

## **ABSTRACT**

Molecular Mechanisms of TMEFF2 Action in Prostate Cancer

By

Xiaofei Chen

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Director of Dissertation: Maria J. Ruiz-Echevarria, Ph.D.

Department of Biochemistry and Molecular Biology

The transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) is an evolutionarily conserved type I transmembrane protein expressed in the embryo and limited adult tissues, mainly the brain and the prostate. The ectodomain of TMEFF2 can be cleaved from the membrane in an ADAM17/ $\gamma$ -secretase-dependent fashion and consists of an epidermal growth factor-like motif and two follistatin motifs. The cytoplasmic portion contains a potential G protein-activating (GA) domain. Given its elevated expression in primary and metastatic prostate cancer, TMEFF2 has been implicated to play a role in this disease. However, the exact biological function of TMEFF2 is rather controversial, with conflicting reports supporting both tumor-suppressing and growth-promoting activities of TMEFF2.

In the present study, we demonstrate a dual mode of action for TMEFF2. Ectopic expression of wild-type full-length TMEFF2 inhibits monolayer and anchorage-independent cell growth, cellular invasion and migration, and increases cellular sensitivity to apoptosis. In contrast, expression of TMEFF2 ectodomain or addition of conditioned medium containing the ectodomain increases cell proliferation, in line with previous results using recombinant TMEFF2

ectodomain protein. Furthermore, we investigate the molecular mechanisms involved in the function of TMEFF2. TMEFF2 interacts with sarcosine dehydrogenase and modulates cellular levels of sarcosine, a differential metabolite that increases during prostate cancer progression. The tumor suppressor activity of TMEFF2 correlates in part with its ability to modulate sarcosine levels. Moreover, TMEFF2 expression decreases prostate cancer cell spreading and migration, particularly on vitronectin, with a concomitant decrease in focal adhesion and stress fiber formation. Consistently, TMEFF2 inhibits RHOA activation – RHOA activation is known to induce stress fiber formation -- and downregulates expression of several integrins including  $\beta 1$  and the major receptor for vitronectin --  $\alpha v\beta 3$ . All these effects require the presence of the GA domain, as expression of a TMEFF2 mutant lacking the GA domain does not affect RHOA activation or integrin expression. Finally, we show that different forms of TMEFF2 differentially regulate AKT and ERK activation. While the full-length TMEFF2 protein promotes ERK phosphorylation in response to growth factors EGF and PDGF-AA, the ectodomain activates AKT and inhibits ERK phosphorylation, which may contribute to the distinct cellular responses of tumor suppression or proliferation. Altogether, these data provide significant knowledge on the molecular mechanisms of TMEFF2 action, shedding light on the seemingly conflicting roles of various TMEFF2 forms.

# **MOLECULAR MECHANISMS OF TMEFF2 ACTION IN PROSTATE CANCER**

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By

Xiaofei Chen

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Xiaofei Chen

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By

Xiaofei Chen

Approved by:

Director of Dissertation: \_\_\_\_\_  
Maria J. Ruiz-Echevarria, Ph.D.

Committee Member: \_\_\_\_\_  
Phillip H. Pekala, Ph.D.

Committee Member: \_\_\_\_\_  
Brett D. Keiper, Ph.D.

Committee Member: \_\_\_\_\_  
Li Yang, Ph.D.

Chair of the Department of Biochemistry and Molecular Biology:

\_\_\_\_\_  
Phillip H. Pekala, Ph.D.

Dean of the Graduate School: \_\_\_\_\_  
Paul J. Gemperline, Ph.D.

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## ABBREVIATIONS

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ADAM	A disintegrin and metalloprotease
cAMP	Cyclic adenosine monophosphate
ADT	Androgen deprivation therapy
AR	Androgen receptor
ATCC	American type culture collection
BMP	Bone morphogenetic protein
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFP	Cyan fluorescent protein
CHD1	Chromodomain helicase DNA-binding protein 1
CMV	Cytomegalovirus
CRPC	Castration-resistant prostate cancer
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium

DMGDH	Dimethylglycine dehydrogenase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAP	GTPase-activating protein
GEF	Guanine nucleotide-exchange factor
GNMT	Glycine N-methyltransferase
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate

HBME	Human bone marrow endothelial
HEK293	Human embryonic kidney 293
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IP	Immunoprecipitation
K-SFM	Keratinocyte serum-free medium
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MS	Mass Spectrometry
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCA3	Prostate cancer antigen 3
PCR	Polymerase chain reaction

PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositide 3-kinase
PIN	Prostatic intraepithelial neoplasia
PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PSA	Prostatic-specific antigen
PTEN	Phosphatase and tensin homolog
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAM	S-adenosylmethionine
SARDH	Sarcosine dehydrogenase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SFK	SRC family kinase
SH2	SRC-homology 2
shRNA	Small hairpin ribonucleic acid

siRNA	Small interfering ribonucleic acid
SPINK1	Serine protease inhibitor Kazal-type 1
SPOP	Speckle-type POZ protein
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
TGF	Transforming growth factor
THF	Tetrahydrofolate
TMEFF	Transmembrane protein with epidermal growth factor and two follistatin motifs
TNF $\alpha$	Tumor necrosis factor alpha
TOF	Time-of-flight
TRE	Tetracycline response element
TRAMP	Transgenic adenocarcinoma of the prostate
YFP	Yellow fluorescent protein

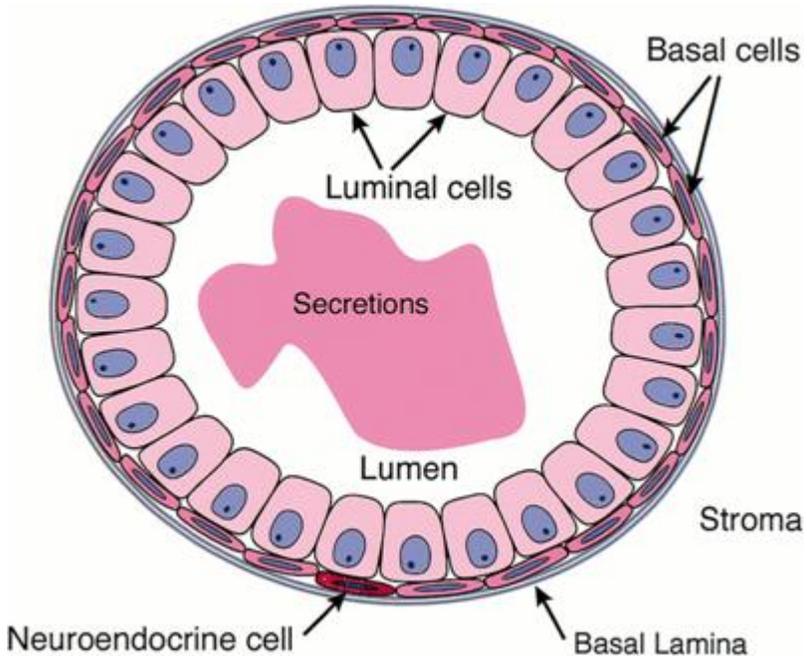
## **CHAPTER ONE: Introduction**

### **The prostate gland and prostate cancer**

As part of the male reproductive system, the prostate gland is a walnut-sized exocrine gland that secretes important components of the seminal fluid. Although the adult prostate lacks discernible lobular structure, it can be divided into three zones: peripheral, transition, and central (McNeal 1988). There are at least three distinct cell types within the prostatic epithelium: luminal, basal, and neuroendocrine (Figure 1). The luminal cells form a continuous layer of columnar cells, serving a secretory function to produce prostatic fluid. Basal cells are located between the luminal cells and the underlying basement membrane. Neuroendocrine cells are rare cells of uncertain origin and function dispersed throughout the basal layer. Both basal and luminal cells have been shown to contain putative prostate stem cells (Goldstein et al. 2008; Leong et al. 2008; Richardson et al. 2004; Wang et al. 2009).

Prostate cancer has become a major public health concern in developed countries. It is the most commonly diagnosed cancer and the second leading cause of cancer deaths in American men. The American Cancer Society has estimated that in 2013, in the United States alone, there will be 238,590 new cases diagnosed, with 29,720 prostate cancer deaths (Siegel et al. 2013).

Prostate cancer originates mostly in the peripheral zone and is a heterogeneous and multifocal disease. Within a given section of prostate cancer tissue, there can be benign glands, preneoplastic foci, and neoplastic foci of varying severity juxtaposed to each other. Moreover, individual neoplastic foci can be genetically distinct (nonclonal) even in close proximity (Abate-Shen and Shen 2000). The vast majority of prostate cancer is classified pathologically as adenocarcinoma, characterized by a luminal phenotype and the disappearance of the basal layer.



**Figure 1.** Schematic depiction of the cell types within the prostatic duct (Abate-Shen and Shen 2000).

If prostate cancer metastasizes, it most frequently goes to bone, followed in frequency by liver, lymph nodes, and lungs (Shah et al. 2004).

Currently the mainstay for early detection of prostate cancer is a blood PSA test. PSA is a kallikrein serine protease secreted by luminal cells of the prostatic epithelium. The blood level of PSA is often elevated in men with prostate cancer, as a consequence of disruption of normal prostate architecture (Lilja et al. 2008); however, other benign prostate conditions such as benign prostatic hyperplasia (BPH) or prostatitis can also cause an elevation in PSA levels. In addition, some men who have prostate cancer do not have elevated PSA, making PSA tests prone to both false-positive and false-negative results. Moreover, the potential risk of over-diagnosis and over-treatment may outweigh the benefits of screening because much of PSA-detected prostate cancer is clinically indolent and requires only conservative management (Linn et al. 2007). Nevertheless, an abnormality found after the PSA test in men at high risk prompts a recommendation that the man undergo prostate biopsy to assess the potential presence of prostate cancer. Following biopsy, the severity of the cancer is evaluated by the Gleason grading system. A Gleason score is the sum of the two most prevalent patterns, which classify tumors from 1 to 5 (well to poorly differentiated). If prostate cancer is diagnosed when it is confined to the prostatic capsule, it is potentially curable by surgical intervention and/or radiation therapy. However, if not detected and left untreated, prostate carcinoma may advance to more aggressive forms characterized by local invasion of the seminal vesicles, followed by metastasis primarily to the bone. Since the tumor is initially dependent on androgens for growth, the standard treatment for advanced prostate cancer is androgen deprivation therapy (ADT), either by surgical or chemical castration. Unfortunately, the disease almost inevitably recurs as castration resistant prostate

cancer (CRPC), which is currently untreatable and finally results in lethality (Abate-Shen and Shen 2000).

There are four major challenges in diagnosing and treating prostate cancer. First, just as mentioned above, although ADT is initially effective in causing tumors to regress, recurrence to CRPC is inevitable and conventional chemotherapeutic regimens for CRPC have been at most palliative. It has become apparent that AR signaling remains active in CRPC through a variety of mechanisms, including: (1) de novo androgen biosynthesis and conversion of adrenal androgen precursors in tumor tissues (Chang et al. 2013; Locke et al. 2008; Montgomery et al. 2008; Stanbrough et al. 2006), (2) AR hypersensitivity via AR gene amplification, protein stabilization, or increased recruitment of AR coactivators (Fujimoto et al. 2007; Gregory et al. 2001; Linja et al. 2001), (3) AR gene mutations leading to promiscuous AR activation by noncognate ligands or ligand-independent activation (Culig et al. 1993; Dehm et al. 2007; Steinkamp et al. 2009; Thin et al. 2003; Zhao et al. 2000), (4) expression of AR alternative splice isoforms that are constitutively active (Dehm et al. 2008; Guo et al. 2009; Hu et al. 2009), and (5) growth factor-mediated ligand-independent AR activation and increased AR transcription activity (Gao et al. 2006; Guo et al. 2006; Jiao et al. 2007; Xu et al. 2009). Based on these findings, novel therapies targeting the AR pathway (i.e. androgen synthesis inhibitors, AR antagonists) have become available and have been proven to significantly increase survival in phase III clinical trials (de Bono et al. 2011; Ryan et al. 2013; Scher et al. 2012).

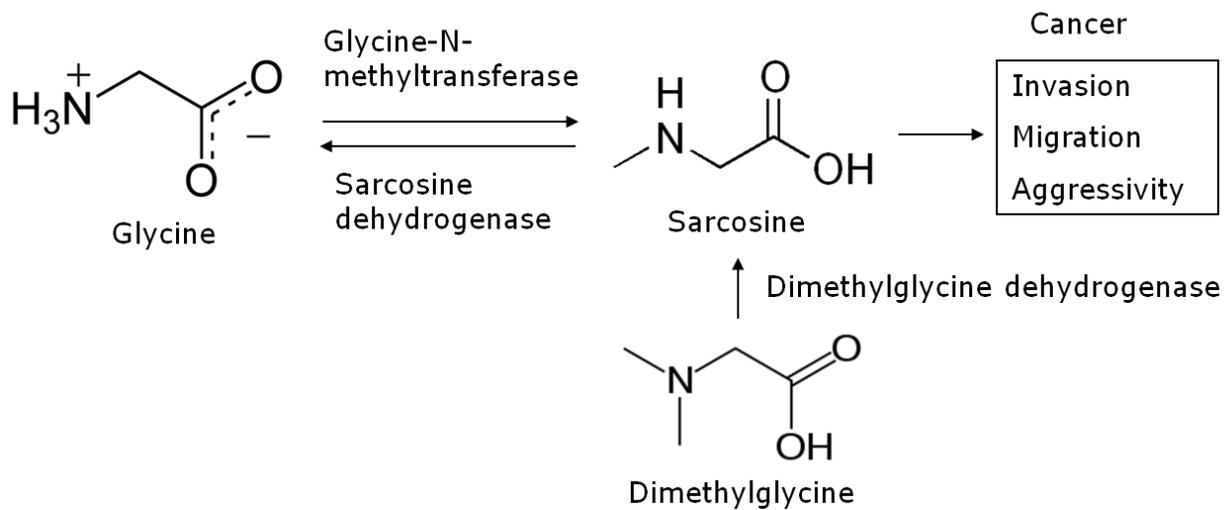
Second, prostate cancer metastasizes primarily to bone with osteoblastic lesions, which is the main cause of patient morbidity and mortality. It has been established that during metastasis, cancer cells carry out the following sequence of events, often referred to as the metastatic cascade: they break away from their neighboring cells and the basement membrane, invade

through the interstitial stroma, intravasate into the lymph or blood system, survive in the circulation, extravasate from the bloodstream, and proliferate in the new microenvironment to form secondary tumors. The molecular mechanisms underlying the bone tropism of prostate cancer is very poorly understood, but is likely to involve bone endothelium attachment and extravasation, and colonization in the bone. In fact, *in vitro* binding experiments have shown that prostate cancer cells have higher affinity to human bone marrow endothelial (HBME) cells than to other endothelial cells (Lehr and Pienta 1998; Cooper et al. 2000). The binding of prostate cancer cells to HBME cells was inhibited by integrin  $\beta 1$  antibody but not antibodies to other integrins, suggesting that interaction between prostate cancer cells and bone marrow endothelium is primarily mediated by integrin  $\beta 1$  (Scott et al. 2001). In addition, bone matrix components such as osteonectin and osteopontin (Jacob et al. 1999; Khodavirdi et al. 2006) attract prostate cancer cells and promote their spreading and growth in the bone. Integrin  $\alpha\beta 3$  is heavily implicated in this process, as it is the receptor for these bone matrix proteins. Functional integrin  $\alpha\beta 3$  promoted prostate cancer cell migration to the bone matrix and enabled tumor growth in the bone in a mouse xenograft model, whereas inactive or constitutively active  $\alpha\beta 3$  mutants did not (McCabe et al. 2007).

Third, there is a lack of accurate biomarkers for prostate cancer diagnosis and prognosis. While the advent of serum PSA screening for prostate cancer has dramatically increased cancer detection, the limitations of serum PSA screening as mentioned earlier have fueled the search for new prostate cancer biomarkers. A number of potential biomarkers have emerged. Notably, urine prostate cancer antigen 3 (PCA3), a noncoding prostate-specific transcript overexpressed in greater than 95% of prostate cancers (Bussemakers et al. 1999) has proven to be useful as an adjunct to serum PSA for prostate cancer detection (Deras et al. 2008; Groskopf et al. 2006).

Furthermore, about 50% of prostate cancers harbor the *TMPRSS2:ERG* fusion gene as a result of chromosomal rearrangement (Tomlins et al. 2009). Urine *TMPRSS2:ERG* transcript was shown to be associated with the presence of clinically significant cancer and the combined measurement of *TMPRSS2:ERG* and *PCA3* in urine outperformed serum PSA for prostate cancer diagnosis (Tomlins et al. 2011).

In 2009, a study (Sreekumar et al. 2009) revealing sarcosine as a promising prostate cancer biomarker aroused considerable enthusiasm in the biomarker field. The study showed that sarcosine, also known as N-methylglycine, was significantly increased in prostate cancer specimens compared to benign adjacent prostate samples, and was even higher in metastatic tissues. Additionally, sarcosine levels were significantly higher in urine samples from biopsy-positive than biopsy-negative individuals, performing better than PSA when restricted to samples having PSA in the clinical grey zone of 2-10 ng/ml. In prostate cell lines, there was a correlation between sarcosine levels and cell invasiveness, and the mere addition of exogenous sarcosine imparted an invasive phenotype to benign prostate epithelial cells. One can also alter cell invasiveness by modulating enzymes regulating sarcosine metabolism (Figure 2), as knockdown of glycine N-methyltransferase (GNMT) or dimethylglycine dehydrogenase (DMGDH), enzymes responsible for sarcosine formation, resulted in a significant reduction in cell invasion, while knockdown of sarcosine dehydrogenase (SARDH), which converts sarcosine back to glycine, increased cell invasion. Since the publication of these findings, the potential use of sarcosine as a prostate cancer biomarker has been tested by many different groups, either challenging (Jentzmik et al. 2010, 2011; Colleselli et al. 2010; Struys et al. 2010; Wu et al. 2011; Bohm et al. 2012) or supporting (Bianchi et al. 2011; Lucarelli et al. 2012, 2013; Koutros et al. 2013) these findings (reviewed in Issaq and Veenstra 2011; Cernei et al. 2013). The conflicting



**Figure 2.** Schematic of the sarcosine pathway and its potential link to prostate cancer

(adapted from Sreekumar et al. 2009).

data in these studies are likely due to a cumulative effect of differences in assay methods, sample selection, handling, storage and analysis. Currently the role of sarcosine as a biomarker for prostate cancer remains unclear.

Lastly, although a high Gleason score is indicative of rapid progression and poor prognosis in prostate cancer and requires immediate treatment, the appropriate treatment for patients with a low Gleason score remains ambiguous. Most of these patients may only require conservative management, since their tumors are relatively indolent, while a small fraction of them progress rapidly to an aggressive disease. Thus, there is a critical need to distinguish most of the low Gleason score tumors that will remain indolent from the few that are truly aggressive. This is part of the challenge in the previous point – lack of reliable biomarker with prognostic information. Much research has been focused on identifying molecular signatures that distinguish indolent versus aggressive forms of prostate cancer. For example, Markert et al. (2011) stratified patients with low Gleason score on the basis of their mRNA microarray signature profiles. Their classification demonstrates that a subset of tumors with stem-like signatures together with TP53 and PTEN inactivation is associated very poor survival outcome, while a second group characterized by the Tmprss2:ERG fusion has intermediate survival outcome. In a recent study using gene set enrichment analysis and decision tree algorithm, Irshad et al. (2013) identified a three-gene biomarker panel -- *FGFR1*, *PMP22*, and *CDKN1A* -- that is predictive of indolent prostate cancer of low Gleason score.

Unlike other epithelial tumors such as breast cancer, prostate cancer lacks distinguishable histopathological subtypes that differ in their prognosis and treatment response. Molecular classification of prostate cancer subtypes could help identify genetic changes that drive tumor progression and potentially benefit prognosis and treatment. In addition to point mutations like

most cancers are associated with, the prostate cancer genome is extraordinarily complex, with large-scale genomic rearrangements and extensive copy number alterations (Barbieri et al. 2012a; Berger et al. 2011). With the help of next generation sequencing, prostate cancer genomic profiling has already identified several molecular subtypes. For example, as mentioned earlier, about 50% of prostate cancers have TMPRSS2:ERG rearrangement. Tumors without TMPRSS2:ERG rearrangement can be further categorized on the basis of SPINK1 overexpression (Tomlins et al. 2008), SPOP mutations (Barbieri et al. 2012b), and CHD1 deletions (Liu et al. 2012), which are often mutually exclusive. Although prognostic implications of these genetic alterations remain to be determined, it is hopeful that prostate cancer might soon transition from a poorly understood, clinically heterogeneous disease to a collection of homogeneous subtypes identifiable by distinct molecular signatures and vulnerable to targeted therapies.

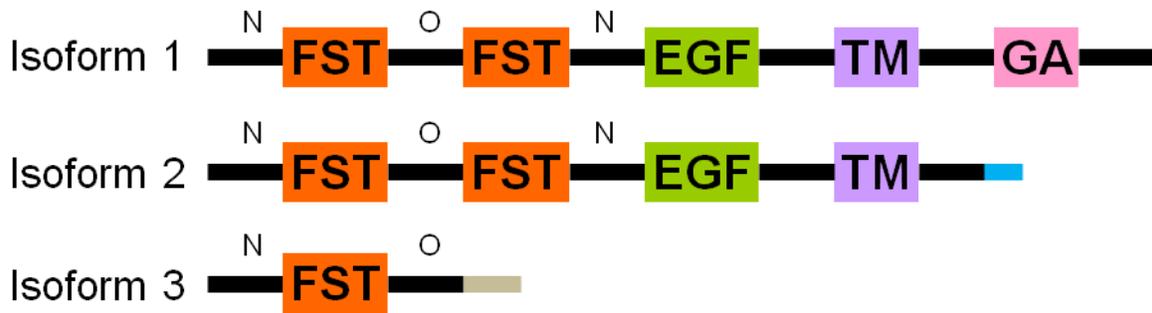
Overall, it is clear that prostate cancer is not driven by one or two gene mutations. It is, rather, due to an accumulation of mutations in tumor-related genes driving transformation of normal prostate epithelium to prostatic intraepithelial neoplasia (PIN), adenocarcinoma, and eventually to metastasis. Developing better biomarkers and therapeutic interventions relies on the understanding of the basic biology of the prostate and the molecular mechanisms underlying prostate cancer initiation and progression. The goal of the present study is to investigate the molecular basis of a protein highly expressed in prostate cancer -- TMEFF2. In the next section we will present the background information about this protein.

