10% of the cellular mRNAs, uORFs are common in the leader regions of mRNAs encoding oncoproteins or proteins involved in the control of cellular growth and differentiation, and they function by modulating translation of these essential genes (72) . After being translated, uORFs generally block translation of the main downstream coding region by hampering translation reinitiation at the main translation initiation codon. However, uORFs can promote selective translation of the downstream coding region under cellular stress or other conditions that increase phosphorylation of the α subunit of the eukaryotic translation initiation factor 2 (eIF2α) (72).

eIF2 in its GTP-bound form is required for the selection of the translation initiation codon. Phosphorylation of the α subunit of eIF2 at Ser-51 (eIF2α-P) inhibits the exchange of eIF2-GDP to eIF2-GTP, preventing recognition of the initiating codon and decreasing global translation initiation (96). However, as mentioned above, uORF-containing mRNAs are actively translated under these conditions. Two mechanisms have been proposed to explain this effect. In the first one, exemplified by the *ATF4* mRNA that contains two uORFs, translation reinitiation at the inhibitory downstream uORF is bypassed under conditions of eIF2α-P, due to the fact that the lower levels eIF2-GTP increase the time required for the scanning ribosomes to re-acquire eIF2-GTP and reinitiate translation (103). In the second one, observed in mRNAs containing a single uORF, scanning ribosomes bypass the inhibitory uORF due to the reduced efficiency of translation at initiation codons with a poor Kozak consensus sequence (79). In both cases, the uORF bypass results in an increased number of ribosomes starting translation at the initiation codon of the main coding sequence, thereby increasing synthesis of that specific protein.

In this study, we demonstrate that TMEFF2 translation is regulated by androgens. Androgen-regulation of TMEFF2 translation requires the presence of the uORFs in the leader region of the *TMEFF2* mRNA and is dependent on eIF2α-P. Further, this effect is mediated by the AR since it is not observed when AR levels are reduced by RNAi or the antagonist bicalutamide, or in cell lines that do not express it. These results support a novel regulatory mechanism of androgen signaling in which uORF-containing mRNAs are translationally activated in response to eIF2α-P.

## Results

### The *TMEFF2* 5'-UTR contains several uORFs that block translation of the TMEFF2 protein

The 5' UTR of the *TMEFF2* mRNA contains several potential uORFs (Fig. 8A) that, if translated would potentially block translation of the TMEFF2 main coding sequence, and therefore contribute to the regulation of *TMEFF2* expression. To investigate the role of the uORFs in regulating TMEFF2 protein expression, we determined whether blocking translation of the uORFs would affect translation of the TMEFF2 protein in human prostate cancer cell lines. A TMEFF2-Gaussia Luciferase (GLuc) reporter was generated for this purpose by cloning the *TMEFF2* 5'-UTR, including four uORFs, upstream of the GLuc sequences (pTM1234-Gluc; Figure 8B). The TMEFF2-Gluc fusion was placed under control of the CMV promoter. The regulatory contribution of the uORFs to *TMEFF2* translation was evaluated by mutating the start codons (AUGs) of the four potential uORFs (AUG to GUG, Fig. 8B) and determining their effect on GLuc expression in the androgen-dependent prostate cancer cell line LNCaP and its bone-metastatic, androgen-independent derivative C4-2B cell line. A six- to seven-fold increase in Luciferase expression was observed when the AUGs from all four uORFs were mutated (pTMXXXX-Gluc; Fig. 8C). Single mutations on the AUGs of the second, third or fourth uORFs promoted a 3-4 fold increase in GLuc expression, while mutating the AUG of the first uORF had a very small effect, suggesting a minimal role, if any, in regulation of TMEFF2 expression. Accordingly, combined mutations of uORFs 2, 3, and 4 resulted in a five- to six-fold increase in Luciferase expression of the reporter, similar to that observed when all four uORFs were mutated (Fig. 8C). Similar results were obtained in other androgen-responsive (22Rv1) and -independent

(PC3) prostate cancer cell lines. Mutation of the uORFs resulted in increased Luciferase expression of the GLuc reporter, albeit at variable fold induction (Fig. 8D). In the constructs used for these experiments, expression of the fusion gene was directed by the CMV promoter, and the luciferase activity was normalized to mRNA levels. Altogether, these results suggest that the uORFs in the 5'- UTR of *TMEFF2* mRNA function synergistically to repress translation of the main downstream ORF.

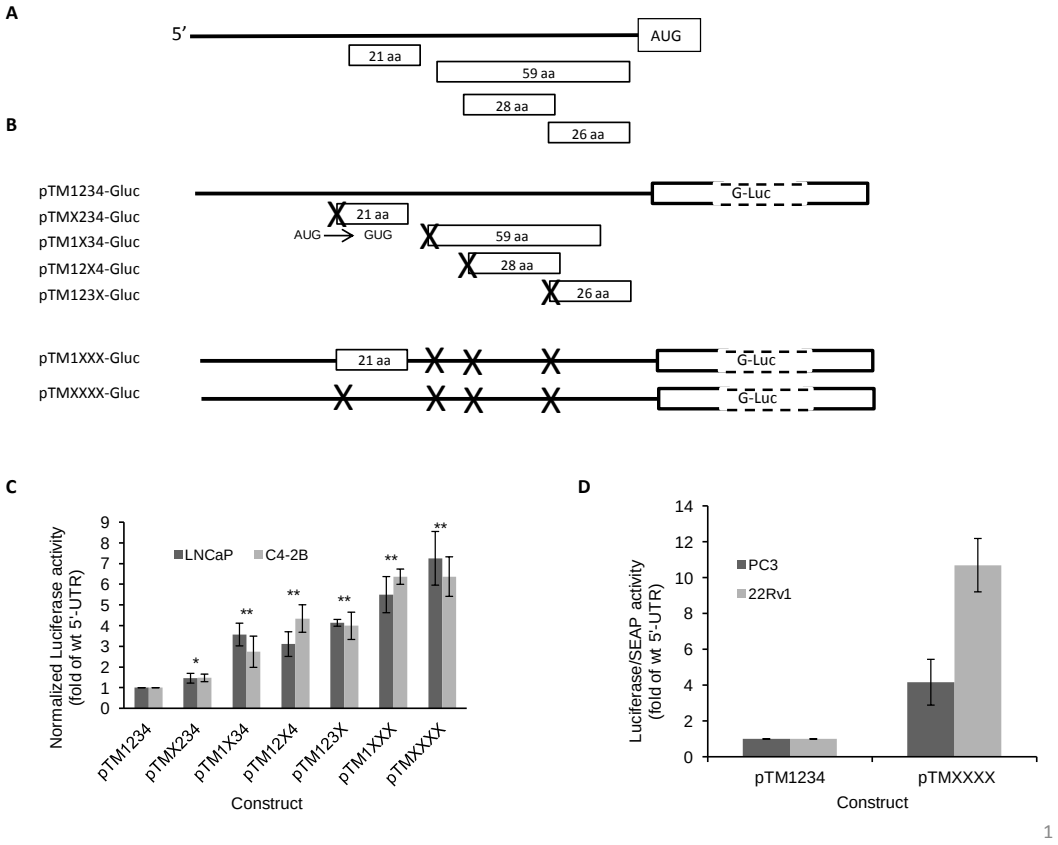


**Figure 8. The uORFs in the 5'-UTR of TMEFF2 inhibit translation of the main coding region. A)** Schematic representation of 394 nt upstream of the *TMEFF2* main coding region and relative localization of four potential uORFs (aa = amino acid) within the 5'-UTR. **B)** Schematic representation of the TMEFF2-Gaussia Luciferase reporter and mutant constructs. The X indicates mutation of the AUG to a GUG to prevent translation of the mutated uORFs. Single and multiple mutations were introduced. **C)** Luciferase activity demonstrated by the pTM1234-Gluc and the different mutant constructs in LNCaP and C4-2 cells. **D)** Luciferase activity demonstrated by the pTM1234-Gluc and multiple mutant construct with all the uORFs mutated (pTMXXXX-Gluc) in PC3 and 22Rv1 cells. In C) and D), luciferase activity was measured in the supernatant and calculated by first normalizing to mRNA levels for each construct and then to the luciferase activity demonstrated by the pTM1234-Gluc reporter construct, which does not

have mutations in the uORFs, considered arbitrarily as 1. Data shown are mean ± S.D. of at least two independent experiments with multiple replicates. *, $p < 0.05$, and **, $p < 0.01$.

**Translation of the TMEFF2-Luciferase reporter is regulated by androgens through a mechanism that requires the presence of the uORFs in the mRNA leader region**

*TMEFF2* transcription is regulated by androgens (35). However, it has been suggested that androgens also affect TMEFF2 expression at the post-transcriptional level (71), prompting us to examine whether TMEFF2 translation was affected by androgens. 22Rv1 cells were selected for these experiments since: i) they have been shown to be a valuable model for AR-mediated reporter gene assays (53), ii) they demonstrated the highest fold increase in Luciferase reporter gene expression when the uORFs were mutated (see Fig. 8D), and iii) they express detectable levels of endogenous TMEFF2. 22Rv1 cells were transfected with the pTM1234-Gluc reporter, grown in phenol red-free media supplemented with charcoal-stripped serum (CSS) -- to remove steroid hormones- and treated with different concentrations of dihydrotestosterone (DHT). Luciferase activity was measured from the supernatants and normalized to mRNA levels. Addition of DHT increased luciferase expression in a dose-dependent manner (Fig. 9A), indicating that androgens stimulate the translation of the main ORF. Importantly, this effect was observed at DHT concentrations within the physiological levels found in human serum (30). Luciferase activity from cells carrying the pTMXXXX-Gluc reporter construct, in which the AUGs from all four uORFs were mutated, did not change in response to DHT, although, as

expected, was much higher (Fig. 9A). These results indicate that translation of the main ORF downstream of the *TMEFF2* 5'- UTR is regulated by androgens in an uORF-dependent manner.

To determine whether the DHT effect on translation is mediated by the AR, the experiments described above were repeated in the presence of bicalutamide to block AR activation. Addition of this drug reduced the DHT-mediated induction of the pTM1234-Gluc reporter luciferase expression to near basal levels, although a small two- to three-fold induction could be observed at 10 nM DHT (Fig. 9B). These results indicate that the effect of DHT on translation of the reporter construct requires AR signaling. Confirming these results, we did not observe DHT-induced translation of the pTM1234-Gluc reporter in PC3 prostate cancer cells that do not express the AR (Fig. 9C). Altogether, these results suggest that DHT-induced translation of TMEFF2 requires activation of the AR and is mediated by the uORFs in the leader region of the *TMEFF2* mRNA.

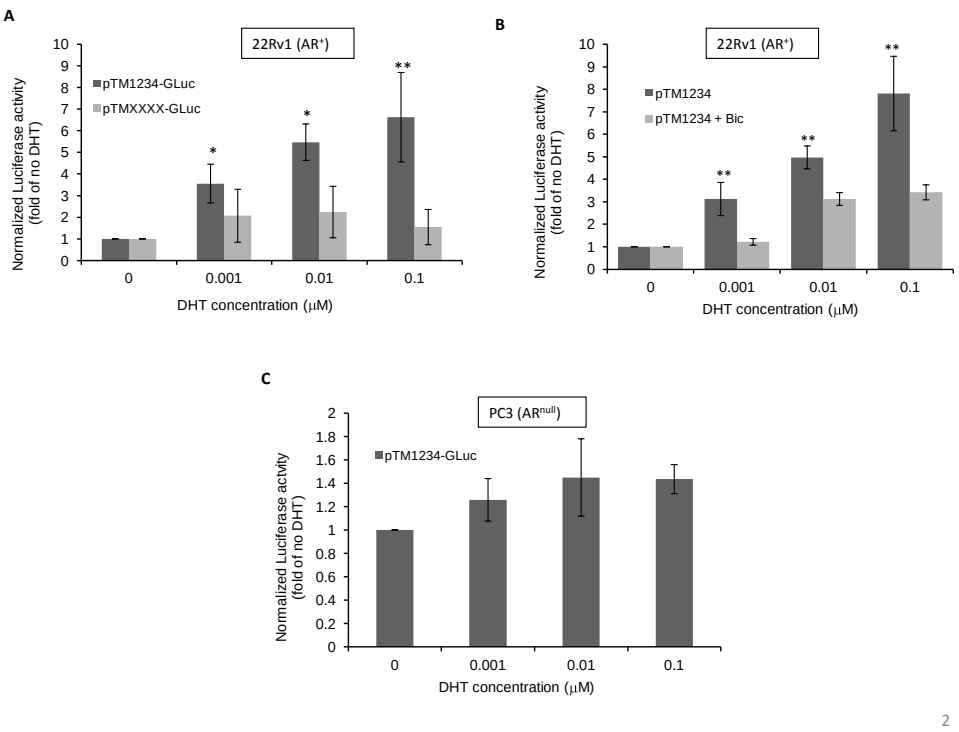


**Figure 9. DHT promotes AR-mediated increased translation of the TMEFF2-GLuc fusion protein in 22Rv1 prostate cancer cells. A)** Luciferase activity demonstrated by the pTM1234-Gluc and the pTMXXXX-Gluc mutant construct in 22Rv1 cells in the presence of different concentrations of DHT. **B)** Luciferase activity demonstrated by the pTM1234-Gluc construct in 22Rv1 cells in the presence of different concentrations of DHT and DHT + 20 μM bicalutamide (Bic). **C)** Luciferase activity demonstrated by the pTM1234-Gluc construct in PC3 cells in the presence of different concentrations of DHT. For all these experiments cells were hormone-starved in phenol red-free media containing charcoal-stripped serum. Luciferase activity was normalized to mRNA levels for each construct and then to the luciferase activity demonstrated by each one of the constructs expressed in cells grown in the absence of DHT, which was set to

1. Data shown are mean ± S.D. of at least three independent experiments with multiple replicates. *, $p < 0.05$, and **, $p < 0.01$.

**Translation of the endogenous TMEFF2 protein is regulated by androgens**

Changes in the expression of the endogenous TMEFF2 protein in response to androgens were also analyzed. For this purpose, 22Rv1 cells were grown in phenol red-free media supplemented with CSS, treated with different concentrations of DHT and lysates analyzed for TMEFF2 expression by western blotting. In the absence of androgens, expression of TMEFF2 was barely detectable. However, addition of DHT increased TMEFF2 expression (Fig.10A), and resulted in the highest levels within the range of physiological DHT concentrations. DHT-induced expression of endogenous TMEFF2 was also observed in the androgen-responsive prostate cancer LNCaP cells (Fig. 10A). The expression of prostate specific androgen (PSA), an AR target used as control for androgen transcriptional activity, was enhanced by the addition of DHT (Fig. 10A). Treatment of the cells with bicalutamide notably inhibited DHT-induced TMEFF2 and PSA expression that was only observed at the highest concentrations of DHT (Fig. 10A). Inhibition of DHT-induced TMEFF2 expression was also achieved after knocking down expression of the AR using siRNA (Fig. 10B). Altogether, these results indicate that the expression of the endogenous TMEFF2 protein is regulated by AR signaling.