## **TMEFF2**

TMEFF2 (Transmembrane protein with epidermal growth factor and two follistatin motifs 2) was first characterized by Uchida et al. in 1999 in search of novel epidermal growth factor (EGF)-like proteins. It is a type I transmembrane protein with several biologically important features (Figure 3): An EGF-like and two follistatin domains are located within the extracellular portion of the protein, the ectodomain, which can be cleaved from the membrane in an ADAM17/ $\gamma$ -secretase-dependent fashion induced by proinflammatory cytokines (Ali and Knaüper 2007; Lin et al. 2003). Also located within the ectodomain are several potential glycosylation sites (Glynne-Jones et al. 2001; Uchida et al. 1999; Horie et al. 2000). The short cytoplasmic tail of TMEFF2 contains a potential G protein-activating domain (Uchida et al. 1999) and the TMEFF2 transmembrane domain has rhodopsin-like G protein-coupled receptor (GPCR) superfamily signature.

TMEFF2 is selectively expressed in the embryo and limited adult tissues, mainly the brain and the prostate, and overexpressed in prostate cancer (Liang et al. 2000; Glynne-Jones et al. 2001; Gery et al. 2002; Afar et al. 2004; Zhao et al. 2005). Because of its restricted expression profile and increased expression in prostate cancer, TMEFF2 has been implicated to play a role in this disease and has received considerable attention as a promising immunotherapeutic target for prostate cancer (Zhao et al. 2005; Afar et al. 2004; Zhao et al. 2008a; Boswell et al. 2012, 2013). However, the exact biological function of TMEFF2 in this disease remains elusive and rather controversial.

Elevated expression associated with higher prostate cancer grade (Zhao et al. 2005; Glynne-Jones et al. 2001; Afar et al. 2004) suggests that TMEFF2 promotes tumorigenesis. Supporting this, soluble TMEFF2 ectodomain was shown to increase survival of primary cultured neurons



**Figure 3.** Schematic representation of three TMEFF2 isoforms. Isoform 1 is the full-length TMEFF2 and has been chosen as the canonical sequence. Isoform 2 differs from isoform 1 in that it is missing the last 27 amino acids that encompass the putative G protein-activating motif, and that it has a KCP to AKL substitution in the three amino acids prior to the deletion. Isoform 3 contains only one follistatin domain and a 29-amino-acid C-terminal specific sequence. FST, follistatin domain; EGF, EGF-like domain; TM, transmembrane domain; GA, G protein-activating domain; N, N-linked glycosylation sites; O, O-linked glycosylation sites.

from the hippocampus and mesencephalon (Horie et al. 2000) and HEK293 human embryonic kidney cell proliferation, while knockdown of endogenous TMEFF2 by siRNA in LNCaP prostate cancer cells or TMEFF2-expressing HEK293 cells inhibited proliferation (Ali and Knaüper 2007).

In contrast, others have demonstrated a tumor suppressive function of TMEFF2. Overexpression of TMEFF2 in HCT116 colon cancer cells blocked tumor growth *in vivo* in nude mice and exerted anti-proliferative effects *in vitro* in cell culture (Elahi et al. 2008). In two prostate cancer cell lines DU145 and PC3, overexpression of TMEFF2 inhibited cell growth (Gery et al. 2002). Moreover, the promoter region of the *TMEFF2* gene is frequently hypermethylated in many cancers (Liang et al. 2000; Lin et al. 2011; Lee et al. 2012; Nagata et al. 2012; Selamat et al. 2011; Park et al. 2011; Tsunoda et al. 2009; Zhao et al. 2008b; Brücher et al. 2006; Suzuki et al. 2005; Ebert et al. 2005; Hanabata et al. 2004; Sato et al. 2002), suggesting a potential role of TMEFF2 as a tumor suppressor.

The complex roles of TMEFF2 in cancer may be attributed to the complexity of the TMEFF2 molecule. TMEFF2 could signal as a ligand precursor, a membrane bound receptor and/or a binding protein for growth factors (Glynn-Jones et al. 2001). Several isoforms of TMEFF2 have been described that differ in their C-termini (Figure 3; Quayle and Sadar 2006; Uchida et al. 1999; Horie et al. 2000) and probably their ability to signal and/or being modified. It is possible that functionally distinct isoforms are differentially expressed throughout prostate cancer establishment and progression. In addition, bioavailability of either the whole molecule or the cleavage products, as well as their ligands/receptors, may explain the opposing results in the context of different cell types.

Besides prostate cancer, studies have been focused on the role of TMEFF2 in pathologic conditions affecting the brain, based on the fact that TMEFF2 is expressed in human adult brain (Horie et al. 2000; Glynne-Jones et al. 2001; Uchida et al. 1999; Gery et al. 2002; Liang et al. 2000; Zhao et al. 2005; Afar et al. 2004). As mentioned earlier, soluble TMEFF2 ectodomain promoted the survival of hippocampal and mesencephalic neurons and stimulated dendrite growth of the latter (Horie et al. 2000), making TMEFF2 an attractive candidate in treating neurodegenerative disorders such as Parkinson's disease. TMEFF2 has also been hypothesized to contribute to the pathogenesis of Alzheimer's disease, as it was found present in plaques in Alzheimer's disease brain (Siegel et al. 2006). Lastly, Lin et al. (2011) showed TMEFF2 was downregulated in human brain cancers and was negatively correlated with *TMEFF2* gene methylation and PDGF-AA expression.

Two independent groups have generated TMEFF2 knockout mice and both reported that TMEFF2 homozygous knockout mice were born normal, but showed retarded growth and died around weaning age (Kanemoto et al. 2001; Chen et al. 2012). The histology of the prostate gland appeared normal in TMEFF2 homozygous knockout mice and they also had structurally normal central, peripheral and enteric nervous systems and normal neuronal differentiation. The cause of growth retardation and lethality of the knockout mice is unknown, but it is speculated that the functions of certain neurons were compromised due to TMEFF2 deficiency, which impaired the ability to feed. The aged heterozygous knockout mice did not develop any cancer (Chen et al. 2012).

Little is known about the molecular mechanism underlying TMEFF2's function in tumorigenesis. Due to the presence of an EGF-like motif in its ectodomain, TMEFF2 was examined for its ability to activate EGF receptor family members. In HEK293 human embryonic

kidney and MKN 28 gastric cancer cells, soluble TMEFF2 ectodomain stimulated ERK1/2 phosphorylation in an erbB-1-dependent manner (Ali and Knaüper 2007) and erbB-4 phosphorylation (Uchida et al. 1999), respectively, consistent with a growth-promoting role of the ectodomain. However, it is worth noting that there is a replacement in the EGF-like domain of TMEFF2 (arginine → histidine; Uchida et al. 1999; Horie et al. 2000) that can drastically reduce the affinity of EGF for its receptor (Engler et al. 1990; Hommel et al. 1991).

As mentioned earlier, TMEFF2 overexpression exerted anti-proliferative effects in HCT116 colon cancer cells, inducing apoptosis and suppressing anchorage-independent growth (Elahi et al. 2008). Gene expression changes associated with TMEFF2 overexpression examined by microarray analysis showed that STAT1 as well as a large number of associated interferon-inducible genes were upregulated. Furthermore, STAT1 knockdown restored proliferation and colony formation potential in soft agar of TMEFF2-overexpressing HCT116 cells, suggesting TMEFF2-mediated tumor suppression requires activation of STAT1 pathways.

More recently, Lin et al. (2011) reported that TMEFF2 interacts with PDGF-AA and the interaction requires N-terminal follistatin domain and cannot be mediated by the EGF-like domain alone. Although PDGF-AA downstream signaling pathways were not directly examined, soluble TMEFF2 ectodomain interfered with PDGF-AA-stimulated fibroblast proliferation, suggesting TMEFF2 modulates PDGF-AA signaling.

As the only other member in the TMEFF family, TMEFF1 shares 35.8% identity with TMEFF2 at the amino acid level (Horie et al. 2000). Similar to TMEFF2, TMEFF1 is a type I transmembrane protein that consists of one EGF-like and two follistatin domains in the extracellular region and a potential G protein-activating domain in the short cytoplasmic tail.

TMEFF1 is expressed in the embryo and adult tissues predominantly the brain (Eib et al. 2000; Gery et al. 2003). Although both TMEFF genes are widely expressed in the brain, they exhibit different patterns of expression, suggesting that they play region-specific roles in the central nervous system (Kanemoto et al. 2001).

Functionally, TMEFF1 was suggested to promote growth in hematopoietic cells since an antibody against it induced apoptosis in a cAMP/PKA-dependent manner (Penning et al. 2006). However, overexpressing of TMEFF1 in brain cancer cells resulted in their growth inhibition and 96% of brain tumors had lower levels of TMEFF1 expression than normal brain tissue (Gery et al. 2003). It is possible that TMEFF1 may function as a tumor suppressor in the brain but not in other tissues.

In addition, TMEFF1 has an important role in development involving TGF- $\beta$  signaling. In early *Xenopus* embryos, TMEFF1 regulates TGF- $\beta$  family members nodal and BMP signaling. TMEFF1 inhibits nodal signaling through binding to the nodal co-receptor Cripto (Harms and Chang 2003) and the inhibition requires the follistatin, EGF-like and transmembrane domains, but not the cytoplasmic region of TMEFF1 (Chang et al. 2003), suggesting TMEFF1 that blocks nodal signaling at the ligand/receptor level. TMEFF1 can also block BMP signaling and the activity is dependent only on the presence of its cytoplasmic tail (Chang et al. 2003), suggesting TMEFF1 may activate an intracellular pathway to inhibit BMP function. During hair follicle regeneration, TMEFF1 was found to be a direct TGF- $\beta$ /SMAD2/3 target gene that mediates the antagonistic crosstalk between TGF- $\beta$  and BMP signaling (Oshimori and Fuchs 2012).

## **The role of TMEFF2 in cell migration involves integrins**

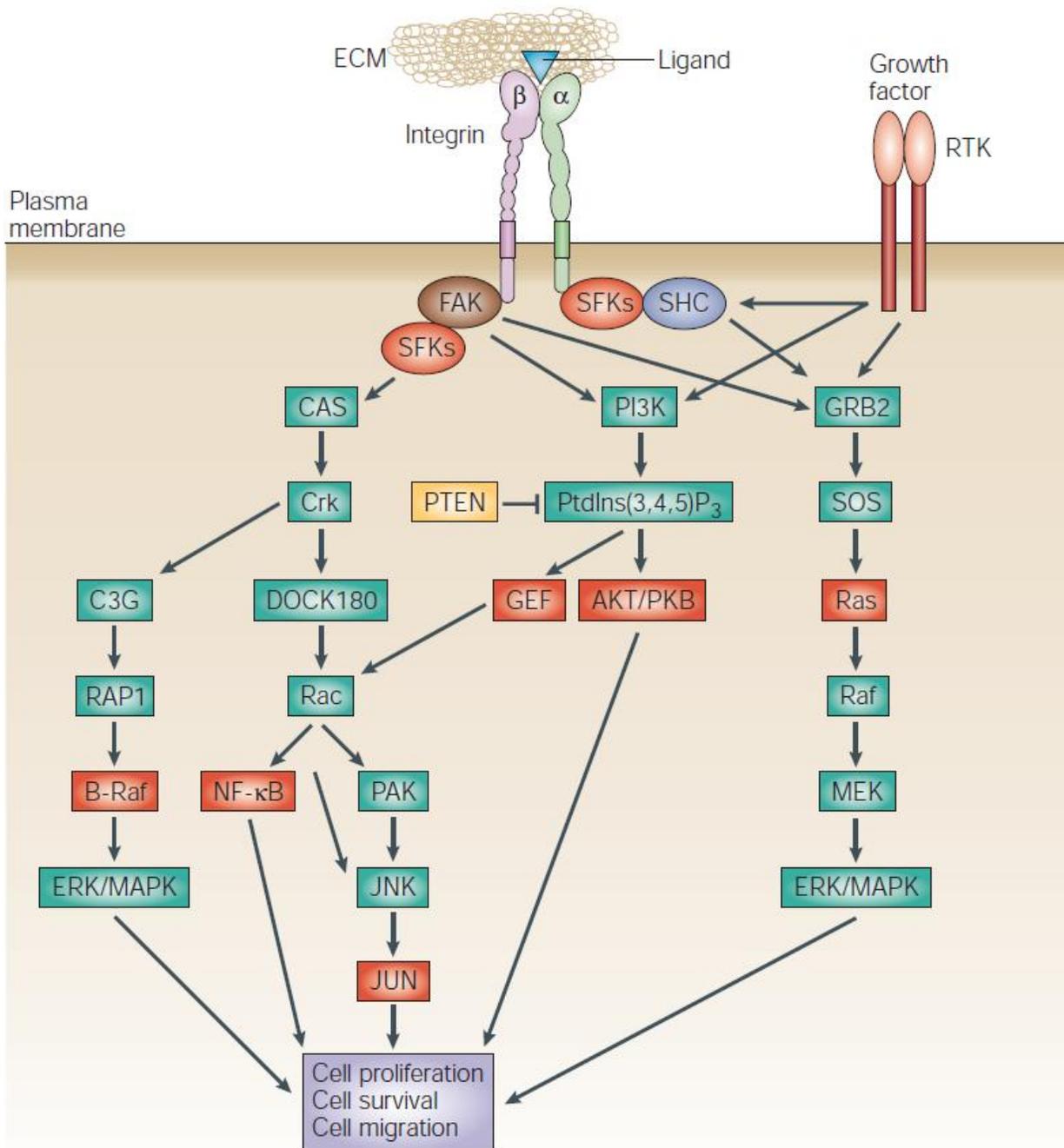
As described in later chapters of the present study, the role of TMEFF2 in tumor biology is phenotypically manifested *in vitro* by cell proliferation, apoptosis, colony formation, and migration/invasion assays. Sustained growth signaling, evasion of apoptosis, and tissue invasion and metastasis are some of the hallmarks of cancer (Hanahan and Weinberg 2011). In Chapter 4, we specifically focus on the role of TMEFF2 in cell migration. Cell migration plays a central role in many biological and pathological processes, such as embryonic morphogenesis, wound healing, immune surveillance, and cancer metastasis (Lauffenburger and Horwitz 1996). In general, migration can be viewed as a multistep cycle, which includes extension of protrusions in the direction of migration, formation of new adhesions at the leading edge, contraction and translocation of the cell body forward, and disassembly of adhesions at the cell rear, allowing it to detach (Ridley et al. 2003). Thus, cell migration requires new adhesions form at the leading edge and old ones break at the trailing edge. Adhesion is mediated primarily by a family of cell-surface molecules known as integrins. In some cases, cell migration requires very little integrin-mediated adhesion on the substratum and can even be integrin-independent, as seen in highly motile cells including neutrophils, dendritic cells, and lymphocytes (Friedl et al. 1998; Lämmermann et al. 2008; Malawista et al. 1997; Woolf et al. 2007).

### *Integrins*

Integrins are transmembrane heterodimers composed of noncovalently associated  $\alpha$  and  $\beta$  subunits. So far, 18  $\alpha$  subunits and 8  $\beta$  subunits have been identified, forming 24 complexes with distinct ligand binding specificities and tissue distribution. In addition, alternatively spliced forms of the  $\alpha$  ( $\alpha3$ ,  $\alpha6$ ,  $\alpha7$ ) and  $\beta$  ( $\beta1$ ,  $\beta3$ ,  $\beta4$ ,  $\beta5$ ) integrin cytoplasmic domains are expressed in a cell- or tissue-type specific manner (Fornaro and Languino 1997).

Two major cellular functions of integrins are cell adhesion and signal transduction. Integrins are the main receptors that mediate cell linkage to the extracellular matrix (ECM). A single integrin heterodimer can bind a variety of ECM proteins, and many ECM proteins have multiple integrin receptors. On the cytoplasmic side, integrins can be linked to actin cytoskeleton via a multi-protein complex that contains over 150 different signaling and adaptor proteins (Zaidel-Bar et al. 2007). Often collectively called focal adhesions, these protein complex structures can be subcategorized into focal complexes, focal adhesions, and fibrillar adhesions (Geiger et al. 2001; Zaidel-Bar et al. 2004; Geiger and Yamada 2011) that are different in size, localization, and morphology. Serving as traction sites by linking extracellular substratum to actin cytoskeleton, integrin-based adhesion plays a crucial role in regulating cell migration.

Although lacking intrinsic catalytic activity, upon adhesion, integrins recruit and activate kinases such as focal adhesion kinase (FAK) and SRC family kinases (SFKs) to relay signals. Ligand binding to integrins induces autophosphorylation of FAK at tyrosine 397, creating a binding site for the SRC-homology 2 (SH2) domain of SRC. Binding of SRC to FAK stabilizes the active conformation of SRC, leading to increased kinase activity which further phosphorylates additional tyrosine residues on FAK, resulting in full activation of both kinases and additional protein binding sites for assembly of focal adhesions (Huveneers and Danen 2009; Legate et al. 2009). Formation of focal adhesion complexes mediates a network of signaling pathways, which affect many cellular processes including proliferation, migration, survival, and differentiation (Figure 4). These signaling pathways can be extremely complicated and roughly divided into the p130CAS-CRK, RAS-RAF-MEK-ERK, and PI3K-AKT signaling pathways (Guo and Giancotti 2004). Notably, the p130CAS-CRK pathway activates DOCK180, an unconventional GEF for RAC (Brugnera et al. 2002; Côté and Vuori 2002; Kiyokawa et al.



**Figure 4.** Integrin signaling – autonomous integrin signaling and joint integrin-RTK signaling (Guo and Giancotti 2004).

1998). Transient activation of RAC upon integrin ligation stimulates membrane protrusions and induces phosphorylation of p190RHOGAP and suppression of RHOA activity, via a reactive oxygen species (ROS)-dependent mechanism (Nimmual et al. 2003), relieving cytoskeletal tension during early cell spreading.

Extensive crosstalk exists between receptor tyrosine kinases (RTKs) and integrins (Huveneers and Danen 2009; Soung et al. 2010; Eliceiri 2001; Chan et al. 2006; Ross 2004; Schwartz and Ginsberg 2002). For example, integrin-mediated adhesion strengthens and lengthens ERK activation from growth factor stimulation; in the absence of adhesion cues, growth factor-mediated ERK activation is weak and transient (Roovers et al. 1999; Miyamoto et al. 1996). Integrin-mediated adhesion can also activate epidermal growth factor receptor (EGFR) independently of EGF binding (Moro et al. 1998), although adhesion-dependent phosphorylation sites on EGFR are different from EGF binding-induced phosphorylation sites (Boeri Erba et al. 2005).

By participating in cellular adhesion and signal transduction, integrins play a critical role in many physiological processes involved in tumor growth and metastasis. It is, therefore, not surprising that cancer cells often have an abnormal integrin repertoire. Changes in integrin expression during tumor progression have been documented in numerous studies and are thought to contribute to tumor growth and metastasis. For example, expression of integrin  $\alpha\beta 3$  is associated with disease progression and metastatic potential of many carcinomas including breast (Sloan et al. 2006; Felding-Habermann et al. 2001; Zhao et al. 2007), cervix (Chattopadhyay and Chatterjee 2001), colon (Vonlaufen et al. 2001), prostate (Zheng et al. 1999; McCabe et al. 2007), pancreas (Hosotani et al. 2002), and ovary (Landen et al. 2008). Interestingly, integrin

$\alpha v\beta 3$  is usually only expressed in macrophages, platelets, endothelial cells, and osteoclasts but not in epithelial cells.

Integrins affect tumor invasion and metastasis by regulating cell migration. Additionally, integrins activate matrix metalloproteinases (MMPs) that are required for ECM degradation and enable tumor cells to adhere to and traverse the blood vessel of the target organ. Moreover, integrin binding to ECM ligands initiates anti-apoptotic pathways to ensure survival of invasive cells to distal tissues (Hood and Cheresh 2002; Guo and Giancotti 2004). However, not all integrins are pro-neoplastic and pro-metastatic. For example, expression of integrin  $\alpha 5\beta 1$  suppresses the tumorigenic properties of several cell lines (Giancotti and Ruoslahti 1990; Varner et al. 1995). Similarly, loss of integrin  $\alpha 2\beta 1$  promotes breast cancer cell metastasis in vivo (Ramirez et al. 2011), and re-expression of  $\alpha 2\beta 1$  in breast cancer cells abrogates the malignant phenotype of the cells (Zutter et al. 1995), suggesting that integrin  $\alpha 2\beta 1$  is a metastasis suppressor. In contrast, integrin  $\alpha 2\beta 1$  promotes prostate cancer cell metastasis to the bone (Hall et al. 2006; Sottnik et al. 2013). These data suggest that the role of integrin in cancer is cell type- and context-dependent.