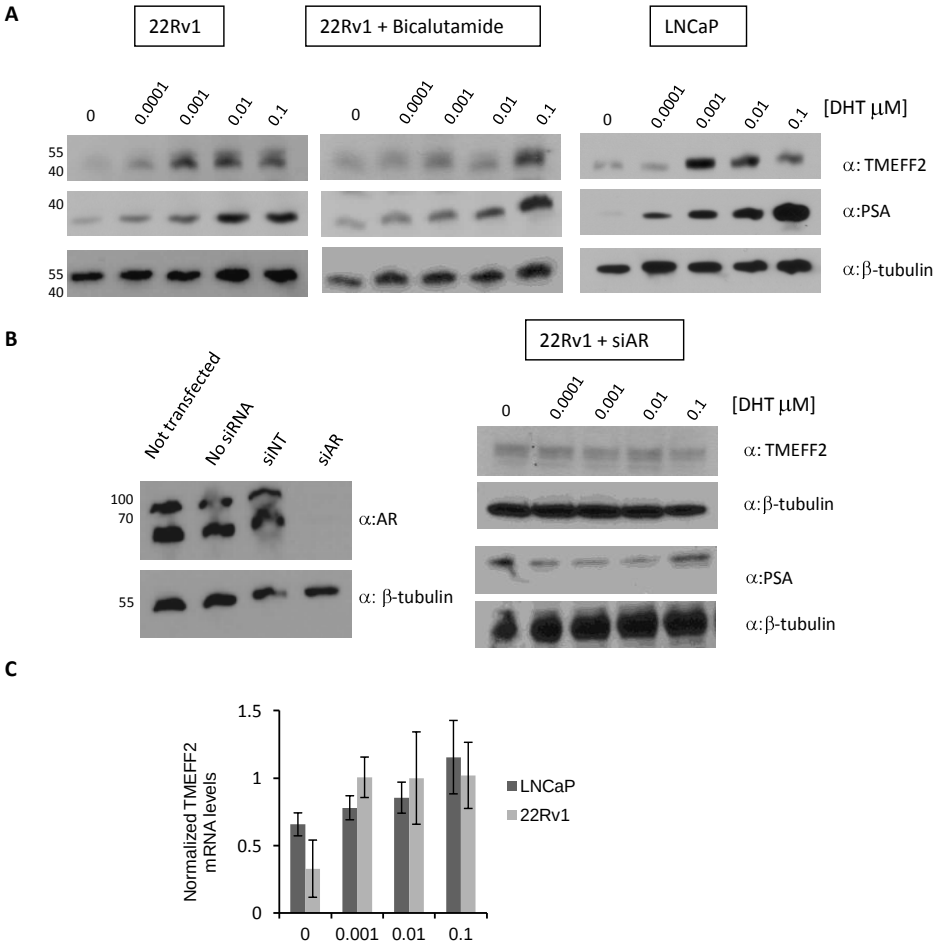


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**Figure 10. DHT promotes AR-mediated increased expression of the endogenous TMEFF2 protein in 22Rv1 and LNCaP prostate cancer cells. A)** Representative western blots indicating an increase in TMEFF2 expression in response to DHT addition in 22Rv1 and LNCaP cells. Simultaneous addition of 20 μM bicalutamide to 22Rv1 cells (middle panel) prevented the increase in TMEFF2 expression observed at physiological concentration of DHT. PSA was used as positive control since its expression is induced by androgen in an AR-dependent manner. β-tubulin was used as a loading control. **B)** Western blot indicating effective knock down of the two forms of the AR in 22Rv1 cells using siRNA (left). Representative western blot indicating

that addition of DHT has no effect on the expression of TMEFF2 in cells in which AR levels were reduced by RNAi. PSA was used as control and, as expected, its expression was not affected by DHT in the AR-siRNA treated cells. β-tubulin was used as a loading control. **C)** Changes in *TMEFF2* mRNA level in LNCaP and 22Rv1 cell lines in response to DHT as measured by qRT-PCR. Values were normalized to β-tubulin mRNA. Each experiment was repeated at least three times and, for the representative images presented, the membranes were stripped and re-probed with the different antibodies or the same samples were re-run in a different gel. β-tubulin was used as a loading control each time the samples were run.

**Androgen signaling promotes eIF2α phosphorylation**

Phosphorylation of eIF2α reduces global translation but also provides a mechanism that selectively enhances translation of uORF-containing mRNAs (72). We therefore hypothesized that the molecular mechanism by which DHT promotes endogenous TMEFF2 translation was through the phosphorylation of eIF2α. The effect of DHT on eIF2α phosphorylation was examined by western blot analysis in prostate cancer 22Rv1 and PC3 cells grown in phenol red-free media supplemented with CSS and treated with different concentrations of DHT. Increased levels of eIF2α-P were clearly detected, in a dose-dependent manner, in lysates from DHT-treated 22Rv1 cells but not in lysates from DHT-treated AR-null PC3 cells (Fig. 11A), indicating that androgens promote eIF2α-P and that this effect is dependent on the presence of a functional AR. Confirming these results, pretreatment of the 22Rv1 cells with the AR antagonist bicalutamide prevented DHT-mediated increases in eIF2α phosphorylation (Fig. 11B). DHT

addition also promoted an increase in the expression of the ATF4 protein, a transcription factor regulated by eIF2α phosphorylation (Fig. 11A).

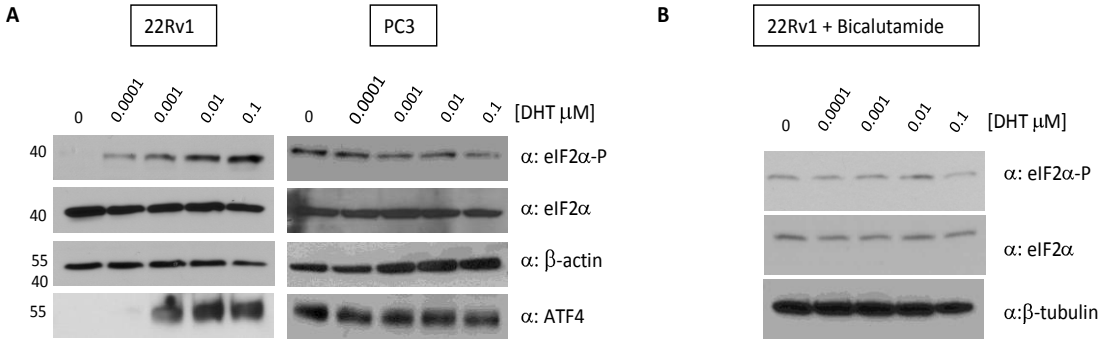


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**Figure 11. DHT induces phosphorylation of eIF2α in an AR-dependent manner.**

**A)** Representative western blots indicating an increase in eIF2α-P in response to DHT addition in 22Rv1 cells. This effect was abrogated in PC3 cells, which do not express AR (right). ATF4 protein levels were measured as a positive control since it is induced by eIF2α-P. β-actin was used as a loading control. **B)** Addition of 20 μM bicalutamide to 22Rv1 cells prevented the increase in eIF2α-P observed after addition of DHT. β-tubulin or β-actin were used as loading controls.

**eIF2α phosphorylation is essential for increased TMEFF2 translation in response to androgens**

To further investigate the role of eIF2α phosphorylation and androgens on the translation of TMEFF2, mouse embryonic fibroblasts (MEFs) expressing either wild-type eIF2α (wt/wt) or an eIF2α S51A (A/A) mutant form, carrying a Ser to Ala mutation that prevents eIF2α-P, were transfected with the pTM1234-Gluc reporter and expression of the reporter was analyzed in the presence and absence of different concentrations of thapsigargin. This experimental system was tested by treating the cells with clotrimazole or thapsigargin, two drugs known to promote eIF2α-P. Both effectively promoted eIF2α-P in cells carrying the wild-type but not the S51A (A/A) mutant eIF2α (Fig. 12A). Cells were grown in phenol red-free media supplemented with CSS and treated with different concentrations of thapsigargin. When expression of the pTM1234-Gluc reporter was analyzed in conditioned media from the wt/wt and A/A MEFs lines, increased luciferase activity was detected in response to thapsigargin in cells carrying the wild-type eIF2α but not in the eIF2α S51A mutant cells (Fig. 12B). Similarly, only the cells carrying the wild-type eIF2α allele were able to induce translation of the ATF4 protein used as a positive control for eIF2α-P (Fig. 12B). Altogether these results confirm that eIF2a-P is required for the DHT-induced translation of TMEFF2.