## **Overview and objectives**

TMEFF2 is a type I transmembrane protein that harbors a potential G protein-activating domain in the cytoplasmic tail and contains an EGF-like and two follistatin motifs in the ectodomain, which is subject to ADAM17/ $\gamma$ -secretase-mediated cleavage. TMEFF2 is predominantly expressed in normal brain and prostate, and is overexpressed in prostate cancer, suggestive of its involvement in this disease. However, the biological function of TMEFF2 is

uncertain, with conflicting reports supporting both tumor-suppressing and growth-promoting activities. In the following chapters, we will examine the molecular mechanisms of TMEFF2 action, with the ultimate goal of elucidating the role of TMEFF2 in prostate cancer and exploring its potential as a therapeutic target.

## CHAPTER TWO: Experimental Procedures

### Plasmid construction

C-terminal MYC-HIS fusions to SARDH, TMEFF2, TMEFF2-ECTO, and TMEFF2-ΔGA, were made by inserting the relevant sequences into the eukaryotic expression plasmid pSecTag2A (Invitrogen). The untagged SARDH expressed under the CMV promoter was constructed using the pCMV-GLuc expression plasmid (NEB) and substituting the GLuc sequence for the SARDH sequence. The untagged TMEFF2 ectodomain (TMEFF2-ECTO) and TMEFF2 used for immunofluorescence analysis were constructed by cloning the respective sequences in the pcDNA5-FRT cloning vector (Invitrogen) under the control of the CMV promoter. Fluorescent protein fusion constructs to the C-terminus of TMEFF2 and SARDH were made in pECFP-N1 and pEYFP-N1 (BD Biosciences) respectively. To inducibly express TMEFF2 and TMEFF2-ΔGA in RWPE1 and RWPE2 cells, TMEFF2 sequences were cloned into the pRetroX-Tight-Pur vector (Clontech). All these constructs were made using PCR and standard cloning strategies (see Table 1) and sequences verified.

**Table 1.** Primers used for plasmid construction.

Construct name	Primer sequence (5'→3')
pCMV-SARDH	a) TAAGATCTCCACTGGGCAAGCCACAC
	b) TATCTAGACCTGTGAGAATGGATGG
SARDH-MYC-HIS	a)TAGCTAGCCCCCATGGCCTCACTGAG
	b)TAGCGGCCGCGTAGATTCCCTTCACCC
TMEFF2-MYC-HIS	a) TAGCTAGCAGTCATGGTGCTGTGGG
	b) TACTCGAGAGATTAACCTCGTGGACG

ECTO-MYC-HIS	a) TAGCTAGCAGTCATGGTGCTGTGGG b) TACTCGAGCATACTGAAATCGTACAGG
$\Delta$ GA-MYC-HIS	a) TAGCTAGCAGTCATGGTGCTGTGGG b) TACCATGGTGTGATGCAGAGGACC c) TACCATGGCAAATAACAGGGCACTAC d) TACTCGAGAGATTAACCTCGTGGACG
TMEFF2-CFP	a) TAGCTAGCAGTCATGGTGCTGTGGG b) TACTCGAGGATTAACCTCGTGGACG
SARDH-YFP	a) TAGCTAGCCCCATGGCCTCACTGAG b) TAAAGCTTGTAGATTCCCTTCACCC
pRetroX-TMEFF2	a) TAGGATCCCTCCACCCTGCCTCCTCG b) TAACGCGTGTCTATAATACTGTATTGTGTAGTC
pRetroX- $\Delta$ GA	a) TAGGATCCCTCCACCCTGCCTCCTCG b) TAACGCGTGTCTATAATACTGTATTGTGTAGTC

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### **Cell culture, transfection, and transduction**

The HEK293T, LNCaP, 22Rv1, RWPE1 and RWPE2 cell lines were purchased from American Type Culture Collection (ATCC). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio Products), 2mM L-glutamine (Gemini Bio Products), 100 U/ml penicillin (Gemini Bio Products), 100  $\mu$ g/ml streptomycin (Gemini Bio Products), and 1.25  $\mu$ g/ml amphotericin B (Gemini Bio Products) at 37°C in 5% CO<sub>2</sub>. LNCaP and 22Rv1 cells were maintained in RPMI-1640 medium (Gibco) supplemented with the same ingredients. RWPE1 and RWPE2 cells were cultured in keratinocyte serum-free medium (K-SFM; Gibco)

supplemented with EGF and bovine pituitary extract provided in the medium kit and 50 U/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. Transfection of HEK293T cells was achieved using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. Stable cell lines were generated by drug resistance followed by clonal selection. The selected clones were further characterized for expression of the specific protein by western blot. Several clones were used to rule out insertion effects. Development of a system for inducible expression of the TMEFF2 gene in RWPE1 and RWPE2 cells was achieved using the Clontech's Tet-On advanced system that requires two consecutive transductions. The first is to introduce the Tet-On plasmid, which contains the regulatory gene encoding the transcription activator under the control of a CMV promoter. The second is to introduce the TMEFF2 gene under the control of the TRE-element and a minimal CMV promoter and that was constructed using standard PCR and cloning strategies. The TRE-element is activated when it binds to the transactivator in a doxycycline dependent manner. To inducibly express TMEFF2 and TMEFF2-ΔGA, cultures were grown in the presence of doxycycline (250 ng/ml; Sigma). 22Rv1 cells transduced with pLKO.1 vectors containing shRNA to TMEFF2 or scramble control were described elsewhere (Green et al. 2013). Briefly, pLKO.1 vectors containing shRNA to TMEFF2 and scramble control were obtained from Open Biosystems and viral stocks were prepared using HEK293T and plasmids psPAX2 and VSV-G (Addgene) for viral packaging. 22Rv1 cells were transduced with each shRNA viral stock and 6 mg/ml polybrene (Millipore). After 48 hr, the transduced 22Rv1 cells were stably selected with 5 mg/ml puromycin (Millipore).

In Chapter 5, for experiments in which conditioned medium was added, RWPE1 cells were growing in basal K-SFM without supplements for 30 min before growth medium was replaced

with the specified conditioned medium. Cells were then incubated for 30 min, unless otherwise specified, before lysis. Conditioned medium was obtained from HEK293T cells stably transfected with TMEFF2-MYC-HIS, ECTO-MYC-HIS, or the empty vector. Cells were grown at 70%-80% confluency, starved overnight and the conditioned medium collected and utilized to replace the growth medium. For PDGF-AA treatment, RWPE1 cells were grown in basal K-SFM without supplements for 30 min before treated with the indicated amount of PDGF-AA for 10 min, unless otherwise specified. For EGF treatment, RWPE1 cells were grown in basal K-SFM without supplements for 3 h before treatment with 10 ng/ml EGF for 10 min.

### **Affinity chromatography and mass spectrometry analysis**

The FreeStyle 293-F cell line was purchased from Invitrogen and maintained in FreeStyle 293 expression medium at 37°C in 8% CO<sub>2</sub>. TMEFF2-MYC-HIS was transfected into FreeStyle 293-F cells using FreeStyle Max reagent (Invitrogen) according to manufacturer's instructions and captured on Nickel Sepharose 6 Fast Flow beads (GE Healthcare) using 20 mM imidazole-containing buffer. Beads were washed in 20 mM imidazole-containing buffer and TMEFF2-MYC-HIS complexes eluted with 500-800 mM imidazole-containing buffer. Eluted proteins were resolved by SDS-PAGE on 4-12% NuPAGE Novex Bis-Tris gel (Invitrogen) and stained with Imperial protein stain (Pierce). Bands of interest were excised and digested with trypsin (Sigma) and subjected to MALDI-Mass Spectrometry proteomic analysis using a Voyager DE Pro-MALDI-TOF Mass Spectrometer. Protein database searching was performed with Mascot Peptide mass Fingerprint (Matrix Science) against the human SwissProt protein database.

## **Co-immunoprecipitation**

Dynabeads Antibody Coupling Kit (Invitrogen) was used according to manufacturer's instructions for the immobilization of antibody on magnet beads. The antibodies used for the immunoprecipitation (IP) were anti-TMEFF2 (Abcam ab77038), anti-MYC (Invitrogen R950-25), anti-EIF4E (Santa Cruz) and anti-4EBP1 (Santa Cruz). HEK293T cells were transfected with the indicated constructs and lysed in Cell Lysis Buffer (Cell Signaling). The lysate (50 µg) was incubated with antibody-coupled Dynabeads for 30 min at 4 °C. Beads were washed with PBS, and the immunoprecipitated proteins, remaining on the beads, were eluted by heating at 70 °C for 10 min in NuPAGE LDS sample buffer (Invitrogen), resolved by SDS-PAGE, and analyzed by western blot using appropriate primary horseradish peroxidase (HRP)-conjugated antibody prepared with Lightning-Link HRP Conjugation Kit (Novus). The antibodies used to conjugate to the HRP were anti-TMEFF2 (Abcam ab50002) and anti-SARDH (Sigma AV42344). For co-immunoprecipitation analysis using LNCaP or 22Rv1 lysates, 100-250 µg of total protein was used and elution of the target protein was in SDS sample buffer. To increase the sensitivity, subsequent western blot analysis was performed using the appropriate primary antibody and a light-chain specific secondary antibody (Jackson ImmunoResearch).

## **Western blotting**

For western blot analysis, cells or mouse prostate lobes were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Sigma P8340), sodium orthovanadate and beta-glycerophosphate. Equal amounts of proteins were resolved by SDS-PAGE and transferred to Immobilon transfer membrane

(Millipore). Blots were blocked in 5% non-fat milk or bovine serum albumin (BSA; Sigma) and probed with the appropriate antibodies. Immunoreactive bands were visualized using ECL plus western blotting detection system (GE Healthcare), SuperSignal West Pico or Femto chemiluminescent substrate (Thermo Scientific). The following antibodies were used: antibodies to TMEFF2 (for cell lines; Abcam ab50002 and Sigma HPA015587), TMEFF2 (for mouse tissues; SDIX, custom antibody), SV40 T-antigen (1:100, Abcam ab16879), ITGAV (1:1000, BD Biosciences 611012), ITGB3 (for human cell lines, 1:1000, BD Biosciences 611140), ITGB3 (for mouse tissue, 1:500, Sigma Ab-773), ITGA5 (1:500, Millipore AB1949), ITGB1 (1:1000, BD Biosciences 610467), RPS6 (1:1000, Cell Signaling 2217), ACTB (1:1000, Cell Signaling 4970), phospho-FAK (Tyr394, 1:1000, Cell Signaling 3283), FAK (1:1000, Cell Signaling 3285), phospho-AKT (Ser473, 1:1000, Cell Signaling 4060), AKT (1:1000, Cell Signaling 9272), phospho-ERK1/2 (Thr202/Tyr204, 1:2000, Cell Signaling 4370), ERK1/2 (1:1000, Cell Signaling 4695), phospho-SMAD2 (Ser465/467, 1:1000, Cell Signaling 3101). Secondary antibodies: goat anti-mouse IgG-HRP (Santa Cruz sc-2005), goat anti-rabbit IgG-HRP (Santa Cruz sc-2004).

### **Immunofluorescence and cell spreading assay**

In Chapter 3, HEK293T cells were seeded on poly-L-lysine and laminin (Sigma)-coated cover glass, transfected with TMEFF2 and SARDH-MYC-HIS expression constructs and incubated for 24-48 h. Cells were fixed with cold 4% paraformaldehyde in PBS (USB Products), permeabilized with 0.1% Triton X-100 (Bio-Rad) in PBS (PBS-T) and blocked with 5% normal goat serum (Invitrogen) in 0.1% PBS-T. Samples were incubated with the indicated primary antibodies overnight at 4°C and then with the corresponding goat anti-mouse FITC (Santa Cruz)

or goat anti-rabbit Alexa 568 (Invitrogen) secondary antibody at room temperature and mounted with medium containing DAPI (Santa Cruz). Images were obtained with a Zeiss LSM 510 confocal microscope. For live cell imaging, HEK293T cells were seeded in collagen coated glass bottom culture dishes (MatTek), transfected with SARDH-YFP and TMEFF2-CFP and incubated for 24-48 h.

In Chapter 4, cover glass (Fisher Scientific) were coated with collagen (40  $\mu\text{g}/\text{ml}$ ; BD Biosciences), laminin (10  $\mu\text{g}/\text{ml}$ ; Sigma), fibronectin (40  $\mu\text{g}/\text{ml}$ ; Millipore), or vitronectin (2  $\mu\text{g}/\text{ml}$ ; Promega) in a 12-well plate at 4 °C overnight and rinsed twice with PBS before 40,000 cells/well were seeded. Following a 3 h incubation, pictures were taken for 10 random fields in each well using an EVOS FL cell imaging system (AMG) and spread/round cells were counted. After counting, cells were then fixed with 4% paraformaldehyde in PBS (USB Products) for 10 min, permeabilized with 0.5% Triton X-100 (Bio-Rad) in PBS (PBS-T) for 5 min, and blocked with 5% normal goat serum (Invitrogen) in PBS-T for 30 min. Samples were incubated with anti-vinculin antibody (1:300; Sigma V9131) at 4 °C overnight followed by goat anti-mouse Alexa Fluor 488 (1:500; Invitrogen) and rhodamine phalloidin (Cytoskeleton) for 1 h. Nuclei were counterstained with DAPI (Santa Cruz). For immunofluorescence analysis with only rhodamine phalloidin staining, cells were seeded in 8 well glass slides (Lab-Tek) and the primary antibody and the corresponding secondary antibody were omitted. Immunofluorescent images were taken on an Optiphot-2 fluorescent microscope (Nikon) with an AxioCam MRm digital camera (Zeiss).

### **Cell migration assay**

Cell migration assays were performed in the presence of 1 µg/ml aphidicolin (Sigma) to prevent proliferation. For wound healing assays, 70 µl of cells were applied to each well of the culture inserts (ibidi) at  $3-7 \times 10^5$  cells/ml. The culture inserts were removed the next day to allow cell migration. Wound healing process was monitored by taking pictures at 0 h, 10 h, 24 h, or 48 h using an EVOS FL cell imaging system (AMG). When specified, 2 µg/ml of CT04 (Cytoskeleton) was added to the fresh medium after insert removal. Cell migration was also assayed using Boyden chambers (BD Biosciences). 50,000 cells were added to the upper chamber and the lower chamber was filled with 500 µl of medium containing 20% FBS. The culture was maintained overnight. For the assay of cell migration towards vitronectin or fibronectin, Boyden chambers were coated with 10 µg/ml indicated ECM proteins in PBS at 4 °C overnight.  $0.5-1 \times 10^5$  cells were added to the upper chamber with basal K-SFM and the lower chamber was filled with the same medium. The culture was maintained for 2 days. Cells were then fixed with 70% ethanol for 10 min, stained with 0.1% crystal violet for 1 h. Migrated cells were then fixed with 70% ethanol for 10 min, stained with 0.1% crystal violet, and photographed.

### **MTT assay**

RWPE1 or RWPE2 cells were seeded at 3,000-5,000 cells/well in 96-well plates. After 48 h of incubation, MTT reagent (Sigma) was added at a concentration of 5 mg/ml in phenol red-free RPMI containing 1% FBS. Following a 3 h incubation at 37 °C, 200 µl of dimethyl sulfoxide (DMSO; Sigma) was added to each well, and optical density was measured at 562 nm.

### **Sarcosine assay**

Cells were washed with PBS, lysed in sarcosine assay buffer (MBL International), and briefly sonicated. Insoluble material was removed by centrifugation at 4 °C. The cellular levels of sarcosine were measured in the supernatant using a sarcosine assay kit (MBL International), and the data were normalized to the level of total L-amino acid present in the same supernatant (L-amino acid quantification kit, MBL International).

### **RT<sup>2</sup> Profiler PCR Array**

The Human Focal Adhesion RT<sup>2</sup> Profiler PCR Array (SABiosciences) was used to determine cellular adhesion-related gene expression affected by TMEFF2 according to the manufacturer's instructions. Briefly, total RNA was extracted from RWPE2 cells expressing TMEFF2 or the vector with RNeasy mini kit (Qiagen) and cDNA was synthesized with RT<sup>2</sup> First Strand Kit (Qiagen). The cDNA was combined with RT<sup>2</sup> SYBR Green Mastermix (Qiagen) and dispensed into the RT<sup>2</sup> Profiler PCR Array. Real-time PCR was performed on an iQ5 instrument (Bio-Rad).

### **G-LISA RHOA activation assay**

RWPE2 cells were incubated in serum free EpiLife CF/PRF medium (Invitrogen) for 3 d and then stimulated with 10% FBS for 2 min. RHOA activity was determined using a colorimetric G-LISA RHOA activation assay biochem kit (Cytoskeleton) according to the manufacturer's instructions.

## **Mouse strain**

Animals were maintained in accordance with the Institutional Animal Care and Use Committee of East Carolina University. Transgenic TMEFF2 mice (129/Sv background, Lineberger Cancer Center Transgenic mouse facility) and maintained in a C57BL/6 background (backcrossed for over 7 generations to C57BL/6). TRAMP mice (FVB background) were purchased from the Jackson laboratory (stock number 008215) and crossed to C57Bl/6J (stock number 000664). The F1 derived from this cross, was then crossed to the transgenic TMEFF2 mouse and TRAMP/TMEFF2 and TRAMP progeny selected after genotyping by PCR of tail genomic DNA.

## **Statistical analysis**

Data are presented as mean  $\pm$  SD (standard deviation). Student's t test (paired, two-tailed) was used to compare two groups of independent samples. P values under 0.05 or 0.01 were considered significant.

## CHAPTER THREE: The Tumor Suppressor Activity of TMEFF2 Correlates with Its Ability to Modulate Sarcosine Levels<sup>1</sup>

### Introduction

TMEFF2 (transmembrane protein with epidermal growth factor and two follistatin motifs 2) is an evolutionarily conserved type I transmembrane protein expressed in the embryo (Heanue and Pachnis 2006; Uchida et al. 1999) and selectively in the adult brain and prostate (Afar et al. 2004; Gery et al. 2002; Glynn-Jones et al. 2001; Zhao et al. 2005; Liang et al. 2000). The extracellular (ecto-) domain can be cleaved from the membrane in an ADAM17/ $\gamma$ -secretase-dependent fashion (Ali and Knaüper 2007; Lin et al. 2003) and consists of an epidermal growth factor-like and two follistatin domains. The cytoplasmic domain contains a potential G protein-activating motif (Uchida et al. 1999). A critical role for TMEFF2 in tumorigenesis is suggested by the fact that it is upregulated in primary and metastatic prostate tumors (Afar et al. 2004; Glynn-Jones et al. 2001; Zhao et al. 2005). In fact, ectopic expression or addition of purified recombinant TMEFF2 ectodomain promotes neuronal cell survival (Horie et al. 2000), cell proliferation (Ali and Knaüper 2007), and phosphorylation of erbB4 and ERK1/2 (Uchida et al. 1999; Ali and Knaüper 2007). However, it has also been suggested that TMEFF2 functions as a tumor suppressor because ectopic expression of full-length TMEFF2 demonstrates *in vitro* anti-proliferative effects (Gery et al. 2002; Elahi et al. 2008) and suppresses tumor growth *in vivo* in nude mouse xenografts (Elahi et al. 2008). Consistent with a tumor suppressor activity, *TMEFF2* has been shown to be hypermethylated in a number of cancer types (Liang et al. 2000; Lin et al.

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<sup>1</sup> This chapter contains previously published work. Xiaofei Chen, Ryan Overcash, Thomas Green, Donald Hoffman, Adam S. Asch and Maria J. Ruiz-Echevarría. 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 Chem. 2011 May 6;286(18):16091-100. doi: 10.1074/jbc.M110.193805. Epub 2011 Mar 10.

2011; Lee et al. 2012; Nagata et al. 2012; Selamat et al. 2011; Park et al. 2011; Tsunoda et al. 2009; Zhao et al. 2008b; Brücher et al. 2006; Suzuki et al. 2005; Ebert et al. 2005; Hanabata et al. 2002; Sato et al. 2002). Results from our lab show that TMEFF2 has a dual mode of action. On the one hand, ectopic expression of wild-type full-length TMEFF2 inhibits monolayer and anchorage-independent cell growth, cellular invasion and migration, and increases cellular sensitivity to apoptosis. On the other hand, expression of TMEFF2 ectodomain or addition of conditioned medium containing the ectodomain increases cell proliferation.

Recently, sarcosine, a glycine derivative, was identified as a potential marker of prostate cancer progression (Sreekumar et al. 2009). Sarcosine levels were highest in metastatic cancer, and in urine its levels were higher in men with prostate cancer than in controls. Importantly, using cell lines, Sreekumar et al. provided evidence that the enzymes involved in sarcosine metabolism act as regulators of cell invasion and therefore as potential therapeutic targets for prostate cancer. The addition of sarcosine or knockdown of sarcosine dehydrogenase (SARDH), the enzyme that converts sarcosine into glycine, in benign prostate epithelial cells enhanced invasion. Conversely, lowering the levels of glycine N-methyltransferase (GNMT), the enzyme that catalyzes the conversion of glycine into sarcosine, in DU145 prostate cancer cells reduced their invasiveness.

In this chapter we present data to demonstrate that TMEFF2 interacts with SARDH and regulates the cellular levels of sarcosine. The data also indicate that there is an association between the ability of TMEFF2 to bind SARDH and modulate the level of sarcosine and its ability to act as a tumor suppressor. Further, this activity requires the transmembrane and/or cytoplasmic portion of the protein. Ectopic expression of TMEFF2 results in monolayer and anchorage-independent growth inhibition and decreased sarcosine-induced cellular motility.

However, the TMEFF2 ectodomain fails to bind to SARDH and to modulate the cellular levels of sarcosine and reverses the tumor suppressor phenotype, demonstrating no effect on anchorage-independent growth and an increase in monolayer growth.