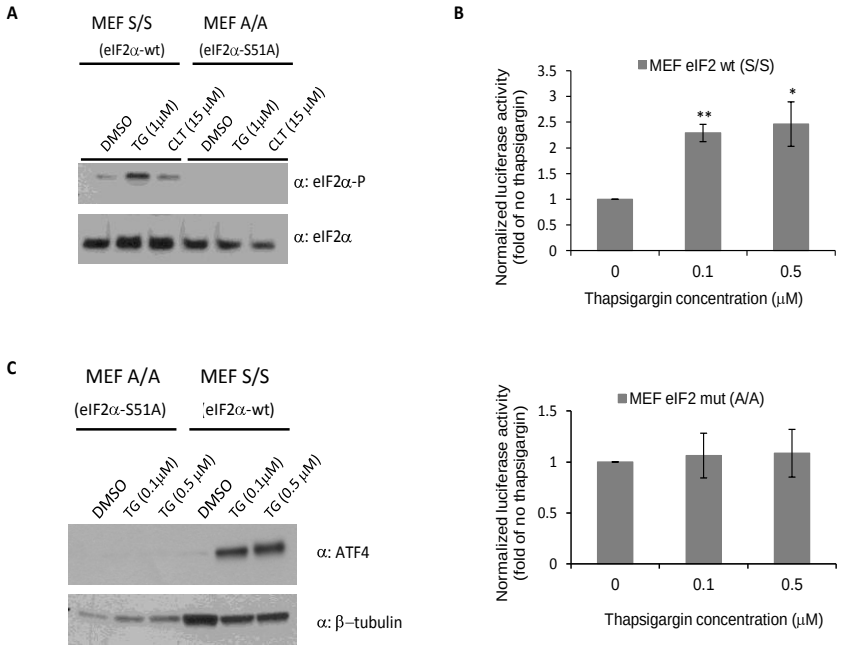


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**Figure 12. Increased TMEFF2 translation in response to androgens requires eIF2α-P. A)** Western blot showing total and phophorylated eIF2α in wild-type and A/A MEF cells treated with the indicated amounts of thapsigargin or clotrimazole. **B)** Gaussia luciferase levels were measured in wt/wt and A/A MEF cells treated with the indicated amounts of thapsigargin for 3 hrs and normalized to Seap expression. Luciferase/Seap values for the vehicle-treated cells are set to 1. **C)** As a positive control for eIF2α-P, western blot analysis demonstrates that ATF4 protein expression is increased in wt/wt MEF cells treated with thapsigargin. β-tubulin was used

as a loading control. Data shown are mean ± S.D. of at least three independent experiments with multiple replicates. *, $p < 0.05$, and **, $p < 0.01$.

**Conditions that promote eIF2α-P result in increased TMEFF2 translation**

In order to determine whether eIF2α phosphorylation is in itself sufficient to regulate translation of TMEFF2, 22Rv1 cells were treated with clotrimazole, a drug that causes depletion of intracellular $Ca^{2+}$ stores resulting in activation of the PKR kinase and subsequent eIF2α-P, and the effect on TMEFF2 translation was analyzed using polysome analysis. Western blot analyses indicated that the clotrimazole treatment resulted in increased phosphorylation of eIF2α in 22Rv1 cells (Fig. 13A). As previously described (4), clotrimazole treatment resulted in reduced polysomes along with an increase in monosomes indicating inhibition of translation initiation (Fig. 13B). Under these conditions, we observed a shift of the *TMEFF2* mRNA towards the heavier polysomal fractions when compared to the DMSO-treated controls (Fig. 13B and 13C), suggesting that clotrimazole treatment increased translation of the *TMEFF2* mRNA. However, the shift was small, likely reflecting the presence of multiple uORFs and a complex translational regulatory mechanism. Similar results were observed in other cell lines (data not shown). Confirming these results, clotrimazole treatment of cells containing the pTM1234-Gluc reporter resulted in a significant increase in luciferase activity (data not shown). In addition, a shift to the heavier polysomal fractions was also observed for the uORF-containing *ATF4* mRNA, known to be preferentially translated upon eIF2α-P (Fig. 13B and 13C; (45)). Taken together, these results

demonstrate that eIF2α phosphorylation, independent of the causative stimulus, is sufficient to enhance translation of TMEFF2.

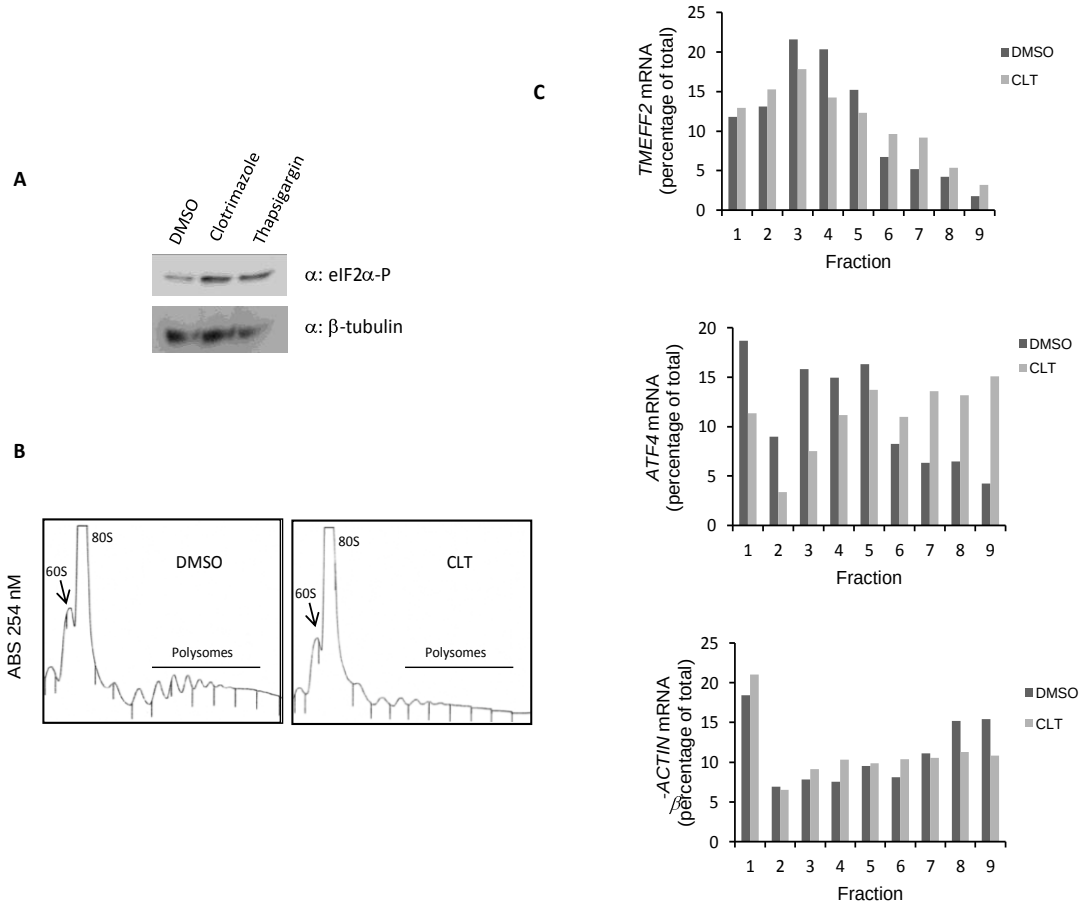


**Figure 13. *TMEFF2* mRNA associates with heavier polysomes in response to ER stress.**

**A)** Western blot indicating phosphorylation of eIF2α in response to ER-stress inducing agents in 22Rv1 cells. **B)** 22Rv1 cells were exposed to 15 μM clotrimazole or vehicle control for 1 hr and lysates subjected to polysome analysis. Total RNA was prepared from the fractions, and the percentage of *TMEFF2*, *ATF4*, and *ACTB* mRNAs present in each fraction were determined by qRT-PCR. A representative example of one of the three independent experiments is shown. **C)** Quantitation of the qRT-PCR results presented in B).