## **Results**

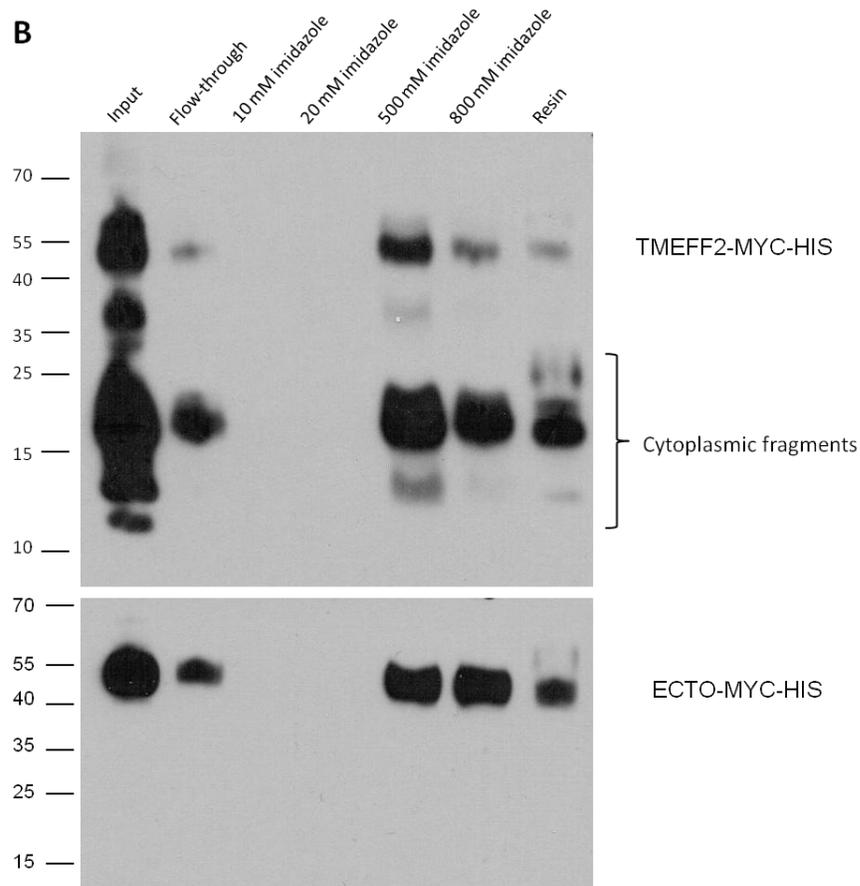
### *TMEFF2 binds specifically to SARDH*

To gain insight into the molecular mechanisms of TMEFF2 action, we sought to search for candidate functional partners of TMEFF2 by screening TMEFF2 affinity complexes using mass spectrometry (MS). FreeStyle 293-F cells were transfected with TMEFF2-MYC-HIS, and TMEFF2 complexes were purified using a histidine affinity column, resolved in a polyacrylamide gel, and subjected to MALDI-TOF/MS analysis. Binding to and elution of the TMEFF2 protein from the column were verified by western blot (Figure 5). To identify specific TMEFF2 interactors, we compared TMEFF2 and empty vector affinity eluates and chose those bands that were mainly present only in the TMEFF2 affinity eluates. Furthermore, to qualify as a specific interactor, a protein had to be identified in at least two out of four independent TMEFF2 affinity/MS analysis. With six peptides displaying a probability-based Mowse score of 53 (Pappin et al. 1993), one of the candidate proteins identified was SARDH. Additional searches provided up to nine different peptides corresponding to SARDH (Table 2). Besides SARDH, TMEFF2 affinity/MS analysis revealed two other TMEFF2-interacting proteins –  $\beta$ -actin (ACTB) and  $\alpha$ -tubulin (TUBA) that are relevant in subsequent studies not in the scope of this chapter (Green et al. 2013).

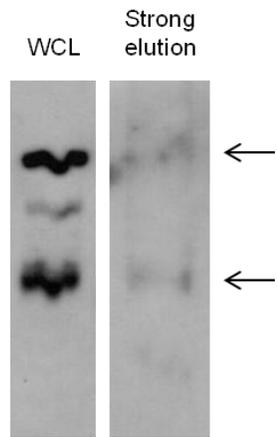
**A**



**B**

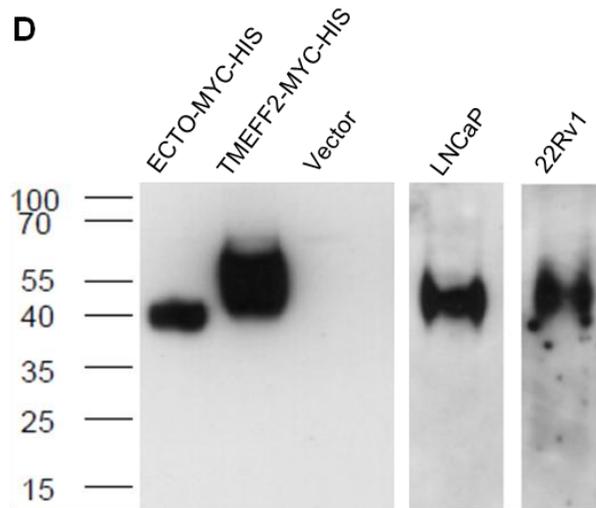


**C** HEK293T/TMEFF2-MYC-HIS



IP: TMEFF2  
IB: SARDH-HRP

**D**



IP: MYC  
IB: TMEFF2

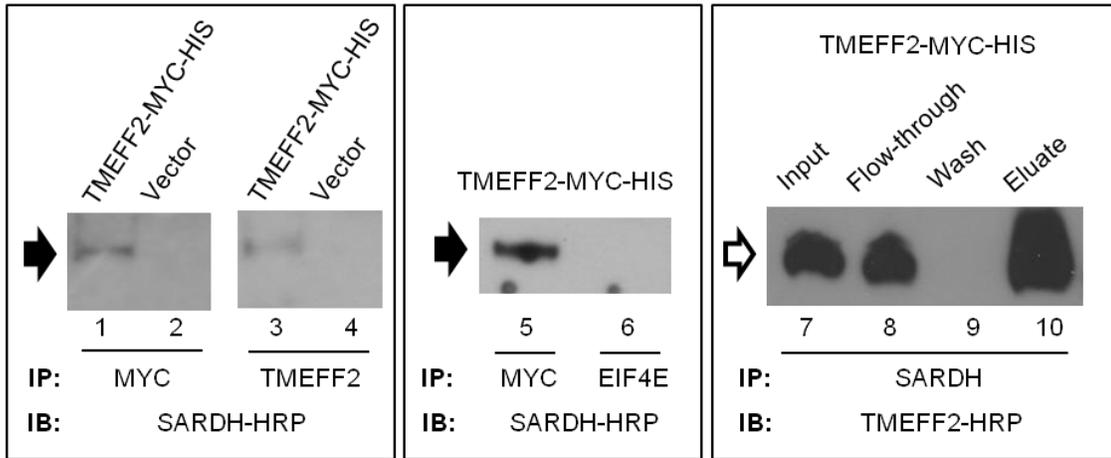
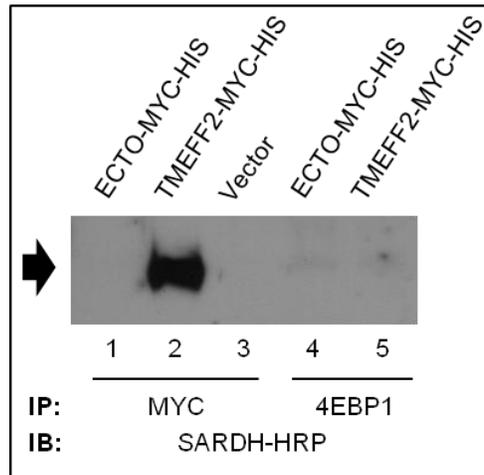
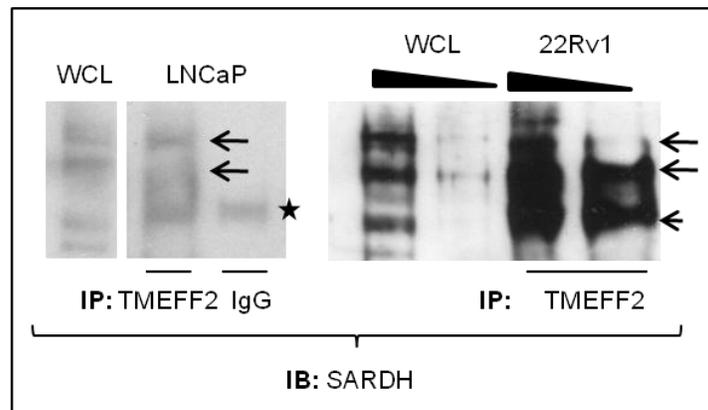
IP: TMEFF2  
IB: TMEFF2

**Figure 5.** Strategies to study co-immunoprecipitation of TMEFF2 and SARDH. **A)** Schematic of the MYC-HIS-tagged TMEFF2 constructs used in this study. FST, follistatin domain; EGF, EGF-like domain; TM, transmembrane domain; GA, G protein-activating domain. **B)** Western blot of TMEFF2-MYC-HIS (top) and ECTO-MYC-HIS (bottom) proteins bound to a nickel affinity column. Whole cell extracts of HEK293T cells expressing TMEFF2-MYC-HIS or ECTO-MYC-HIS (input) were applied to a nickel column. The column was washed with 10-20 mM imidazole and bound proteins were eluted with 500-800 mM imidazole. The presence of TMEFF2 sequences in the eluate was detected with an anti-MYC antibody. The smaller bands are the cytoplasmic fragments that result from TMEFF2 shedding. **C)** Co-immunoprecipitation of SARDH using TMEFF2 specific antibodies. Cell lysates from HEK293T cells overexpressing SARDH and TMEFF2-MYC-HIS were immunoprecipitated (IP) with anti-TMEFF2 antibody and immunoblotted (IB) with anti-SARDH horseradish peroxidase (HRP) conjugated antibody. The size of the bands corresponding to SARDH (black arrows) after strong elution (50 mM glycine pH 2.8 and LDS buffer) lies between the 55-70 kDa and the 70-100 kDa markers. WCL, whole cell lysate. **D)** Analysis of protein binding to antibody affinity columns. Cell lysates from HEK293T cells overexpressing TMEFF2-MYC-HIS, ECTO-MYC-HIS or the empty vector as a control were immunoprecipitated (IP) with anti-MYC antibody coupled dynabeads and immunoblotted (IB) with anti-TMEFF2 antibody. In addition, cell lysates from LNCaP or 22Rv1 cells were immunoprecipitated (IP) with anti-TMEFF2 antibody coupled dynabeads and immunoblotted (IB) with anti-TMEFF2 antibody.

**Table 2.** SARDH peptides identified by mass spectrometry analysis of TMEFF2 affinity complexes.

Start to end	<i>m/z</i>	Peptide sequence
167-173	804.0	RLMSLGK
174-188	1,588.8	AYGVESHVLSAETK
189-222	3,771.0	TLYPLMNVDDLTYGTLYVPHDGTMDPAGCTTLAR
228-241	1,514.4	GAQVIENCPVTGIR
242-251	1,250.1	VWTDDDFGVRR
286-300	1,737.8	VPLVAMHHAYVETER
312-320	1,076.2	DHDASVYLR
467-484	2,101.4	NYSVVFPHDEPLAGRNM
566-587	2,398.8	GAAAVFDMSYFGKFYLVGLDAR

An interaction between TMEFF2 and SARDH was confirmed by co-immunoprecipitation (co-IP) analysis (Figure 6). A plasmid expressing SARDH from a CMV promoter (pCMV-SARDH) was transiently transfected into HEK293T cells stably expressing the TMEFF2-MYC-HIS protein or the empty vector, and anti-TMEFF2 immunocomplexes were analyzed for the presence of SARDH by western blotting. SARDH was clearly detected by the SARDH antibody in the TMEFF2 immunoprecipitates from cells expressing the TMEFF2-MYC-HIS construct (Figure 6A, lanes 1 and 3), but not the empty vector (Figure 6A, lanes 2 and 4), demonstrating that the presence of TMEFF2 is required to detect SARDH. Two different antibodies, MYC and a TMEFF2-specific antibody, were used to immunoprecipitate the TMEFF2-MYC-HIS protein with the same results (Figure 6A, compare lanes 1 and 2 with lanes 3 and 4). As negative controls, two isotype-matched, anti-4EBP1 (now shown) and anti-EIF4E (Figure 6A, lane 6)

**A****B****C**

**Figure 6.** TMEFF2 interacts with sarcosine dehydrogenase. **A)** TMEFF2 associates with SARDH in cells. Cell lysates from HEK293T cells overexpressing SARDH and TMEFF2-MYC-HIS or the empty vector as a control were immunoprecipitated with the indicated antibodies (IP) anti-MYC, anti-TMEFF2, anti-SARDH, or anti-EIF4E (control) and immunoblotted (IB) with anti-SARDH or anti-TMEFF2 HRP-conjugated antibodies. The size of the band corresponding to SARDH (black arrowhead) lies between the 55-70 kDa marker. The size of the band corresponding to TMEFF2 (empty arrowhead) lies between the 40-55 kDa marker. **B)** TMEFF2 ectodomain fails to associate with SARDH in cells. Cell lysate from HEK293T cells overexpressing SARDH and ECTO-MYC-HIS were immunoprecipitated with anti-MYC or anti-4EBP1 (control) and immunoblotted with anti-SARDH-HRP antibody. SARDH is indicated with a black arrowhead. **C)** Association of endogenous TMEFF2 and SARDH proteins. Cell lysates from LNCaP or 22Rv1 cells were immunoprecipitated with anti-TMEFF2 antibody or IgG as a control and immunoblotted with anti-SARDH antibody. The wedge shape indicates increasing amount of lysate used (150-250  $\mu$ g of total protein). The arrows indicate the position of the major SARDH bands (between the 55-70 and 70-100 kDa markers). The small arrowhead indicates a band with migration similar to the IgG heavy chain (star). WCL, whole cell lysate.

antibodies were utilized for the IP step. SARDH was not co-immunoprecipitated when either one of these antibodies was used for the IP step, demonstrating that the interaction between SARDH and TMEFF2 is specific. Reciprocal co-IP experiments using anti-SARDH for immunoprecipitation and anti-TMEFF2-HRP for western blot were also performed (Figure 6A, lanes 7–10) and confirmed the TMEFF2-SARDH interaction (Figure 6A, lane 10).

To further validate the specificity of the TMEFF2-SARDH interaction, we performed co-IP studies as described above with cells that ectopically express the TMEFF2 ectodomain (ECTO-MYC-HIS; Figure 5A). The results indicate that SARDH was not present in the co-immunoprecipitates obtained from the ECTO-MYC-HIS-expressing cells (Figure 6B, lanes 1–5). The ECTO-MYC-HIS protein is readily secreted into the medium, but it is also abundant in the cellular lysates and able to bind to the nickel or antibody affinity columns (Figure 5), ruling out that the lack of interaction reflects the absence of intracellular protein or failure to bind to the columns.

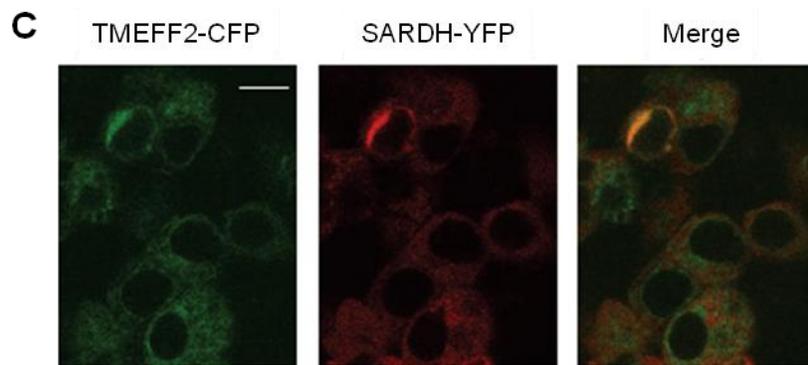
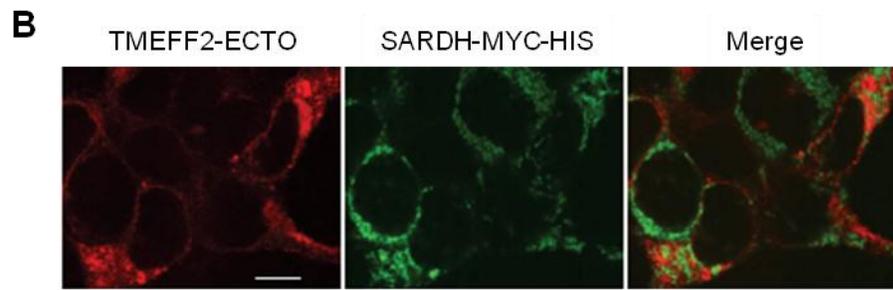
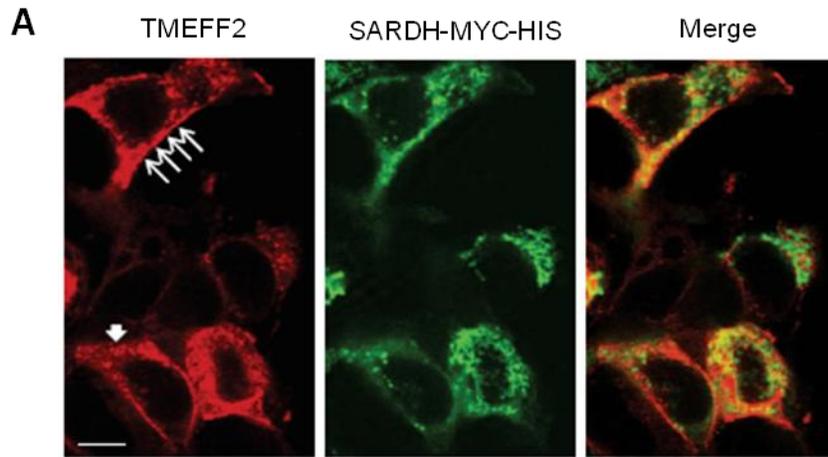
To better establish the physiological relevance of the above results, the TMEFF2-SARDH interaction was also analyzed in two prostate cancer cell lines, LNCaP and 22Rv1, known to express some endogenous TMEFF2. Co-IP studies indicated that SARDH is present in the TMEFF2 co-immunoprecipitates obtained using LNCaP or 22Rv1 cell lysates and therefore that the interaction occurs and is detectable even with the low endogenous levels of these proteins (Figure 6C). Polyclonal rabbit immunoglobulin G (IgG) was used as a negative control. The ability of endogenous TMEFF2 to bind to the antibody affinity column was verified (Figure 5D). Collectively, these results indicate that TMEFF2 specifically interacts with SARDH and that the presence of the transmembrane and/or cytoplasmic domains is essential for this interaction.

### *TMEFF2 co-localizes with SARDH*

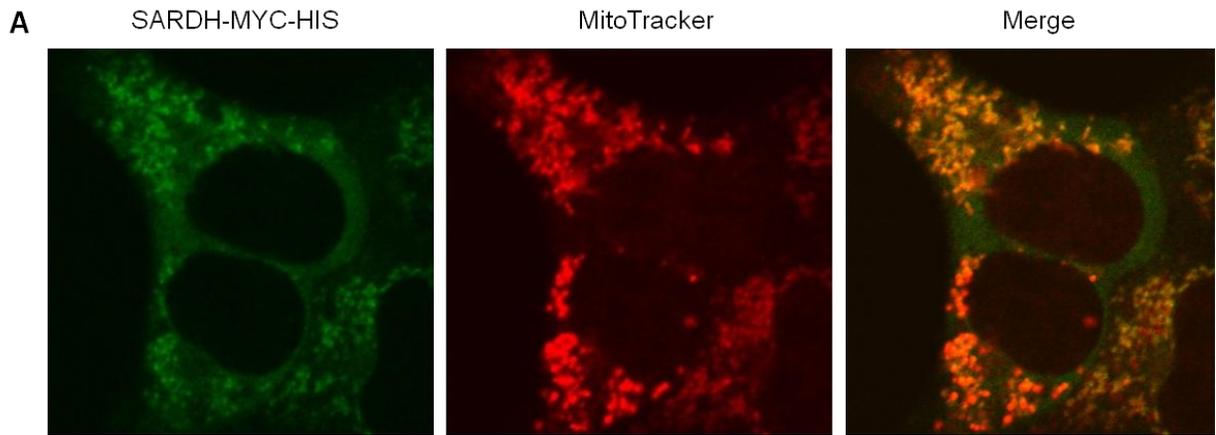
The possibility that TMEFF2 and SARDH can temporarily localize to the same cellular compartment was examined by immunofluorescence co-localization studies. HEK293T cells ectopically expressing TMEFF2 were transiently transfected with a tagged SARDH-MYC-HIS construct and incubated with polyclonal TMEFF2 antibody (to detect TMEFF2) and monoclonal MYC antibody (to detect SARDH). The localization of the proteins was subsequently visualized by confocal microscopy using Alexa Fluor 568 and FITC-conjugated secondary antibodies. Cells expressing TMEFF2 exhibited fluorescence concentrated at the plasma membrane but also in the cytoplasm in a punctate pattern (Figure 7A, 8B). Confirming published observations, SARDH mainly localized to the mitochondria (Bergeron et al. 1998), with less fluorescence detected in the cytoplasm (Figure 8A). Co-localization of the proteins in the cytoplasm was evidenced by overlapping fluorescence signals (Figure 7A, 8B). As anticipated based on the results from the co-IP analysis, the ectodomain of TMEFF2 did not demonstrate co-localization with SARDH (Figure 7B).

Co-localization of TMEFF2 and SARDH was also examined in living cells. For this purpose, TMEFF2 and SARDH were C-terminally tagged with cyan (CFP) and yellow fluorescent proteins (YFP) to generate TMEFF2-CFP and SARDH-YFP, respectively. The resulting fusion proteins were expressed in HEK293T cells, and the localization of the fluorescent proteins was imaged by confocal microscopy (Figure 7C, 8C). Interestingly, when TMEFF2-CFP and SARDH-YFP were co-expressed, they co-localize in an area surrounding the nuclear envelope that could correspond to the Golgi apparatus.

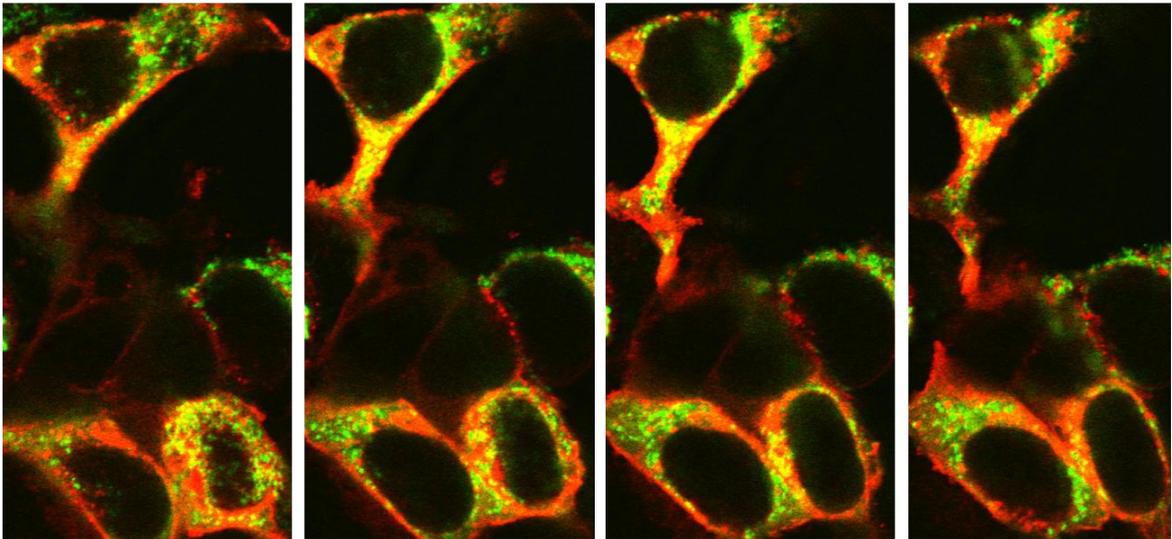
*Overexpression of the full-length TMEFF2 results in decreased cellular sarcosine levels*



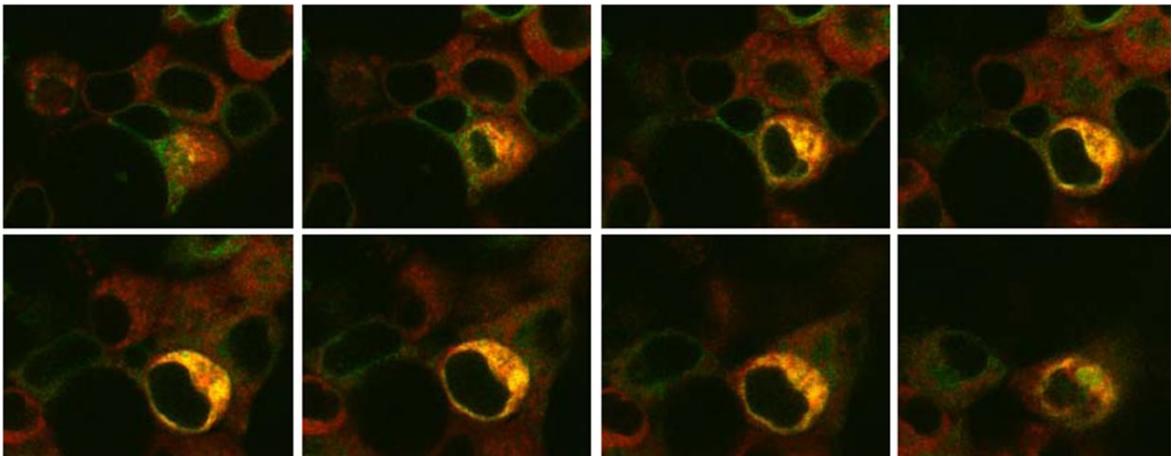
**Figure 7.** TMEFF2 co-localizes with sarcosine dehydrogenase. **A)** HEK293T cells ectopically expressing TMEFF2 and SARDH-MYC-HIS were fixed and stained with anti-TMEFF2 and anti-MYC (to detect SARDH) antibodies. Thin and thick filled arrows point to the membranous and vesicular localization of TMEFF2, respectively. **B)** HEK293T cells ectopically expressing the TMEFF2 ectodomain (TMEFF2-ECTO) and SARDH-MYC-HIS were treated and processed for Immunofluorescent staining as in A. **C)** In vivo co-localization of TMEFF2 and SARDH. HEK293T cells were transfected with TMEFF2-CFP and SARDH-YFP fusion constructs, and living cells were observed 24 h later. CFP fluorescence is shown as green signal, YFP fluorescence is shown as red signal, and co-localization of CFP and YFP is illustrated by yellow signal. The confocal images in each panel are representative of more than 40 fields observed over four or five different experiments. Scale bars, 10  $\mu$ m.



**B** Fixed cells (SARDH = green, TMEFF2 = red)



**C** Live cells (SARDH-YFP = red, TMEFF2-CFP = green)



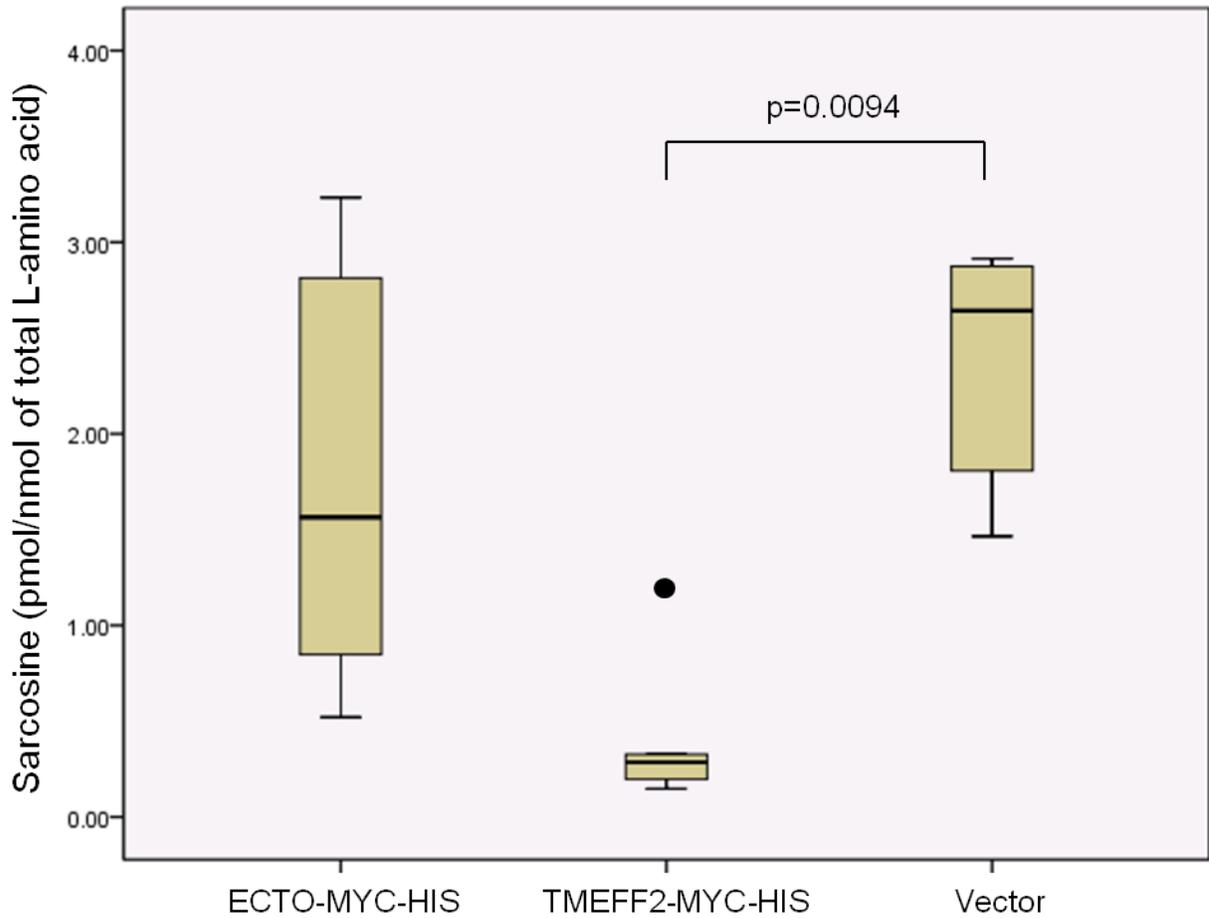
**Figure 8.** TMEFF2 and SARDH co-localize in HEK293T cells. **A)** Confocal microscopy of HEK293T/SARDH-MYC-HIS cells stained with anti-MYC. Mitochondria were stained with MitoTracker Red. The merge image demonstrates the localization of SARDH in mitochondria (yellow signal). **B)** Confocal Z-stack sequential images (left to right) of the co-localization (yellow) of TMEFF2 (red) and SARDH-MYC-HIS (green) in HEK293T fixed cells. **C)** Confocal Z-stack sequential images (left to right, top to bottom) of the *in vivo* co-localization (yellow) of TMEFF2-CFP (green) and SARDH-YFP (red) in HEK293T cells.

SARDH catalyzes sarcosine to glycine conversion and, consequently, siRNA to SARDH results in increased sarcosine levels. Interestingly, this also results in an increase in the invasion potential of the cells, linking sarcosine metabolism with tumorigenesis (Sreekumar et al. 2009). Because TMEFF2 interacts with SARDH, we hypothesized that TMEFF2 modulates SARDH activity to promote changes in cell growth and/or invasion. We analyzed changes in sarcosine levels in response to TMEFF2 overexpression. Lysates were prepared from HEK293T cells stably transfected with the TMEFF2-MYC-HIS and ECTO-MYC-HIS expression constructs or with the empty vector as a control, and sarcosine levels were determined in the resulting cell lines. Overexpression of the ectodomain (ECTO-MYC-HIS) does not affect sarcosine levels (Figure 9). However, overexpression of TMEFF2 resulted in a significant decrease in the amount of sarcosine with respect to the lysates expressing the empty vector control (Figure 9), whereas having no effect on SARDH expression as measured by western blot (not shown). Although it is possible that the observed decrease in sarcosine levels is due to an indirect effect of TMEFF2, the physical interaction between SARDH and TMEFF2 demonstrated above suggests that the reduction in sarcosine may be due to an increase in SARDH activity mediated by its interaction with TMEFF2.

(The following experiments were performed by other members of the lab but are included here for better understanding of this chapter.)

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

To investigate the function of TMEFF2 in tumorigenesis, we determined whether ectopic expression of this protein could affect cellular proliferation. HEK293T cells stably expressing

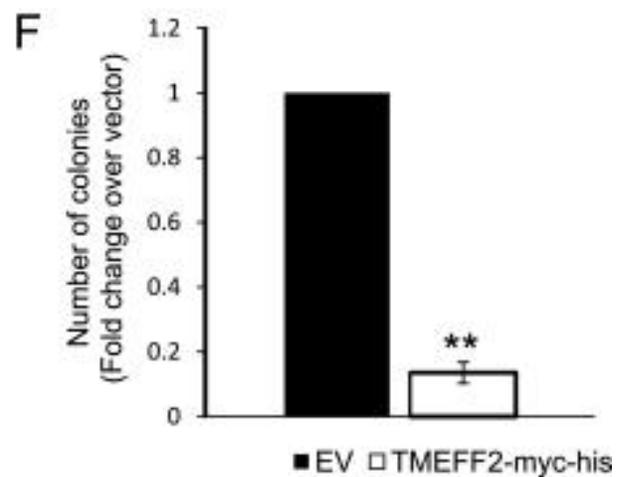
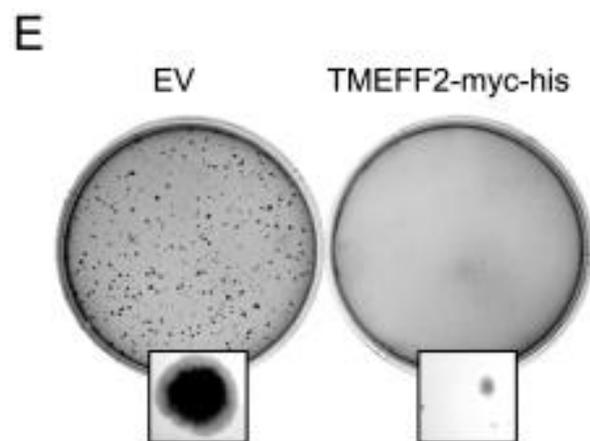
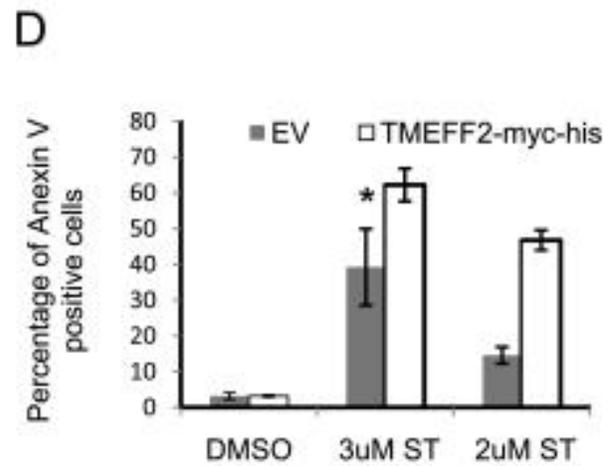
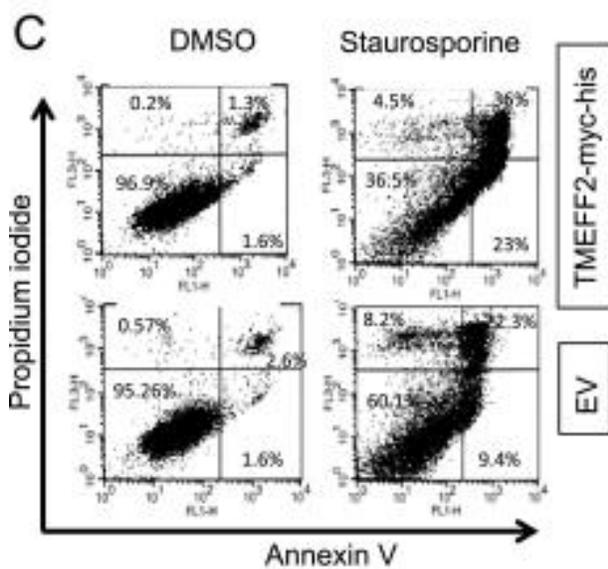
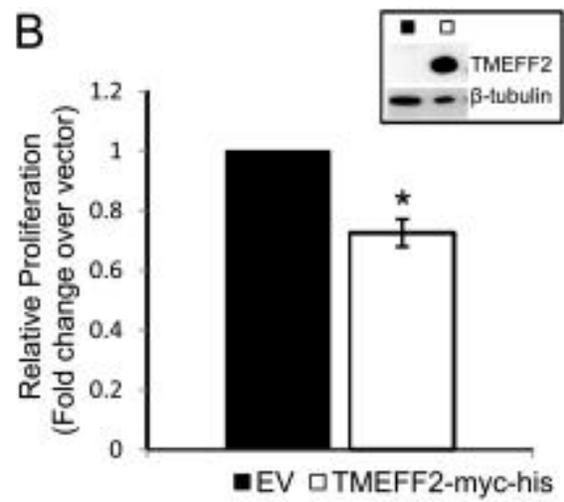
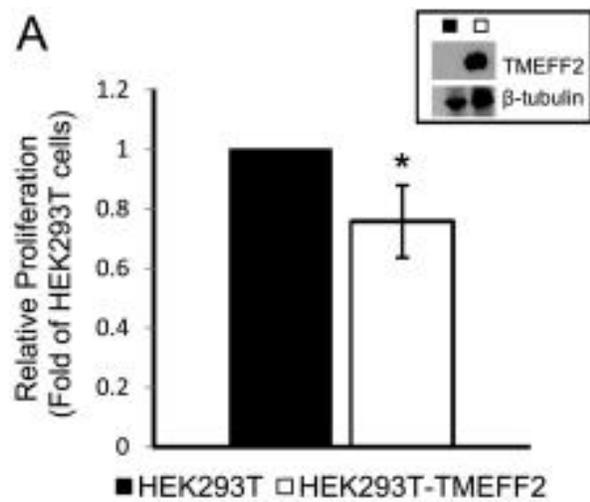


**Figure 9.** TMEFF2 affects the levels of cellular sarcosine. Overexpression of TMEFF2 significantly reduces the levels of sarcosine in HEK293T cells. Dots on the box plot indicate outliers. Data shown are the result of six different experiments with multiple replicates.

untagged or MYC-HIS-tagged TMEFF2 proteins, along with control cells transfected with empty vector or untransfected cells, were generated for this purpose. Overexpression of either untagged (Figure 10A) or C-terminal MYC-HIS-tagged TMEFF2 (Figure 10B) 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 MYC-HIS tag did not change the effect of TMEFF2 on cell growth. Therefore, subsequent experiments were done using the MYC-HIS-tagged form of the protein.

To further characterize the nature of the alteration in proliferation rate, FACS analysis was used to investigate the effect of TMEFF2 in apoptosis and cell cycle progression. 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 (Belmokhtar et al. 2001; Zhang et al. 2004). The presence of TMEFF2 in HEK293T cells had no effect on the number of apoptotic cells. However, it increased the sensitivity of the cells to staurosporine-induced apoptosis when compared with empty vector transfected cells (Figure 10C, D). TMEFF2 had a small effect on cell cycle, resulting in a slightly increased cell number in G1 (not shown). Supporting this observation, our preliminary array data indicate that overexpressing TMEFF2 resulted in increased expression of the cyclin-dependent kinase inhibitor p15 and decreased expression of cyclin E2 (not shown).

To further 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 (Figure 10E, F). Thus, TMEFF2 suppresses the formation and the



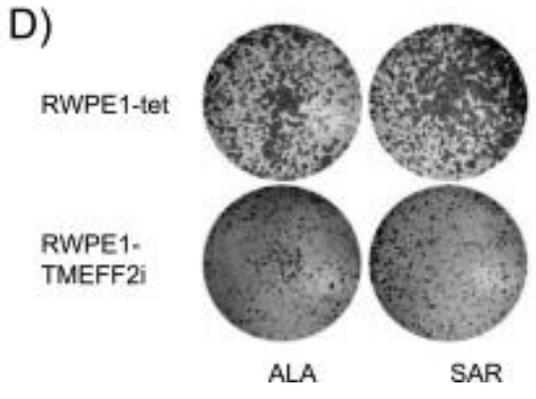
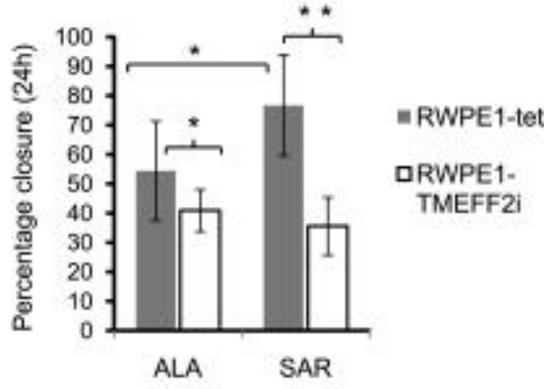
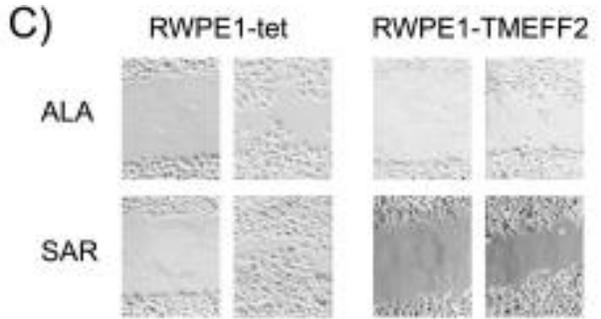
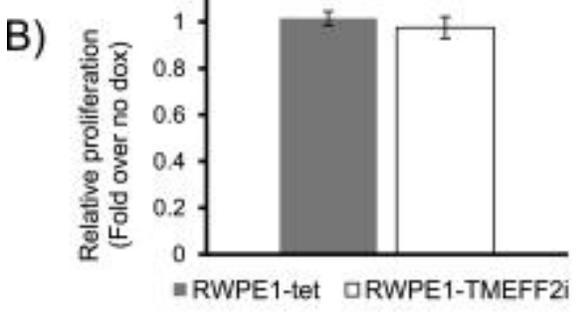
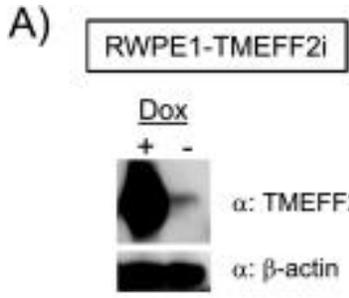
**Figure 10.** TMEFF2 inhibits proliferation and anchorage-independent growth and sensitizes cells to apoptosis. Stable expression of untagged (A) or MYC-HIS (B-F)-tagged TMEFF2 decreases proliferation of HEK293T cells (A, B), sensitizes the cell to an apoptotic stimulus (C, D), and inhibits anchorage-independent growth (E, F). **A, B**) Overexpression of TMEFF2 was confirmed by western blot analysis. The effect of TMEFF2 on growth was determined using an MTT assay after 96 h of growth. The A562 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) or the cell line carrying the empty vector (EV; B). **C, D**) The effect of TMEFF2 on apoptosis of HEK293T cells 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. Shown are representative images of the flow cytometry analysis (C) and percentage of apoptotic cells (D). **E, F**) Representative images showing anchorage-independent growth (E) and number of colonies formed by HEK293T cells stably expressing TMEFF2-MYC-HIS or the empty vector as a control (F) after 14 d of growth. Data are mean  $\pm$  SD 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 \*\*,  $p < 0.01$ .