## Discussion

Changes in the expression of TMEFF2 protein have been documented in prostate cancer, and a potential role for TMEFF2 in this disease has been proposed (3, 35, 39, 71). However, the molecular mechanisms leading to the deregulation of TMEFF2 expression in prostate cancer are not known. The present study reveals a novel role for androgens in the regulation of TMEFF2 translation that could account for the changes in TMEFF2 expression observed in prostate cancer. We demonstrate that androgen signaling promotes increased TMEFF2 translation through a mechanism that involves the phosphorylation of eIF2α and is dependent on the presence of uORFs in the 5'-UTR of the *TMEFF2* mRNA.

The main androgen, testosterone, and its metabolite, DHT, largely mediate their effect through binding to the AR, a ligand-inducible transcription factor. The bound AR can recognize and bind specific androgen response elements in the promoter regions of target genes, causing activation or repression of transcription, leading to subsequent changes in protein synthesis (8). However, post-transcriptional regulation of gene expression by androgens has also been documented, affecting mRNA stability, protein localization, polyA-tail length, and translation efficiency (75, 80). The effect of androgens on translation is mediated through several mechanisms including changes in miRNA expression or processing (32), phosphorylation of translation factors like eEF2 (43), or mTOR activation-mediated phosphorylation of p70S6 and 4E-BP1 (108). The results presented here indicate that physiological concentrations of DHT promote eIF2α phosphorylation in prostate cancer cell lines to influence translation.

Phosphorylation of eIF2α ultimately leads to the inhibition of global translation initiation along with stimulation of translation of specific mRNAs, i.e. uORF-containing mRNAs. These

changes facilitate cellular adaptation to the stress conditions promoting either survival or initiation of apoptosis (85). Using TMEFF2-Luciferase reporter constructs in which the 5'-UTR of *TMEFF2* was fused to the Gaussia Luciferase gene, we demonstrated that, in addition to eIF2α-P, DHT also promotes translation of TMEFF2. Importantly, this effect requires the presence of uORFs in the 5'-UTR of the *TMEFF2* mRNA, as DHT showed no effect on the translation of a TMEFF2-Luciferase reporter in which the AUGs for each of the uORFs were mutated. Supporting these results, translation of the TMEFF2 endogenous protein and the uORF containing ATF4 transcriptional regulator, (known to be regulated by eIF2α-P) were also increased in response to DHT stimulation. Moreover, as previously demonstrated for ATF4 (81), using polysome analysis we observed that translation of TMEFF2 increased in response to clotrimazole, a translation inhibitor that promotes ER stress and subsequent eIF2α-P. In addition, the DHT effect on TMEFF2 translation was not observed in cells carrying a non-phosphorylatable form of eIF2α. These results suggest that the effect of DHT on TMEFF2 expression was mediated by eIF2α-P. We also demonstrated that the DHT effect on TMEFF2 expression required the presence of a functional AR, but was partially independent of transcriptional activity. To our knowledge, this is the first report demonstrating a transcription-independent effect of DHT on translation of uORF-containing mRNAs through phosphorylation of eIF2α.

Phosphorylation of eIF2α in response to different forms of stress is mediated by one of the following four kinases (19): protein kinase R (PKR), heme-regulated eIF2α kinase (HRI), protein kinase R-like kinase (PERK), or GCN2 (general control nonderepressible-2. Several different kinases might be responsible for eIF2α phosphorylation in response to DHT. Androgens regulate the transcription of many genes, including some of those involved in amino acid

metabolism (46, 75), and it is therefore possible that DHT-induced eIF2α phosphorylation results from GCN2 activation driven by metabolic changes. However, under our experimental conditions, DHT stimulated the translation of TMEFF2 in the presence of actinomycin D (data not shown), suggesting that the DHT-induced phosphorylation of eIF2α is, at least partly non-genomic. While this manuscript was in preparation, Dai and collaborators (22) reported increased apoptosis of human liver cells in response to DHT administration through a PKR-eIF2α-P dependent mechanism. This effect was sensitive to the AR antagonist flutamide and it seemed to involve CHOP (GADD153) activation. These results suggest that DHT functions by activating PKR, however, the experiments were performed in the presence of 100 nM DHT, a concentration much higher than physiological levels (1-10 nM). In this regard, studies in mice indicate that excess androgens can promote oxidative stress (67), which can induce PKR activity and phosphorylation of eIF2α (33). In cell lines, addition of physiological levels of DHT can promote increased cytosolic calcium ($Ca^{2+}$), which can promote PRK activation either directly or indirectly by increasing oxidative stress (33). Therefore, DHT could indirectly promote PKR activation through its ability to promote accumulation of intracellular calcium, a well-known transcription independent, non-genomic effect of androgen.

What is the role of AR-mediated phosphorylation of eIF2α in prostate cancer? A correlation between translation initiation and prostate cancer can be postulated from the observation that several translation initiation factors are overexpressed or activated in this disease (21). For example, eIF3h and eIF4E are frequently overexpressed in advanced prostate cancers, together with increased phosphorylation of eIF4E and eIF4E-BP1, which support increased translation (91). However, the role of eIF2α-P in prostate or other cancers is not clear. While expression of a nonphosphorylatable form of eIF2α or overexpression of a dominant-

negative form of PKR have been shown to inhibit apoptosis and cause malignant transformation, other reports indicated that reduced PKR levels correlated with less aggressive tumors (91). In general, it has been suggested that the role of eIF2α-P is dependent on the stage and grade of the disease, occurring at the earliest stages as a response to stress, while downregulation of eIF2α-P will occur later on as a result of the selective pressure imposed by the tumor cell's need to proliferate (21). Androgen signaling is critical for the progression of prostate cancer, and changes in the sensitivity to androgen signaling after prostate cancer regression drives the cancer to the advanced stages. It is possible that early in prostate cancer, or after regression, androgen signaling triggers PKR activation and subsequent eIF2α-P, leading to anti-proliferative effects, apoptosis, and the activation of tumor suppressor mechanisms. In this respect, it has been reported that the anti-proliferative and pro-apoptotic effects of PTEN, a tumor suppressor protein frequently mutated in prostate cancer, require activation of the PKR-eIF2α-P pathway (74). Persistent AR signaling along with downregulation of eIF2α-P may lead to AR-mediated transcriptional events and tumor progression.

In summary, our findings reveal a novel role for AR signaling as a translational regulator of TMEFF2 through the phosphorylation of eIF2α. We have previously demonstrated that the tumor suppressor ability of TMEFF2 partly correlates with its ability to interact with SARDH and modulate the levels of sarcosine (17). SARDH and GNMT, the enzymes that catalyze the forward and reverse conversion of sarcosine into glycine, are under transcriptional control mediated by the androgen receptor. Treatment of prostate cancer cell lines with androgens resulted in an increase in GNMT expression and a simultaneous decrease in SARDH levels (93). Since TMEFF2 is both transcriptionally (35) and translationally regulated by androgens, these observations potentially link androgens with the regulation of sarcosine metabolism and changes

in TMEFF2 expression, and may suggest a model by which in the early stages of tumorigenesis the increased expression of TMEFF2 in response to androgens is a cellular response to overcome tumorigenesis. Persistent AR signaling leads to an increase in GNMT and a simultaneous decrease in SARDH expression that outweigh the effect of increased TMEFF2 levels. Therefore, TMEFF2 upregulation in response to AR signaling may initially serve as a barrier for malignant progression of prostate cancer.

# CHAPTER 5

# GENERATION AND CHARACTERIZATION OF A TRANSGENIC MOUSE MODEL OVEREXPRESSING TMEFF2 IN THE PROSTATE

## Introduction

The complex biology of the TMEFF2 protein has made it difficult to fully understand the biological function of TMEFF2 in the prostate and in prostate tumorigenesis. Although previous reports using cell lines and primary cells have provided important clues into its function and mechanism of action as a tumor suppressor, it is critical to evaluate the function of TMEFF2 in a mammalian model and an environment that is biologically similar to that of the human prostate. The mouse prostate divided into four lobes, the anterior, ventral, dorsal, and lateral lobes. The dorsal and lateral lobes are usually grouped together as the dorsolateral lobe. The dorsolateral lobe is believed to be most similar to the human peripheral zone where the majority of prostate tumors originate. Although mice do not spontaneously develop prostate tumors naturally, most genetic mouse models of prostate cancer develop PIN and prostate cancer in the dorsolateral lobe that shares many of the histological features and often similar genetic signatures as human prostate cancer (59, 105).