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).

*TMEFF2 inhibits sarcosine-induced cell migration 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 (Bello et al. 1997), which express very low levels of endogenous TMEFF2 as demonstrated by quantitative real time 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 level of expression of TMEFF2 in the RWPE1-TMEFF2i cell line upon the addition of doxycycline was demonstrated (Figure 11A). 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 (Figure 11B).

The addition of sarcosine to RWPE cells increases the migration and invasion ability of these cells (Sreekumar et al. 2009). We therefore tested whether TMEFF2 can reverse the sarcosine-induced migration effect. Briefly, RWPE1-TMEFF2i cells were grown in the presence of sarcosine or alanine and doxycycline to induce TMEFF2 expression before their migration potential was analyzed using a wound-healing assay. The effect of TMEFF2 was investigated by

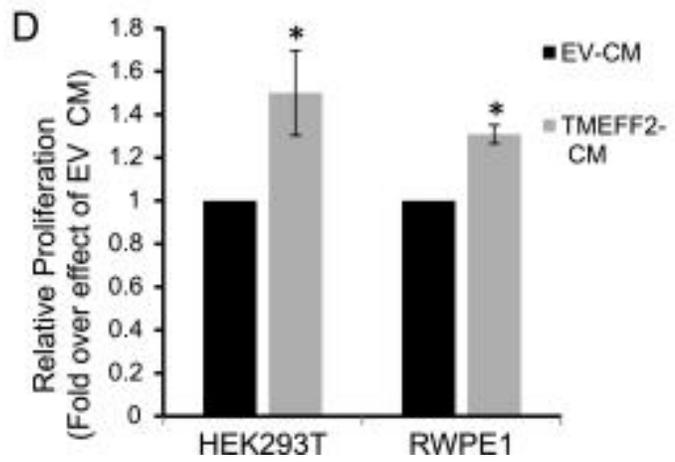
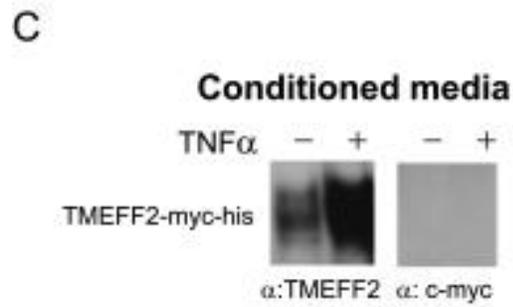
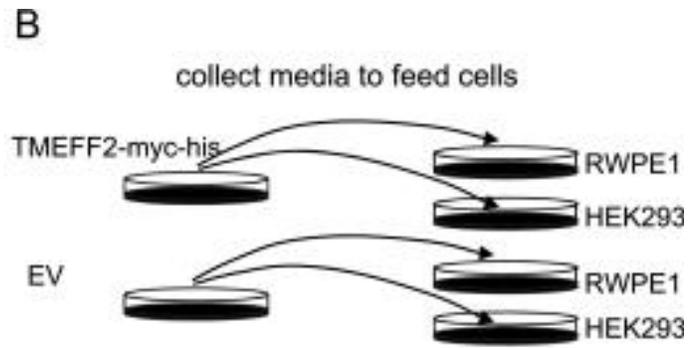
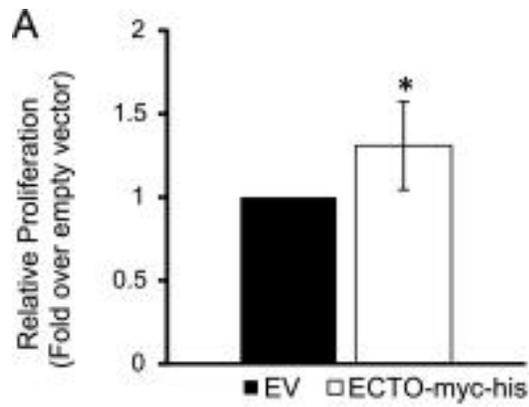


**Figure 11.** TMEFF2 inhibits migration of RWPE cells. **A)** Western blot demonstrating the induction of TMEFF2 expression in response to doxycycline (Dox; 250 ng/ml) in the RWPE1-TMEFF2i cell line.  $\beta$ -Actin was used as a loading control. **B)** The effect of TMEFF2 overexpression on the growth of RWPE1 cells was determined using an MTT assay after 96 h of growth. The A560 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. **C)** The effect of TMEFF2 on migration was determined using a 24-h wound-healing assay. The cells were grown in the presence of 50  $\mu$ m alanine (ALA) or sarcosine (SAR) and 250 ng/ml doxycycline to induce the expression of TMEFF2. The RWPE1-tet cell line was used as a control. A representative image (top) and quantification of the results (bottom) are shown. **D)** Migration of cells from a random experimental repeat was also analyzed using Boyden chambers. Cells adhering to the bottom of the membrane were fixed, stained with crystal violet, and photographed. Single cell clones were analyzed and gave similar results. Data shown are mean  $\pm$  SD of three independent experiments with multiple replicates. \*,  $p < 0.05$ , and \*\*,  $p < 0.01$ .

comparing the migration of RWPE1-TMEFF2i cells with the migration ability of the control cell line, RWPE1-tet, both in the presence of doxycycline. The addition of sarcosine resulted in an increase in migration of the RWPE1-tet cells (Figure 11C) when compared with cells grown in the presence of alanine. Overexpressing TMEFF2 in these cells blocked the increased migration associated with the addition of sarcosine (Figure 11C). The addition of alanine also had a small effect on migration that was also reversed by TMEFF2 overexpression. Migration data obtained using Boyden chambers confirmed the results of the wound assay (Figure 11D). These results suggest that TMEFF2 can block the intrinsic and the sarcosine-induced migration potential of RWPE1 cells. In addition, using a Boyden chamber invasion assay, we observed that TMEFF2 overexpression was also able to reverse the intrinsic and the sarcosine-induced invasion ability of the cells (not shown). It is worth noting that although in HEK293T cells TMEFF2 negatively affects monolayer and anchorage-independent growth but has no effect on migration or invasion, the reverse seems to be true when TMEFF2 is overexpressed in RWPE cells, indicative of the cell line-specific effect of TMEFF2.

*The ectodomain region of TMEFF2 acts as a ligand to promote cell growth*

Based on our findings, we also hypothesized that because of the inability of the TMEFF2 ectodomain to interact with SARDH and to affect the levels of cellular sarcosine, it would not demonstrate a tumor suppressor phenotype. We therefore expressed the ectodomain form of TMEFF2 (ECTO-MYC-HIS) in HEK293T cells and analyzed its effect on cell growth. As reported previously (Ali and Knaüper 2007), overexpression of the ectodomain resulted in increased monolayer growth when compared with empty vector transfected cells (Figure 12A). In contrast to full-length TMEFF2, overexpression of the ectodomain did not have any effect on anchorage-independent growth in soft agar or cellular invasion (not shown).



**Figure 12.** TMEFF2 ectodomain promotes cell growth. **A)** The effect of the TMEFF2 ectodomain on growth was determined in HEK293T cells stably transfected with the ECTO-MYC-HIS construct using an MTT assay. After 96 h of growth, the A562 was measured and normalized first to the value obtained at zero time and then to the value obtained for the cell line carrying the empty vector (EV) as control. **B)** Schematics of the experiment used to determine the effect of the secreted ectodomain on cell growth (shown in D). **C)** Detection of ectodomain sequences in the conditioned medium using the specified antibodies. Because the ectodomain region is produced by TMEFF2 shedding from the membrane, it is not detected by the MYC antibody to the C-terminal region. Specificity was further confirmed by the addition of TNF $\alpha$  to induce shedding. **D)** Effect of the secreted ectodomain on the growth of HEK293T and RWPE1 cells was determined by MTT assay after 48 h of growth on conditioned medium obtained from cells expressing TMEFF2-MYC-HIS (TMEFF2-CM) or the empty vector as control (EV-CM). The A562 at 48 h was normalized first to the value obtained at zero time and then to the value obtained for the same cell line grown in the empty vector conditioned medium. Data shown are mean  $\pm$  SD of three independent experiments with multiple replicates. \*,  $p < 0.05$ .

The ectodomain region expressed throughout these experiments corresponds essentially to the naturally shed ectodomain of the TMEFF2 protein. Because it lacks a transmembrane domain, it is directly secreted and can be detected in the conditioned medium (not shown). It was therefore likely that the observed effect on monolayer growth was due to the secreted form of the ectodomain acting as a ligand from the outside of the cell. To examine this possibility, we determined the effect that conditioned medium collected from cell cultures overexpressing full-length TMEFF2 had on the growth of two different cell lines, HEK293T and RWPE1 (Figure 12B-D). Exponentially growing HEK293T cells transfected with the TMEFF2-MYC-HIS construct were starved for 24 h, and the conditioned medium was collected and supplemented with 0.4% FBS. The presence of TMEFF2 sequences in the conditioned medium was analyzed by western blot using antibodies against the TMEFF2 ectodomain or against the MYC tag (Figure 12C). The collected conditioned medium was used to replace the growth medium of cultures of HEK293T and RWPE1 cells, and the growth rate of these cells was measured at different time intervals after the medium replacement. As a control, conditioned medium collected from vector transfected HEK293T cultures was used. The addition of conditioned medium from the TMEFF2-MYC-HIS-overexpressing cultures resulted in a significant growth increase of HEK293T and RWPE1 cells (Figure 12D). Similar results were observed when conditioned medium from ECTO-MYC-HIS-overexpressing cells was used to feed the HEK293T or RWPE1 cell cultures (not shown). These results suggest that the ectodomain may act as a ligand to promote increased growth rate of HEK293T and RWPE1 cells.

## Discussion

Deregulated expression of TMEFF2 has been documented in a variety of tumor types. However, the relationship of TMEFF2 to the biology of tumor development or suppression and the molecular bases of these activities remain unknown. The study in this chapter reveals a novel functional and physical interaction between TMEFF2 and SARDH, an enzyme involved in sarcosine metabolism, and characterizes the role of this interaction in the tumorigenic activity of TMEFF2. We demonstrate that TMEFF2 expression results in a decrease in the level of cellular sarcosine and that this effect correlates with its ability to act as a tumor suppressor.

SARDH is responsible for conversion of sarcosine to glycine and is therefore one of the regulators of sarcosine levels in the cell. Because sarcosine is formed as a result of glycine methylation, the decrease in sarcosine levels that we observed with TMEFF2 overexpression could be due to an effect on the global cellular amino acid metabolism and/or methylation activity or to an effect on the activity of the SARDH enzyme. Our data indicating that TMEFF2 physically interacts with SARDH favor the latter possibility. This interpretation bestows an active role for sarcosine in tumorigenesis consistent with data indicating that in cell culture, the addition of sarcosine promotes cell invasion of prostate epithelial cells (Sreekumar et al. 2009). Our results demonstrate that the ability of TMEFF2 to decrease the cellular sarcosine levels correlates with its function. Expression of full-length TMEFF2 decreases cellular sarcosine levels and leads to a tumor suppressor phenotype, whereas expression of the ectodomain does not alter sarcosine levels and leads to reversion of the tumorigenic phenotype. These results also suggest that the effect of TMEFF2 on sarcosine levels requires the presence of a transmembrane domain and/or the cytoplasmic tail and could possibly be mediated by the G protein-activating domain present in this region.

As described in Chen et al. 2011, the ectodomain promotes cellular proliferation, confirming previous reports (Ali and Knaüper 2007; Horie et al. 2000). Cleavage of the extracellular domain of TMEFF2 is induced by pro-inflammatory cytokines and regulated by ADAM17 (Ali and Knaüper 2007; Lin et al. 2003) and this effect could contribute to the opposing results described for TMEFF2, such as growth suppression dependent on full-length TMEFF2, its ability to modulate sarcosine levels, and proliferation dependent on regulated release of the ectodomain, which is unable to modulate sarcosine levels. Interestingly, a soluble isoform of TMEFF2 has also been described (Quayle and Sadar 2006), and although its role is unknown, we predict that it will not modulate sarcosine levels. The identification of proteins with both pro-oncogenic and anti-oncogenic activities has been previously described, emphasizing the complexity of cellular events that occur during tumorigenesis (Genander et al. 2009; Kang et al. 2008; Murray-Zmijewski et al. 2006; Niu et al. 2008; Zhu et al. 2007). Several mechanisms account for the switch in oncogenic activity including the cellular context, the type of tumor, the activation of different pathways, or the presence of different isoforms with opposing roles (Genander et al. 2009; Kang et al. 2008; Murray-Zmijewski et al. 2006; Niu et al. 2008; Zhu et al. 2007).

Similar to TMEFF2, TMEFF1, the only other known member of the TMEFF family, demonstrates different activities depending on the presence of the cytoplasmic tail and its anchorage to the membrane. In *Xenopus*, TMEFF1 inhibits TGF- $\beta$  signaling by blocking the nodal co-receptor Cripto (Harms and Chang 2003). The follistatin and EGF motifs contribute to this effect; however, anchorage to the membrane is also essential for this function. Conversely, TMEFF1 blocking of BMP-mediated signaling requires the cytoplasmic domain of the protein, whereas deletion of either the follistatin or the EGF motifs does not interfere with this function (Chang et al. 2003). A function for the TMEFF1 ectodomain has not been described. TMEFF1

and TMEFF2 grossly differ in their tissue distribution. TMEFF1 is more widely distributed than TMEFF2, and even in brain, where both proteins are expressed, they exhibit distinct distribution patterns (Kanemoto et al. 2001). Nevertheless, their high level of sequence similarity suggests that TMEFF1 and TMEFF2 could be playing similar roles in different tissues and/or developmental stages.

Using confocal microscopy, we have observed TMEFF2 localization to the membrane but also to the cytoplasm, where it appears in a punctate pattern. This vesicle-like immunoreactivity of TMEFF2 has also been described in neurons (Siegel et al. 2002, 2006), and it has been proposed to correspond to vesicles translocating newly synthesized TMEFF2 to the cell surface and/or cleaved TMEFF2 to the nucleus. SARDH has been described essentially as a mitochondrial enzyme (Bergeron et al. 1998); however, our results suggest that it can also be found in the cytosol and/or the Golgi apparatus, where it could be interacting with TMEFF2 during trafficking. We predict that this interaction modifies the activity of SARDH and, ultimately, the mitochondrial and/or cytosolic levels of sarcosine. Several mitochondrial enzymes have been described as tumor suppressors, for example, succinate dehydrogenase and fumarate hydratase (King et al. 2006) or a mitochondrial form of the sirtuin deacetylases, SIRT3 (Kim et al. 2010). Similar to these enzymes, SARDH should be considered a tumor suppressor because its inactivation leads to sarcosine accumulation and increased invasion. Interestingly, the level of SARDH protein has been reported to decrease in hepatocellular carcinoma (Lim et al. 2002). By binding to SARDH, TMEFF2 could be modulating the activity of SARDH and therefore the cellular level of sarcosine, suggesting that it may function as a “secondary” tumor suppressor. Whether the tumor suppressor activity of TMEFF2 depends entirely on SARDH or on additional interacting protein/signaling pathways is currently under investigation.

The results presented here indicate that the role of TMEFF2 in tumorigenesis correlates, at least partially, with its ability to bind to SARDH and modulate the cellular levels of sarcosine. However, how does sarcosine promote tumor invasion? Sarcosine is an endogenous amino acid with several important biological functions: a) it participates in one-carbon metabolism essential for protein and nucleotide synthesis and DNA methylation (Stover 2009), and b) it is a competitive inhibitor of the type I glycine transporter (GlyT1), a glycine transporter found in brain (Zhang et al. 2009). It is therefore possible that the addition of exogenous sarcosine and its subsequent metabolism affect the methylation and/or synthetic ability of the cell; alternatively, sarcosine may have a yet unidentified role, for example, similar to its role on GlyT1, as an agonist/antagonist of a factor involved in tumorigenesis. This last possibility is supported by the fact that blocking sarcosine metabolism also promotes cellular invasion due to sarcosine accumulation.

Increasing evidence links sarcosine metabolism with disease state, including cancer (prostate, liver) and brain disease. Although the phenotypic expression of altered sarcosine metabolism is pleiotropic in several cell types, these cell type and isoform-specific differences suggest that a broad range of biologic responses is mediated by TMEFF2 and sarcosine. Because TMEFF2 is also differentially expressed in brain, it is reasonable to hypothesize that its role there may also be related to sarcosine metabolism. In fact, TMEFF2 was identified in a genome-wide association study as a factor involved in schizophrenia. Whether the role of TMEFF2 in schizophrenia is related to its ability to modulate the level of sarcosine and therefore the activity of GlyT1 is not yet certain, but sarcosine is currently being investigated as a treatment for schizophrenia (Javitt 2009). The complex biology of TMEFF2 offers insight into metabolic

regulation of cancer and perhaps other disease states. The contribution of different TMEFF2 isoforms to this biology and the regulation of their expression remain to be defined.

## **CHAPTER FOUR: The TMEFF2 Tumor Suppressor Modulates Integrin Expression, RHOA Activation and Migration of Prostate Cancer Cells<sup>2</sup>**

### **Introduction**

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths in American men (Siegel et al. 2013). While organ-confined prostate cancer is successfully treated by surgical methods, no effective treatment is available for the metastatic form of the disease, which is responsible for the majority of prostate cancer deaths. Prostate cancer cells are known to metastasize to numerous organs, with the bone, liver, and lymph nodes being the most common (Shah et al. 2004); however, the molecular mechanisms that drive the metastatic cascade in prostate cancer are poorly understood. Understanding these mechanisms and the molecules involved in the metastatic cascade is critical to developing strategies for metastasis prevention and therefore for efficient treatment of prostate cancer.

Integrins are members of a family of transmembrane glycoprotein receptors that mediate cell-cell and the interactions with the extracellular matrix (ECM). By interacting with actin-associated proteins, integrins provide a link between the extracellular environment and the cytoskeleton inside the cells (Schoenwaelder and Burridge 1999). Integrins are heterodimers composed of non-covalently associated  $\alpha$  and  $\beta$  subunits. So far, 18  $\alpha$  and 8  $\beta$  subunits, and 24 complexes have been identified that can recognize and bind multiple ECM ligands, triggering a variety of signal transduction events that modulate diverse cellular responses including proliferation, survival, gene expression, adhesion and migration (Hynes 2002; Desgrosellier and

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<sup>2</sup> This chapter contains work submitted as Xiaofei Chen, Joshua M. Corbin, Greg J. Tipton, Li V. Yang, Adam S. Asch and Maria J. Ruiz-Echevarría. The TMEFF2 tumor suppressor modulates integrin expression, RhoA activation and migration of prostate cancer cells.

Cheresh 2010; Hood and Cheresh 2002). As a result, integrins and their ligands play critical roles in biological processes like development, immune response, hemostasis, and cancer. In fact, alterations in integrin signaling have been shown in several types of cancer, including prostate cancer, and correlate with tumor growth, increased invasion, and metastatic potential (Goel et al. 2008; Fornaro et al. 2001).

Several integrins,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha v\beta 1$ ,  $\alpha I I b\beta 3$ , and  $\alpha v\beta 3$  are expressed in prostate cancer cells (Fornaro et al. 2001; Lee et al. 2013). Of those,  $\alpha v\beta 3$  and  $\beta 1$  seem to play important roles in the formation of metastasis to bone, the main site of metastatic prostate cancer (Lee et al. 2013; Cooper et al. 2002; McCabe et al. 2007). Although not expressed in normal epithelial cells (Cooper et al. 2002), integrin  $\alpha v\beta 3$  is expressed in prostate cancer, and its expression correlates with disease progression and metastatic potential (Goel et al. 2008; Fornaro et al. 2001; Cooper et al. 2002), and with prostate cancer cell adhesion to, and migration on, vitronectin, a major extracellular component of mature bone (Zheng et al. 1999). Moreover,  $\beta 1$  integrins were also shown to be upregulated in specimens from prostate cancer patients, and antibodies against  $\beta 1$  integrins inhibited binding of PC3 cells to human bone marrow endothelial cells (Lee et al. 2013; Scott et al. 2001), suggesting that  $\beta 1$  integrins mediates bone metastasis.

Integrins are unique in that they may signal bidirectionally. Intracellular stimuli (inside-out signaling) can potentially promote a conformational change in the integrin that lead to higher affinity for its ligand (Kim et al. 2011; Shen et al. 2012; Ginsberg et al. 2005; Harburger and Calderwood 2009). Ligand binding (outside-in signaling) ultimately leads to integrin activation and clustering into large mature focal adhesions able to affect downstream signaling events in a temporal fashion (Guo and Giancotti 2004; Ginsberg et al. 2005; Harburger and Calderwood 2009). One of the changes that take place early after integrin activation involves cytoskeletal

rearrangements that regulate stress fiber formation and promote cell spreading and initiation of migration, which are critical steps in metastasis (DeMali et al. 2003). These processes require integrin-mediated activation of specific cellular kinases and inhibition of the small GTPase RHOA (Shen et al. 2012; Huvneers and Danen 2009; Schwartz and Shattil 2000). RHOA is essential to remodeling actin fibers, regulation of actomyosin contractility, and rear cell detachment during motility (Sah et al. 2000; Burridge and Wennerberg 2004). Interestingly, RHOA is also activated via G protein-coupled receptors (GPCRs) that couple to the heterotrimeric G12/13 proteins, highlighting a cooperative relationship between integrin and GPCR signaling, also described for integrin and growth factor receptor signaling (Eliceiri 2001; Ivaska and Heino 2011; Miyamoto et al. 1996), to affect cell spreading, migration, growth and survival.

The transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) is an evolutionarily conserved type I transmembrane protein expressed in the embryo and selectively in the adult brain and prostate (Liang et al. 2000; Glynne-Jones et al. 2001; Gery et al. 2002; Afar et al. 2004; Zhao et al. 2005). 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 (Glynne-Jones et al. 2001; Afar et al. 2004; Zhao et al. 2005). We have described that 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 (Chen et al. 2011). TMEFF2 overexpression blocked basal and sarcosine-induced cellular invasion of prostate epithelial RWPE1 cells, while TMEFF2 knockdown in 22Rv1 prostate cancer cells, which naturally express TMEFF2, increased cellular migration/invasion (Green et al. 2013). While these results highlight a role for TMEFF2 in

invasion of prostate cells, the molecular mechanisms involved in this process are not known. Using different ECM substrates, here we report that TMEFF2 expression inhibits spreading and migration of RWPE2 prostate cancer cells on vitronectin and laminin but not fibronectin or collagen. This correlates with a defect in focal adhesion and stress fiber formation and in RHOA activation and requires the presence of the cytoplasmic tail of TMEFF2. Importantly, TMEFF2 expression promoted downregulation of  $\alpha_v$  and  $\beta_3$  integrin subunits in RWPE2 cells and TMEFF2 knockdown caused an increase in  $\alpha_v$  integrin subunit in 22Rv1 cells, indicating that the motility effects observed are integrin-mediated. Decreased expression of integrins was also observed in the prostate of a TRAMP mouse model of prostate cancer that has been engineered to overexpress TMEFF2 exclusively in the prostate. All these results point to an important role of TMEFF2 in modulating integrin signaling and prostate cell motility.

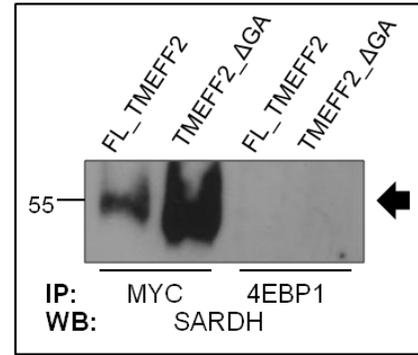
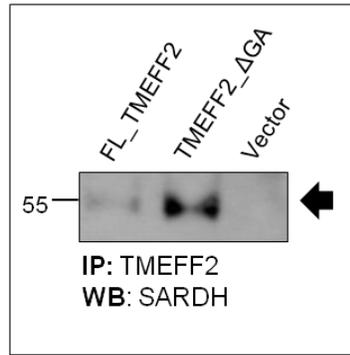
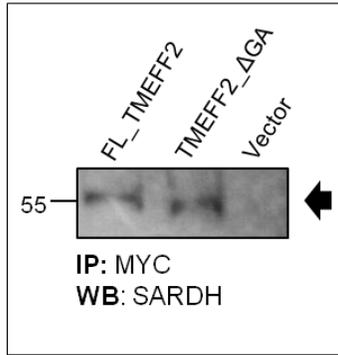
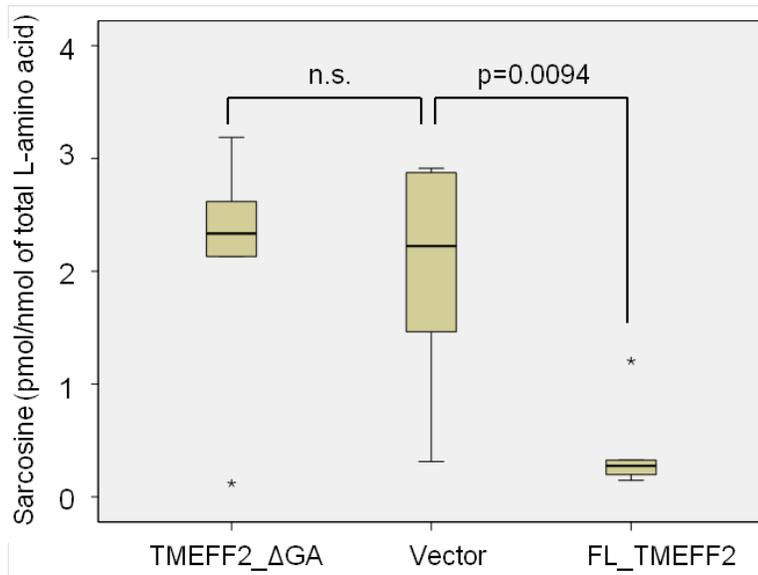
## **Results**

### *The cytoplasmic domain of TMEFF2 is necessary for its tumor suppression role*

The transmembrane and cytoplasmic domains of TMEFF1 and TMEFF2, the two members of the TMEFF family of proteins, are very well conserved at the amino acid level, and contain potential GPCR-signaling motifs in the membrane and cytoplasmic domains. For TMEFF1, the relevance of these domains is underscored by data that point to different functions of the protein depending on their presence/absence (Chang et al. 2003). For TMEFF2, we have shown that while the full-length protein interacts with SARDH and promotes tumor suppression, a soluble form of the TMEFF2 protein lacking the transmembrane and cytoplasmic domains does not interact with SARDH and promotes cellular proliferation (Chen et al. 2011). These observations

suggest that signaling from the membrane and/or the cytoplasmic domain is important for the tumor suppressor role of TMEFF2. To examine the role of the TMEFF2 cytoplasmic domain, we used a deletion mutant lacking 13 consecutive basic-rich amino acids in the C-terminus of the protein (TMEFF2\_ΔGA) and determined its ability to interact with SARDH and its tumor suppressor function. A plasmid expressing SARDH from a CMV promoter (pCMV-SARDH) was transiently transfected into HEK293T cells stably expressing a MYC-HIS tagged full-length TMEFF2 (FL\_TMEFF2) or TMEFF2\_ΔGA protein and anti-TMEFF2 immunocomplexes were analyzed for the presence of SARDH by western blotting. SARDH was detected in the TMEFF2-immunoprecipitates with lysates obtained from cells expressing the TMEFF2-MYC-HIS construct (Figure 13A), supporting previous results (Chen et al. 2011). In addition, SARDH also associated with the TMEFF2\_ΔGA-MYC-HIS protein, indicating that the cytoplasmic tail of TMEFF2 is not essential for its interaction with SARDH (Figure 13A). We next analyzed the effect of expressing the TMEFF2\_ΔGA mutant on cellular sarcosine levels. Cell lysates were prepared from HEK293T cells stably transfected with the TMEFF2-MYC-HIS, TMEFF2\_ΔGA-MYC-HIS expression constructs or with the empty vector as a control, and sarcosine levels were determined in the resulting cell lines (Figure 13B). As previously reported, overexpression of TMEFF2 resulted in a significant decrease in the amount of sarcosine with respect to the lysates expressing the empty vector control (Chen et al. 2011); however overexpression of the TMEFF2\_ΔGA mutant did not affect sarcosine levels (Figure 13B).

As we have hypothesized that TMEFF2 inhibits tumorigenic behavior through its ability to regulate SARDH activity and therefore the levels of cellular sarcosine, we predicted that the TMEFF2\_ΔGA protein would not demonstrate these inhibitory effects. Addition of sarcosine to prostate epithelial RWPE1 cells promotes their tumorigenic potential by increasing the migration

**A****B**

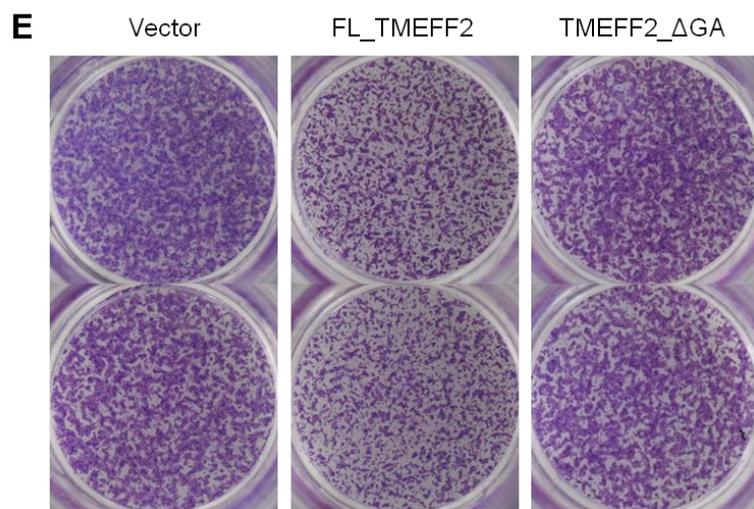
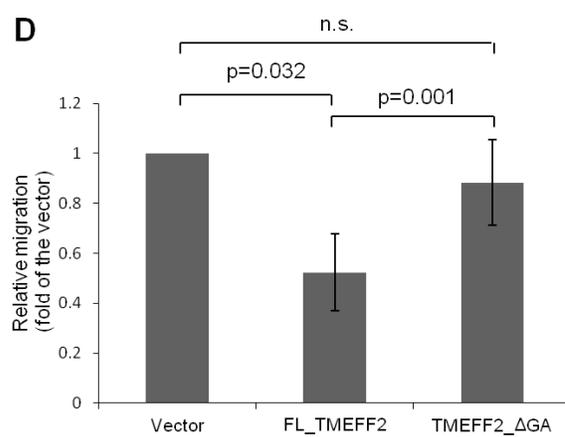
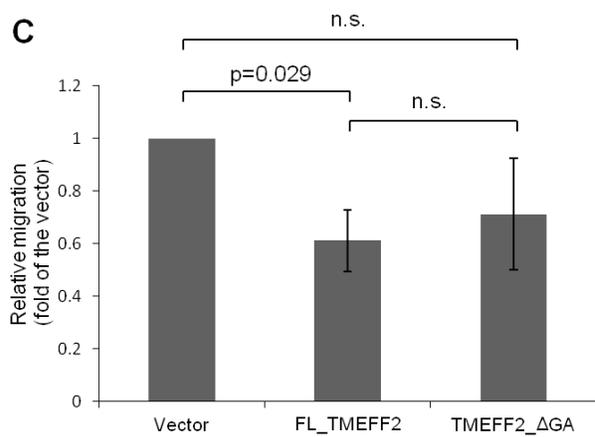
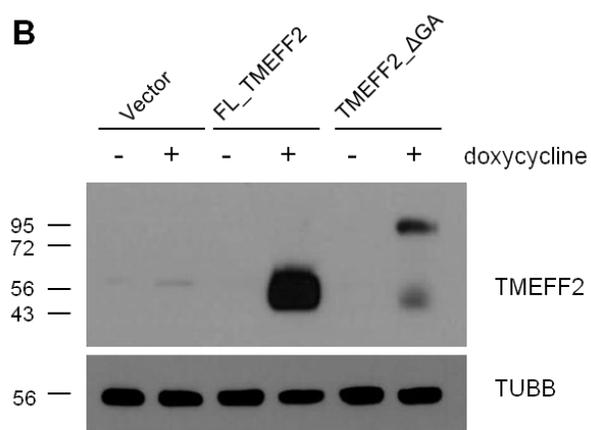
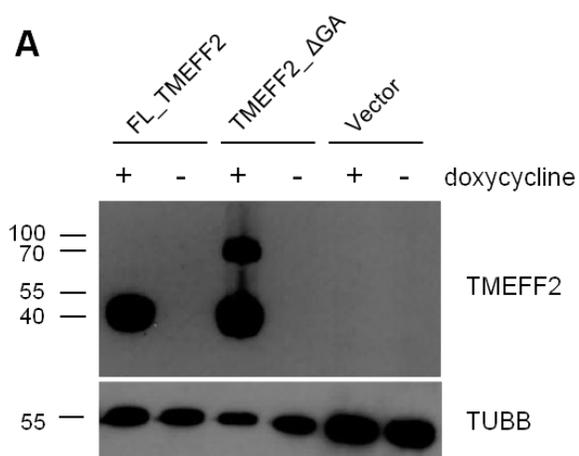
**Figure 13.** TMEFF2\_ΔGA interacts with SARDH but does not affect the levels of cellular sarcosine. **A)** TMEFF2\_ΔGA associates with SARDH. Cell lysates from HEK293T cells overexpressing SARDH and MYC-tagged FL\_TMEFF2, TMEFF2\_ΔGA, or the empty vector control, were immunoprecipitated (IP) with anti-MYC (left, right), anti-TMEFF2 (middle) or control anti-4EBP1 (right) antibodies and immunoblotted (WB) with anti-SARDH-HRP antibody. The size of the band corresponding to SARDH is marked with a black arrowhead. Note that the results in these blots do not represent quantitative differences. **B)** TMEFF2\_ΔGA does not affect intracellular sarcosine levels. HEK293T cells overexpressing FL\_TMEFF2, TMEFF2\_ΔGA, or the empty vector were lysed in sarcosine assay buffer and sarcosine was measured in the lysate using a sarcosine assay kit. Data were normalized to the level of total L-amino acid and are shown as a box plot of six independent experiments. Asterisks indicate outliers.

and invasion ability of these cells (Sreekumar et al. 2009; Chen et al. 2011) and TMEFF2 is able to inhibit the intrinsic and sarcosine-induced migration potential of these cells (Chen et al. 2011). We therefore tested whether TMEFF2\_ΔGA can also inhibit migration of the RWPE1 or RWPE2 cells, a Ki-ras transformed derivative of RWPE1. For this purpose, we generated cell lines that inducibly express FL\_TMEFF2 or TMEFF2\_ΔGA with the addition of doxycycline to the growth medium (Figure 14A, B). Control cells were transduced with the transactivator construct only (vector). Using a wound-healing assay, we observed that, as opposed to cells expressing the full-length TMEFF2 protein, cells expressing the TMEFF2\_ΔGA protein did not significantly inhibit migration of RWPE1 or RWPE2 cells (Figure 14C, D). Similar results were obtained using Boyden chambers (Figure 14E). These results suggest that the cytoplasmic tail of TMEFF2 is necessary for its ability to inhibit migration of prostate epithelial and cancer cells. Interestingly, we observed a tendency towards slower migration of cells expressing TMEFF2\_ΔGA than cells expressing the vector control. This may reflect more than one mechanism to inhibit migration. All together these results suggest that the cytoplasmic tail of TMEFF2 is important for its tumor suppressor function.

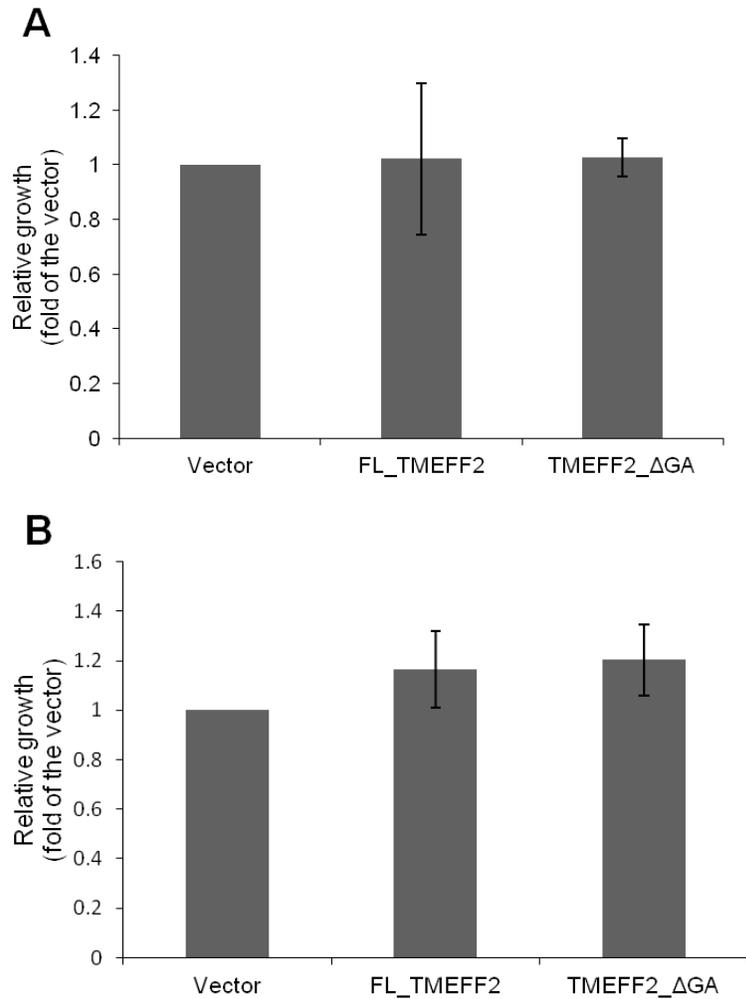
To further evaluate the contribution of the TMEFF2 cytoplasmic domain to tumorigenesis, we determined the effect of the TMEFF2\_ΔGA mutant protein on RWPE1 and RWPE2 cell growth. As previously reported for TMEFF2, the TMEFF2\_ΔGA mutant did not demonstrate an effect on monolayer growth of RWPE1 or RWPE2 cells (Figure 15).

#### *TMEFF2 promotes cell rounding and reduces cell spreading*

In order for cells to migrate they must attach and spread. While conducting the experiments described above, we observed that expression of TMEFF2 induces cell rounding of RWPE2 and



**Figure 14.** TMEFF2 inhibits migration in RWPE1 and RWPE2 cells. **A, B)** Cells were grown in the presence of 250 ng/ml doxycycline to induce the expression of FL\_TMEFF2 or TMEFF2\_ΔGA. Overexpression of these proteins in RWPE1 (A) and RWPE2 (B) cells after doxycycline treatment was assessed by western blot. **C, D)** Cell migration of RWPE1 (C) or RWPE2 (D) cells expressing the different forms of TMEFF2, was determined using a wound healing assay, 48 (RWPE1) or 10 hours (RWPE2) after the wound was made. Quantification of relative migration (fold over the vector) is presented as mean ± SD of three independent experiments. **E)** TMEFF2 inhibits RWPE2 cell migration as measured using a Boyden chamber transwell assay. Cells adhering to the bottom of the membrane were fixed, stained with crystal violet and photographed. Shown are representative images of cells after overnight migration.

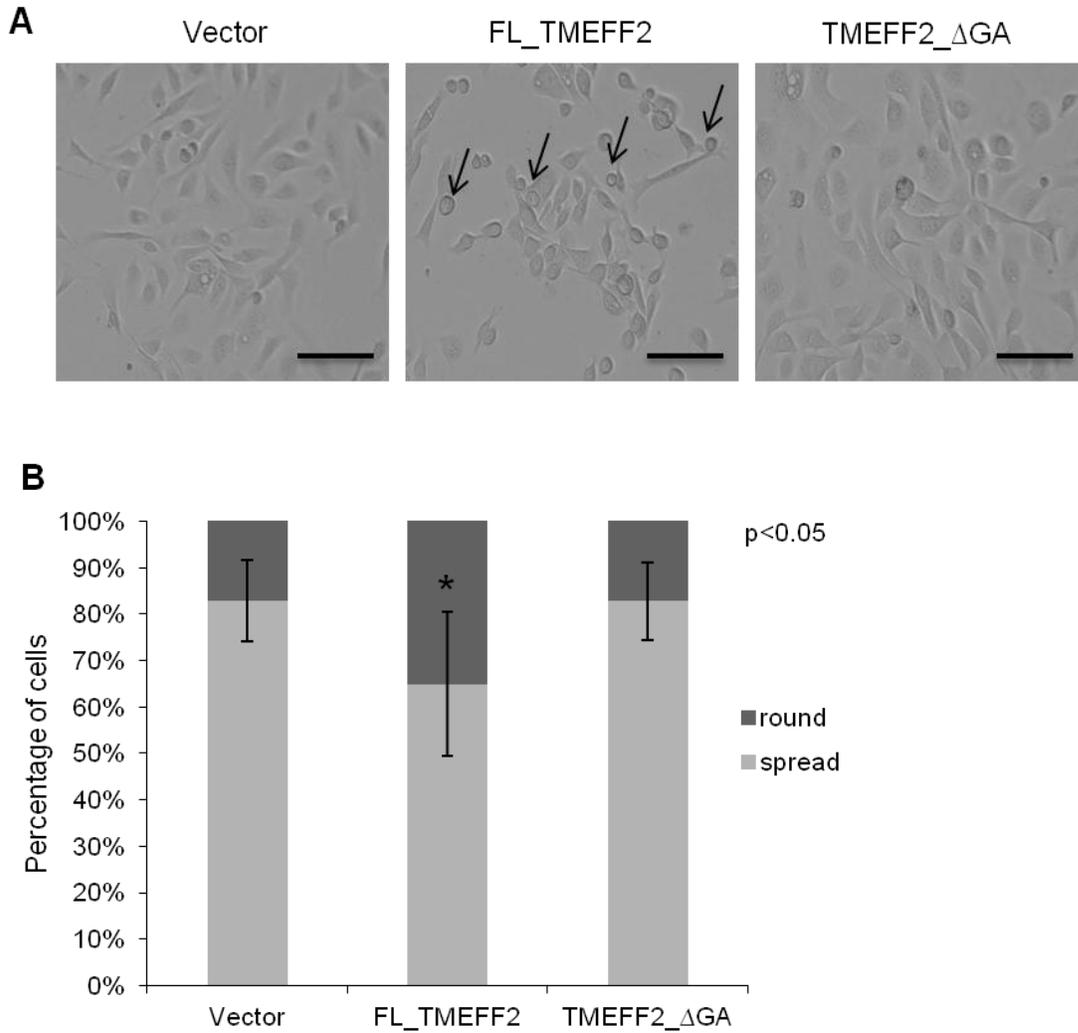


**Figure 15.** TMEFF2\_ΔGA does not affect cell monolayer growth in RWPE1 and RWPE2 cells. The effect of TMEFF2 on growth in **A**) RWPE1 and **B**) RWPE2 cells was determined using an MTT assay after 48 h of growth. The 562 nm absorbance at 48 h was normalized first to the value obtained at 0 time (to correct plating variability) and then to the value obtained for the vector cells. Data are presented as mean  $\pm$  SD of four independent experiments