TMEFF2 expression in the adult mouse is restricted to the brain (39), however it is also expressed in the mid to late stages of embryogenesis, suggesting it may play a role in development (99). A potential role for TMEFF2 in embryonic development is intriguing as developmental pathways are often reactivated in several types of cancer including prostate cancer (7), and may provide insight into the mechanism(s) of TMEFF2 action in tumorigenesis. As mentioned, the other TMEFF family member, TMEFF1, can modulate Nodal signaling, an embryonic developmental pathway that is re-activated in human cancers and promotes tumorigenicity (13, 36). An evaluation of TMEFF2 function in a transgenic mouse model may provide important information into its role in development/tumorigenesis.

TMEFF2 knockout mice have been developed by two separate groups and in both cases resulted in pups that died at weaning (16, 52). The mice survived to birth, but the phenotypic analysis of the TMEFF2 knockout was limited due to the very short lifespan of the pups. However, it was observed that the mice had empty stomachs at the time of death and it was suggested that this may have been due to a neuronal defect affecting the ability to masticate the mouse diet (52). Chen et al. showed that the TMEFF2 knockout mice were of a significantly smaller size than wild-type mice, but otherwise found no structural or molecular defects in the brains of these mice or changes in prostate morphology (16). Although the results of this model are intriguing with respect to its role in brain development, it offered little insight into its role in prostate tissue or in tumorigenesis. In order to study the role of TMEFF2 in the development/homeostasis of the mouse prostate and its role in prostate cancer, we constructed a novel mouse model with prostate-specific TMEFF2 overexpression driven by the androgen-regulated probasin promoter (PB-TMEFF2).

Here we describe the generation of the PB-TMEFF2 transgenic mouse model and its initial characterization. Two PB-TMEFF2 mouse lines were established that express high levels of TMEFF2 in the prostates of transgenic mice. These mice display a normal development and no gross phenotypic effects as a result of TMEFF2 overexpression, and an examination of prostate tissue sections from control and transgenic mice revealed no abnormalities in the architecture of the glands. These sections were also examined for evidence of hyperplasia, PIN, or prostate carcinoma to rule out the possibility that TMEFF2 alone can promote prostate tumor initiation. Transgenic prostates did not show evidence of tumorigenesis being initiated as a result of TMEFF2 expression.

## Results

### Construction of the PB-TMEFF2 Transgenic Expression Construct

To evaluate the functional role of TMEFF2 in prostate development and in prostate tumorigenesis, we developed a novel transgenic mouse model which overexpressess TMEFF2 specifically in prostate tissue. The objective in designing the transgene expression vector was to achieve high levels of TMEFF2 expression exclusively in the prostate and to ensure the highest chance of success for transgene integration into the genome. The pTg1 expression vector (provided by Dr. R. Thresher, UNC-Chapel Hill) contains two multiple cloning sites separated by an intronic sequence of the mouse transthyretin gene. To achieve high levels of prostate-specific TMEFF2 expression we utilized a modified version of the rat probasin (*PB*) gene promoter, the $ARR_2PB$ composite probasin promoter (from Dr. R. Matusik, Vanderbilt University), that has been extensively characterized in the production of transgenic animals (109). The $ARR_2PB$ promoter was inserted into Exon 1 position, which is followed by the transcription start site, and human full-length TMEFF2 cDNA was inserted into Exon 2 position. Immediately downstream from Exon 2 is an SV40 early-region polyadenylation signal sequence (Fig. 14A).

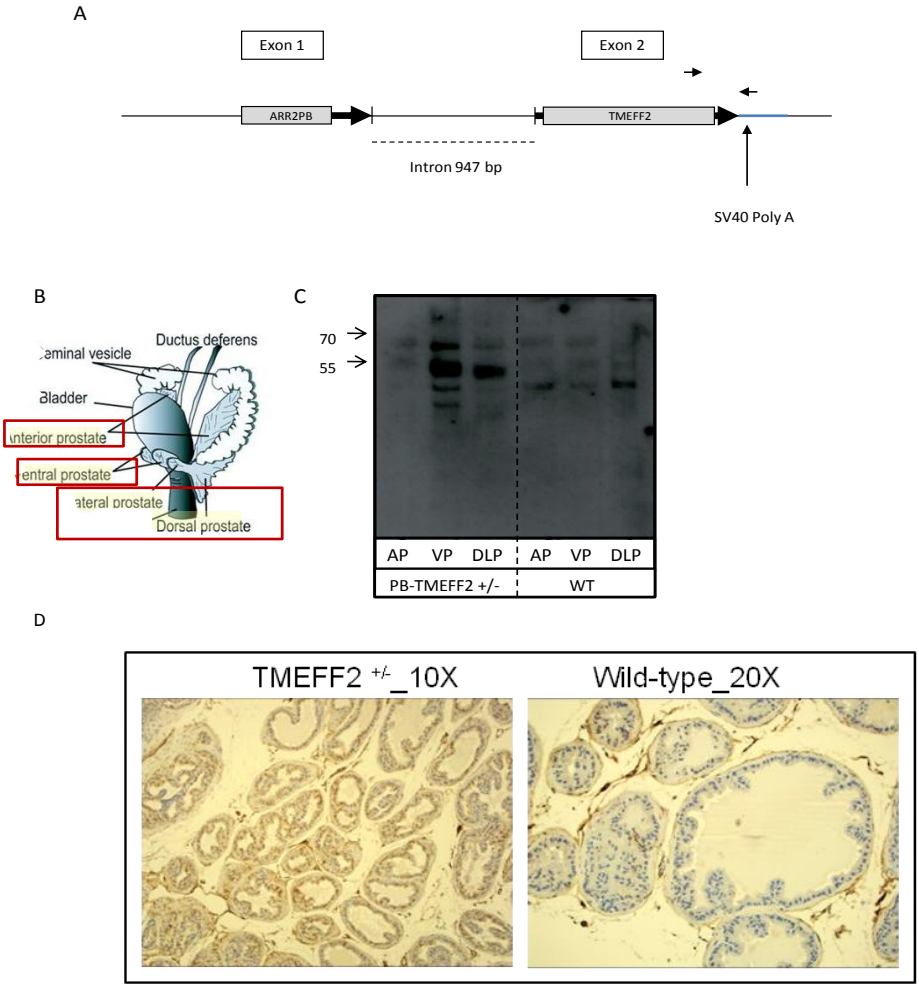


**Figure 14 . Generation and characterization of PB-TMEFF2 transgenic mice. A)** Linear transgene expression vector used to inject into mouse embryos. Arrows indicate the positions of primer binding for genotyping by PCR. **B)** Illustration depicting the lobes of the mouse prostate and other parts of the urogenital system. Prostatic lobes are highlighted in yellow. The dorsal and lateral prostatic lobes are often grouped together as the dorsolateral prostate. **C)** Western blot showing TMEFF2 expression in the anterior, ventral, and dorsolateral prostatic lobes of a

heterozygous (PB-TMEFF2 +/-) and wild-type (WT) littermate from line 478. **D)** Immunohistochemistry staining for TMEFF2 protein in a heterozygous PB-TMEFF2 ventral prostate (left) and a wild-type littermate (right).

**Generation and Identification of PB-TMEFF2 Transgenic Mice**

The transgenic expression vector was injected into C57BL/6 X DBA2 hybrid embryos to produce transgenic founder mice by the UNC-Chapel Hill animal models core facility according to established methods (49). Transgenic F0 mice (first generation following pronuclear injections) were identified by DNA extraction from tail snips followed by PCR with primers specific for the PB-TMEFF2 transgene (Fig. 14A), and 8 mice were initially identified that carried the transgene out of a total of 31 FO mice (resulting from 2 pronuclear injections and subsequent implantations). All 8 transgene-positive F0 mice were male, and each was crossed with a wild-type C57BL/6 female to produce F1 offspring that are heterozygous for the transgene if it is transmitted. Transgenic DNA must be incorporated into the germ cells of the F0 mice in order for the foreign DNA to be transmitted to offspring of the F1 generation. We therefore used PCR genotyping to identify the F1 pups that inherited the transgenic DNA. Two of the founder mice (#478 and #485) transmitted the transgenic DNA and resulted in the generation of two independent mouse lines to study TMEFF2 function.

**Necropsy and Preliminary Evaluation of TMEFF2 Effects on Prostate Tissue**

Post-pubescent mice from lines 485 and 478 were sacrificed and the prostates removed and separated into individual lobes. To assess levels of TMEFF2 protein expression in the individual lobes of the mouse prostates, protein was prepared from each prostatic lobe for

western blotting, consisting of the anterior, ventral, and dorsolateral lobes (Figure 14B). We observed TMEFF2 protein expression in each lobe of prostates from transgenic mice and no TMEFF2 expression in prostatic lobes from wild-type control mice (Fig. 14C). A greater amount of TMEFF2 expression was observed in the prostates of transgenic mice of line 478 than in line 485. The pattern of expression in the different lobes, however, was the same in both transgenic lines. The highest levels of TMEFF2 protein expression were detected in the ventral lobe, followed by the dorsolateral lobe, and minimal expression was detected in the anterior prostatic lobe. This pattern of expression in the prostatic lobes is consistent with previously reported transgenic mouse lines with probasin promoter-driven gene expression (109). TMEFF2 overexpression was also demonstrated by immunohistochemistry in the ventral lobe of a heterozygous PB-TMEFF2 mouse and no TMEFF2 was detected in a wild-type littermate (Fig. 14D).

To examine the effect of TMEFF2 overexpression on the prostate development, heterozygous male transgenic mice were sacrificed at various ages ranging from 6 to 70 wks along with male age-matched, wild-type littermates as controls. Transgenic mice from each litter were identified by PCR with transgene-specific primers (Table 1). There were no apparent developmental/behavioral abnormalities in the transgenic mice, and the litter sizes produced by transgenic males were normal relative to wild-type males. Additionally, a gross examination of transgenic urogenital organs likewise reveals no differences with the control groups. A pathological examination of prostate tissue sections did not reveal an effect of TMEFF2 overexpression on the development of the prostate, and the glandular architecture in each lobe was normal relative to those of wild-type littermates. Furthermore, there was no induction of PIN or cancer in the transgenic mice, suggesting that TMEFF2 alone does not promote cancer

initiation. The lack of tumor initiation is consistent with a role for TMEFF2 as a tumor suppressor; however, its function in later stages of prostate cancer remains to be evaluated.

## Discussion

Here we show the generation of a novel transgenic mouse model overexpressing TMEFF2 in the prostate and initiate its characterization. This model was constructed with the hopes of enhancing our understanding of the functional role of TMEFF2 in the prostate and in prostate tumorigenesis. The results presented here represent a preliminary evaluation of the functional role of TMEFF2 in the development of the mouse prostate, and an initial step in an examination of the role of TMEFF2 in prostate cancer. A role for TMEFF2 in the development or function of the prostate was not identified in this study as no abnormalities were found in either gross or microscopic examinations of transgenic prostate tissue. It is important to note, however, that the induction of transgene expression using the $ARR_2PB$ promoter remains at very low levels until about 5 wks of age in mice (109), and therefore an influence of TMEFF2 on the early stages of prostate development may not be detected using this model. No induction of PIN or any evidence of prostate cancer suggests that TMEFF2 on its own does not have a role in the initiation of prostate cancer, consistent with our proposed role for TMEFF2 as a tumor suppressor. However, the effects of TMEFF2 overexpression on the entire course of prostate tumorigenesis were not addressed in these studies.

The PB-TMEFF2 mouse model will be a valuable tool to study the role of TMEFF2 in prostate tumorigenesis by crossing it with genetically engineered mouse models of prostate cancer that progress through all stages of prostate tumorigenesis. For instance, the transgenic TRAMP mouse model develops prostate cancer foci as a result of the prostate-specific expression of the SV40 T antigen. Pathologically the tumors that develop in this model closely mimic the human disease state and rapidly progress to invasive carcinoma and metastasis (38).

The PB-TMEFF2 transgenic model presented here will be crossed with the TRAMP model in future studies in order to evaluate its role in the development and progression of prostate cancer in a mammalian model. Based on our results that TMEFF2 overexpression does not lead to tumor development in the mouse prostate, coupled with the tumor suppressor effects observed for full-length TMEFF2 in cell culture (see chapter 3), we propose that in normal tissue and early stages of cancer development TMEFF2 may function as a tumor suppressor. We therefore expect that TMEFF2 will initially suppress tumor growth and delay the progression of the cancer. However, excess levels of inflammatory cytokines in the growing tumor would likely induce the cleavage of TMEFF2. If this is the case, the upregulation of TMEFF2 would have the opposite effect as the full-length TMEFF2 and instigate tumor progression in the later stages. This theory is in line with the evidence that TMEFF2 is overexpressed in prostate cancer tissue relative to benign tissue (39, 71), which is generally an occurrence that is associated with oncogenes. The PB-TMEFF2 model can also be utilized to study the influence of TMEFF2 overexpression on prostate tissue regeneration following castration. As mentioned, castration leads to massive apoptosis in the prostate which will re-form upon the return of androgen signaling. The delayed expression of TMEFF2 (until ~ 5 wks) in the PB-TMEFF2 model limits our ability to assess the effects of TMEFF2 overexpression on prostate development, much of which occurs during the 1-5 wk period after birth (14). An examination of the effects of TMEFF2 overexpression on prostate tissue regeneration can be performed in post-pubescent (TMEFF2 expressing) mice and may reveal a role for TMEFF2 in prostate development.

# CHAPTER 6

## DISCUSSION

An influential role for TMEFF2 in the establishment and/or progression of prostate cancer is suggested by its upregulation in a significant fraction of prostate cancers and its limited expression in other tissues (39, 71, 99). The potential for TMEFF2 to influence signaling pathways in prostate cancer cells is highlighted by its EGF-like and follistatin domains, capable of modulating growth factor pathways with well established roles in tumorigenesis (5, 99), as well as a putative G-protein-activating motif at its C-terminus. Although some significant insights into its function have been revealed since it was originally cloned, its role in prostate cancer has remained poorly understood. In order for the treatment of prostate cancer to move towards novel therapies, substantial improvements in understanding the molecular basis of disease progression are needed, particularly of the factors that drive its progression and the pathways that lead to their deregulation.

In the studies presented here, an evaluation of the phenotypic effects of TMEFF2 overexpression *in vitro* and *in vivo* demonstrates its tumor suppressor function. Results from phenotypic assays show that its tumor suppressive activity is pleiotropic, capable of blocking cell invasion, reducing proliferation rates, antagonizing cell survival, and inhibiting anchorage-independent growth. The potential for TMEFF2 to suppress several tumorigenic hallmark traits makes it difficult to point to a certain stage/phase of disease progression in which the tumor suppressor primarily functions. With the described functional effects, TMEFF2 could presumably influence multiple disease stages and suppress tumor progression, from the earliest

tumor stages to metastasis. These *in vitro* results are supported by the inhibition of subcutaneous TRAMP-C2 tumor development in an allograft model when TMEFF2 is expressed.

Although our results demonstrate a role for the full-length TMEFF2 as a tumor suppressor, it is reasonable to suspect that the function of TMEFF2 may change throughout the progression of prostate cancer. This possibility is based on previous results that have demonstrated opposing influences of the full-length and ectodomain forms of TMEFF2 on cancer cells, suggesting a potential dual function in prostate cancer (5, 17). An abundance of genetic and environmental changes that occur during the course of tumorigenesis can modulate the function of some genes, and several genes have been demonstrated to have dual roles in tumorigenesis with functions that can be dependent on the cancer stage, activated oncogenic pathways, or other environmental factors (2, 20). Ultimately, we believe that the effects of TMEFF2 overexpression on prostate tumorigenesis are dependent on whether the full-length (membrane-bound) form predominates or if it exists mostly as a cleaved ectodomain in the tumor environment. A potential pro-tumorigenic role at later stages of disease progression would be consistent with the observation that TMEFF2 is upregulated in advanced prostate cancer, generally a characteristic of an oncogene.

The observation that androgens can stimulate a post-transcriptional increase in TMEFF2 expression suggests a possible mechanism by which TMEFF2 is overexpressed in prostate cancer. Both the normal prostate and prostate cancer are critically reliant on androgen signaling pathways, and evidence has surfaced in recent years that the post-transcriptonal regulation of gene expression by androgens may have a central role in prostate carcinogenesis (48, 108). We therefore investigated the post-transcriptional mechanisms controlling TMEFF2 expression and the regulatory connection to androgen signaling. Our results demonstrate that TMEFF2 is

translationally regulated by androgen signaling through several uORFs in its 5' UTR. Upon androgen stimulation, its translation is selectively increased through the AR-dependent induction of eIF2α-P in prostate cancer cells. Although the functional effects of eIF2α-P in tumorigenesis are not well understood, modulating its activity could have a profound impact on cell behavior through the selective regulation of uORF-containing transcripts. A regulatory connection between AR signaling and the major translational regulators may represent a novel means to block the tumorigenic effects of androgens and the AR in prostate cancer. This is exemplified by the recent development of a novel mTOR inhibitor, INK128, that demonstrated a strong inhibition of prostate cancer invasion and metastasis *in vivo* by blocking 4EBP1 phosphorylation and is currently in clinical trials (48). As previously mentioned, mTOR is activated by androgen signaling (108) and drives prostate cancer cell invasion through the modulation of eIF4E availability (48). These results highlight the promise of identifying and therapeutically targeting specific translational components, particularly the major regulators, that are influenced by androgen signaling.

We propose a model in which TMEFF2 is expressed in normal prostate tissue and exists primarily in a membrane-bound form that functions as a tumor suppressor (Fig. 15). Based on previous data, its role as a tumor suppressor may be mediated in part by modulating intracellular sarcosine levels and/or modulating PDGF signaling. Upon tumor initiation/progression TMEFF2 expression is increased, potentially as a means to suppress tumor growth or progression. This effect is mediated by AR transcriptional and/or translational stimulation. However, the inflammatory conditions in the tumor microenvironment would presumably stimulate TMEFF2 cleavage by ADAM proteinases and lead to increased ectodomain levels. The cleavage of TMEFF2 causes a switch in TMEFF2 function from a tumor suppressor to an

oncogene, promoting the advance to late stages of prostate cancer by increasing proliferation rates/survival of the cancer cells. Based on the model presented here, it is critically important to better understand the mechanism of TMEFF2 shedding from the membrane and at what stage in prostate tumor progression this occurs. Since the full-length TMEFF2 suppresses tumorigenicity while the ectodomain may drive it, the inhibition of its cleavage may represent a promising therapeutic strategy.

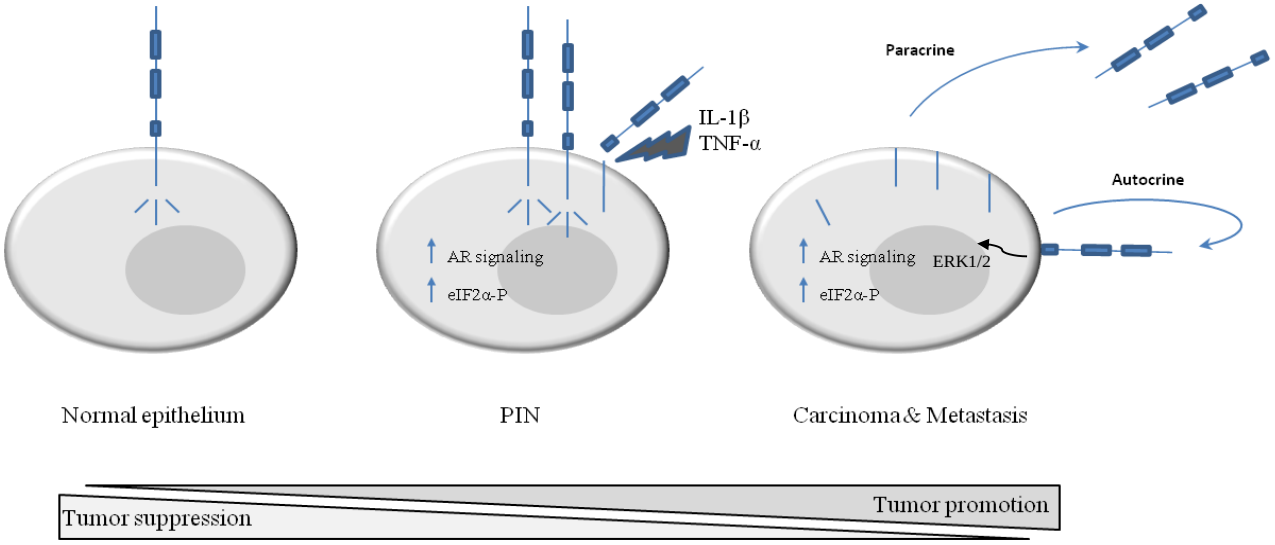


**Figure 15. Model of TMEFF2 action in the course of prostate tumorigenesis.** TMEFF2 is primarily membrane-bound and signals as a tumor suppressor in normal prostate epithelium and early stages of tumorigenesis. Upon the growth and progression of prostate tumor foci, AR activity leads to increased TMEFF2 expression either transcriptionally or at the level of translation through increased eIF2α-P. TMEFF2 likely functions as a tumor suppressor until pro-inflammatory cytokines build up in the tumor environment, stimulating TMEFF2 cleavage from the membrane by ADAM proteinases. Its cleavage from the membrane may represent a

change in TMEFF2 function from a tumor suppressor to an oncogenic role. Advanced, castration-resistant prostate cancers are characterized by deregulated androgen signaling, which would lead to elevated TMEFF2 expression and potentially promote its progression to terminal stages.

Finally, we have presented the generation and initial characterization of the PB-TMEFF2 transgenic mouse model overexpressing TMEFF2 specifically in prostate tissue. The PB-TMEFF2 model will be a crucial tool in the examination of TMEFF2 function in prostate cancer. Crossing the PB-TMEFF2 model with the TRAMP prostate cancer model will allow for the evaluation of TMEFF2 function in the entire course of prostate cancer progression, from disease initiation to metastasis. We expect that the TRAMP mice that overexpress TMEFF2 will have a delayed onset of tumor progression as a result of its tumor suppressor activity; however, upon tumor development we believe that TMEFF2 will accelerate tumor progression and metastasis as a result of increased levels of TMEFF2 ectodomain signaling. The PB-TMEFF2 x TRAMP model will provide valuable information on the levels of TMEFF2 ectodomain that are shed from the membrane throughout tumor progression by its detection in plasma and/or urine, and increases in ectodomain levels in these fluids should coincide with the onset of disease progression. Additionally, its restricted tissue distribution and cleavage from the membrane also make TMEFF2 a promising diagnostic or prognostic biomarker, and this possibility will be evaluated using the PB-TMEFF2 mouse model.

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## APPENDIX A

East Carolina University

**Animal Care and Use Commitee**
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

**252-744-2436** office
**252-744-2355** fax

October 1, 2010

Maria Ruiz-Echevarria, Ph.D.
Department of Medicine
Brody 3E
ECU Brody School of Medicine

Dear Dr. Ruiz-Echevarria:

Your Animal Use Protocol entitled, "The Role of TMEFF2 in Prostate Cancer Development," (AUP #J205) was reviewed by this institution's Animal Care and Use Committee on 10/1/10. The following action was taken by the Committee:

"Approved as submitted"

***Please contact Dale Aycock at 744-2997 prior to biohazard use***

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

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