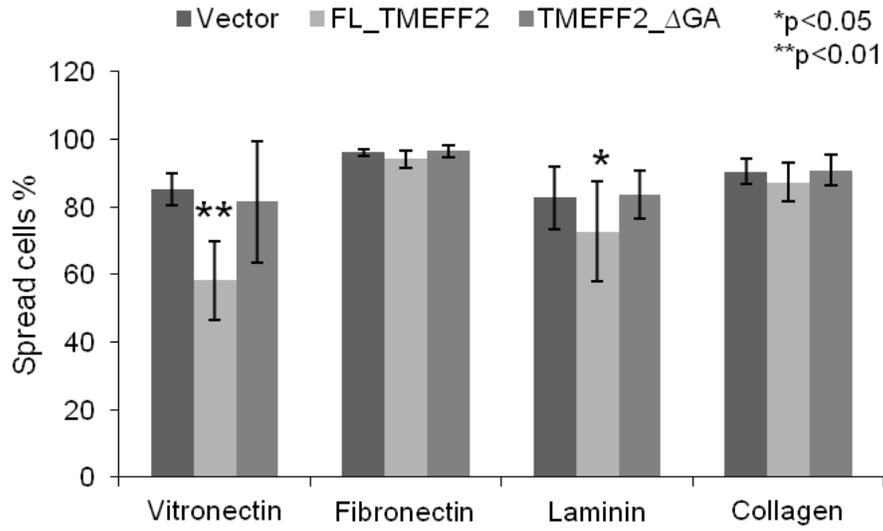
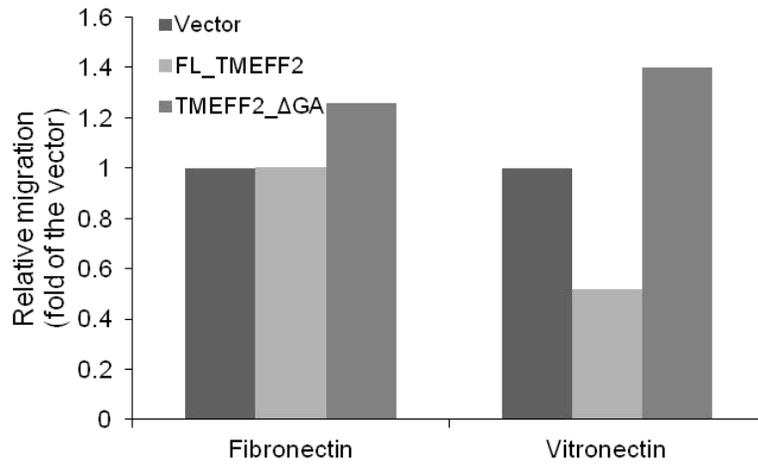
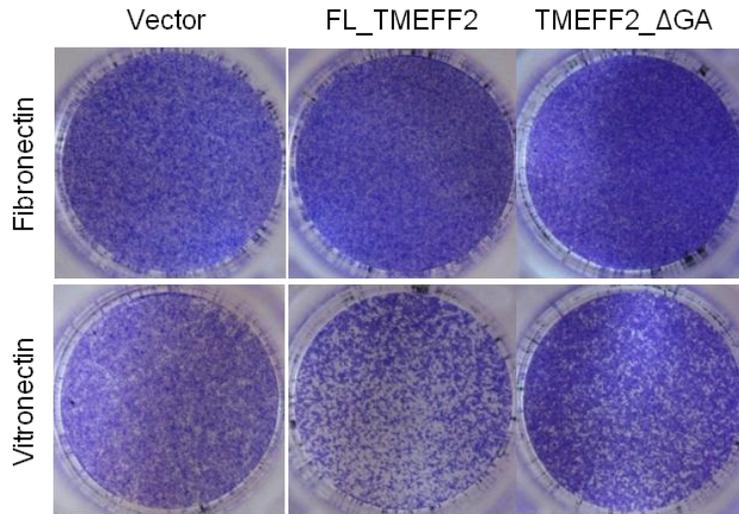
several other cell lines (Figure 16A and not shown), suggesting a potential problem on the ability of TMEFF2 expressing cells to attach/spread to the substrate, and this could account for the TMEFF2 effect on migration. Therefore, we examined attachment/spreading of RWPE2 cells expressing the inducible FL\_TMEFF2, the TMEFF2\_ΔGA proteins or the empty vector as a control, to tissue culture plates. As shown in Figure 16B, when grown in the presence of doxycycline to induce TMEFF2 expression, over 35% of the RWPE2 cells expressing the FL\_TMEFF2 have a round shape. In contrast, 85-90% of the cells expressing the TMEFF2\_ΔGA or the empty vector demonstrate typical epithelial cell morphology with 10-15% presenting rounded shape. We next examined the spreading to the ECM proteins vitronectin, fibronectin, laminin and collagen type I. RWPE2 cells expressing the empty vector control or the TMEFF2\_ΔGA behaved similarly, attaching slightly better to fibronectin and collagen type I than to vitronectin and laminin (Figure 17A). However, when compared to these cells, expression of FL\_TMEFF2 significantly reduced the ability of cells to spread on laminin or vitronectin coated surface (Figure 17A), this effect being more pronounced in vitronectin. This suggests that TMEFF2 blocks spreading to specific ECM substrates, and this could ultimately affect migration. Supporting these results, migration of cells expressing FL\_TMEFF2 is significantly reduced in vitronectin but not in fibronectin (Figure 17B), when compared to cells expressing TMEFF2\_ΔGA or the empty vector control as measured by transwell assays. All together these results suggest that TMEFF2 negatively affects the cell's ability to spread to specific substrates and it requires the presence of the cytoplasmic tail for this inhibitory effect.

#### *TMEFF2 reduces stress fiber and focal adhesion formation and activation*

Since the presence of focal adhesions correlates with cell attachment and spreading, we investigated the ability of FL\_TMEFF2 to inhibit formation of these macromolecular structures.



**Figure 16.** TMEFF2 inhibits RWPE2 cell spreading on tissue culture plates. **A)** Representative images of cell morphology of RWPE2 cells expressing different forms of the TMEFF2 protein, or the empty vector, on tissue culture surface. Arrows point to rounded cells. Scale bars, 100  $\mu$ m. **B)** Quantification of round or spread cells at 3 h after seeding 40,000 cells/well in a 12-well plate. Data are presented as mean  $\pm$  SD of three independent experiments.

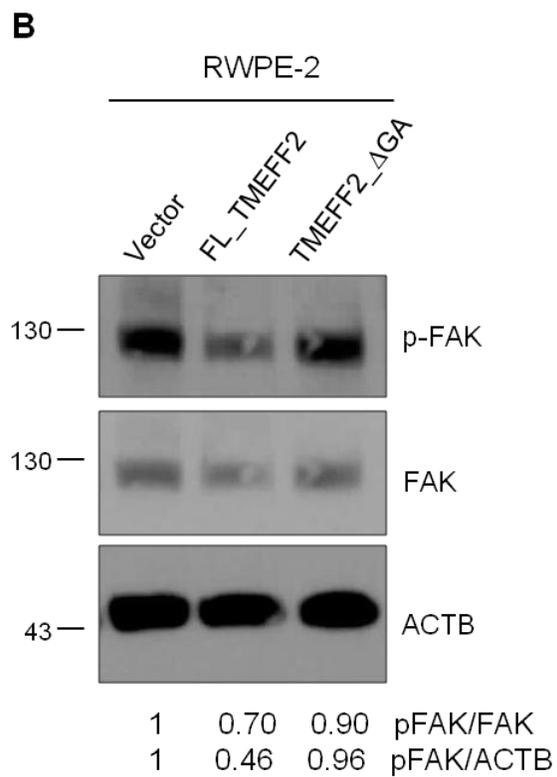
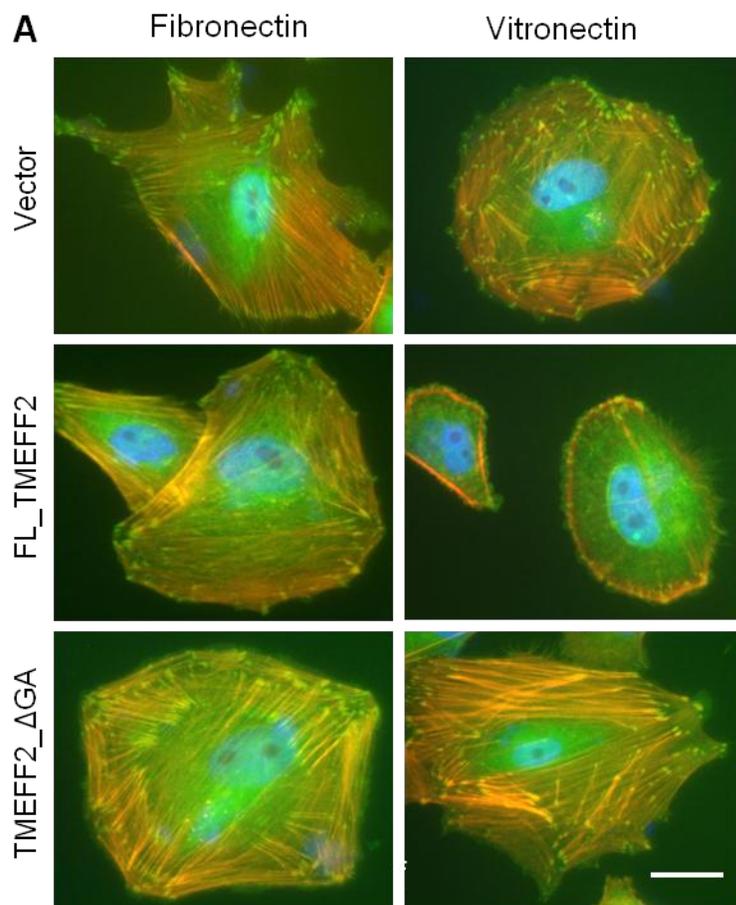
**A****B**

**Figure 17.** Effect of TMEFF2 in different ECM substrates. **A)** TMEFF2 significantly inhibits cell spreading on vitronectin and laminin-coated surfaces. RWPE2 cells expressing FL\_TMEFF2, TMEFF2\_ΔGA, or the vector were plated onto cover glass coated with the different ECM substrates. Percentages of spread cells were analyzed 3 h after seeding. Data are presented as mean ± SD of five independent experiments. **B)** TMEFF2 inhibits RWPE2 cell migration towards vitronectin but not fibronectin as measured in a Boyden chamber assay. Shown are representative images of RWPE2 cells expressing the different TMEFF2 constructs after 48 h migration (top). Quantification of those images by densitometric analysis using ImageJ (bottom). The experiment was repeated twice with similar results.

RWPE2 cells expressing FL\_TMEFF2, TMEFF2\_ΔGA or the empty vector control were grown on cover glass coated with fibronectin or vitronectin and stained with rhodamine-phalloidin to detect polymerized actin and vinculin antibodies to detect focal adhesions. As described above, expression of FL\_TMEFF2, but not the mutant lacking the cytoplasmic tail, resulted in an increase in rounded cells, more so in cells grown in vitronectin. These rounded cells revealed a cortical actin cytoskeleton, without stress fibers, and lack of focal adhesions as demonstrated by diffuse vinculin staining (Figure 18A). Conversely, the normal attachment of FL\_TMEFF2 expressing RWPE2 cells to fibronectin correlated with stress fiber formation and the presence of focal adhesions at the ends of the fibers (Figure 18A). Expression of the TMEFF2\_ΔGA mutant, did not affect stress fiber or focal adhesion formation when grown in either vitronectin or fibronectin. Finally, using western blot analysis, we measured autophosphorylation of focal adhesion kinase (FAK) at tyrosine 397 (Y397), which is required for focal adhesion formation and cell migration. The results, shown in Figure 18B, indicate that FL\_TMEFF2 but not TMEFF2\_ΔGA inhibit FAK-Y397 phosphorylation. All together these results indicate that TMEFF2 interferes with focal adhesion formation/activation and, as a consequence, it inhibits cell spreading and migration. The cytoplasmic tail of TMEFF2 is required for these effects.

#### *TMEFF2 inhibits RHOA activation*

RHOA activation promotes stress fiber formation and maturation of focal adhesions. Since our results indicated that expression of TMEFF2 affects these processes, we determined whether TMEFF2 was able to affect RHOA activation. RHO is active in the GTP-bound state, which is catalyzed by the Dbl family of guanine nucleotide-exchange factors (GEFs). The amount of activated RHOA was determined using the G-LISA kit in RWPE2 cells expressing the different forms of TMEFF2 and compared to the activity of cells expressing the vector as control. Cells

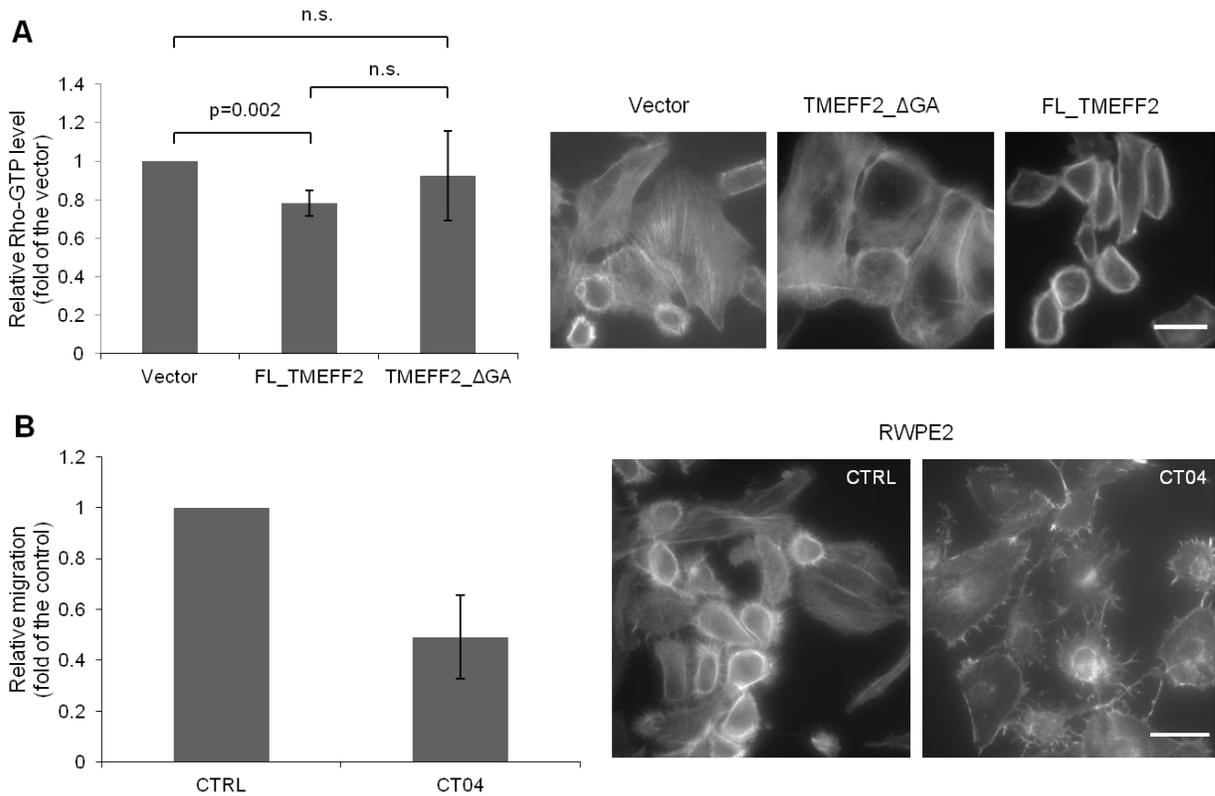


**Figure 18.** TMEFF2 reduces stress fiber and focal adhesion formation and activation. **A)** RWPE2 cells expressing FL\_TMEFF2, TMEFF2\_ΔGA, or the vector were cultured on cover glass coated with fibronectin or vitronectin for 3 h and then stained with anti-vinculin (green), rhodamine phalloidin (orange), and DAPI (blue). Scale bar, 20 μm. **B)** Immunoblotting of phosphorylated FAK (Y397), FAK and β-actin (ACTB) in RWPE2 cells. Numbers under the western blots represent the densitometry quantifications using ImageJ.

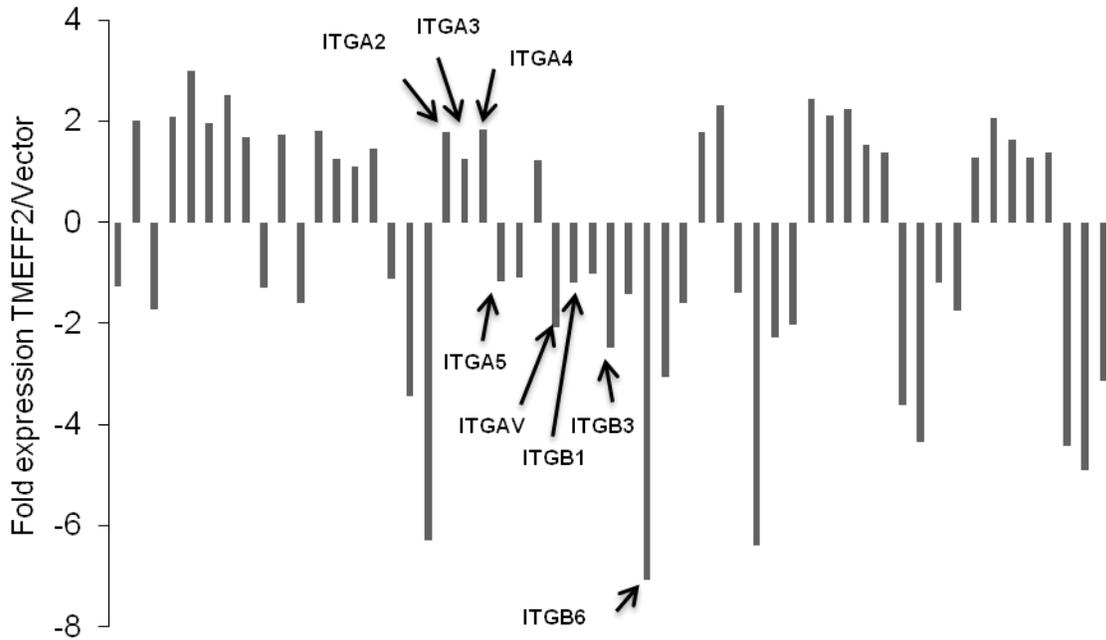
were grown in serum-free media and the stimulated with fetal bovine serum (FBS). TMEFF2 expression significantly reduced the level of active RHOA by 20% of that in the control cells (Figure 19A). Deletion of the cytoplasmic tail of TMEFF2 recovered the level of active RHOA, to 90% of the RHOA activity of control cells. Although this difference was not significant, we observed a tendency towards less RHOA activation in cells expressing TMEFF2\_ΔGA. Changes in RHOA activation correlated with the formation of stress fibers, since expression of FL\_TMEFF2, but not TMEFF2\_ΔGA, also inhibited stress fiber formation (Figure 19A). These results indicate that TMEFF2 decreases RHOA activation in FBS-stimulated conditions and that this is in part due to the cytoplasmic tail of TMEFF2. Since RHOA plays a role in migration, it is possible that the observed TMEFF2-mediated inhibition of migration is due to its effect on RHOA activation. In support of this, treatment of RWPE2 cells with CT04, a RHO inhibitor, dramatically reduced stress fiber formation and inhibited cellular migration by 50% (Figure 19B).

#### *TMEFF2 inhibits expression of integrins*

Integrins are the main ECM receptors. They are involved in motility and migration and integrin signaling can modulate RHOA activation. We therefore analyzed whether the effect of TMEFF2 on migration and RHOA activation is mediated by an effect on integrin expression. The human extracellular matrix and adhesion molecules RT<sup>2</sup> profiler PCR array was used to investigate differences in expression of 21 different integrin subunits (15  $\alpha$  and 6  $\beta$ ) -- other genes important for cell-cell and cell-matrix interactions are also included in the array -- in RWPE2 cells. Expression of FL\_TMEFF2 but not TMEFF2\_ΔGA in RWPE2 cells reduced the levels of some integrin's mRNA, including  $\alpha_v$ ,  $\beta_1$  and  $\beta_3$  (Figure 20). For those integrins, the observed decrease in mRNA levels corresponded with a decrease in total protein levels as



**Figure 19.** TMEFF2 inhibits RHOA activation. **A)** RWPE2 cells expressing FL\_TMEFF2, TMEFF2\_ΔGA, or the vector were incubated in serum-free EpiLife CF/PRF medium for 3 days and then stimulated with 10% FBS for 2 min. Levels of GTP-bound RHOA were determined by G-LISA RHOA activation assays. Data are presented as mean ± SD of five independent experiments (left). Rhodamine phalloidin staining of the cells stimulated with 10% FBS for 10 min (right) demonstrating lack of stress fibers in the cells expressing FL\_TMEFF2. Scale bar, 25 μm. **B)** Inhibition of RHO by CT04 blocks RWPE2 cell migration and stress fiber formation. 24 h migration of RWPE2 cells treated with 2 μg/ml CT04 or control medium was measured by wound healing assays using ibidi culture inserts. Data are presented as mean ± SD of two independent experiments (left). Rhodamine phalloidin staining of RWPE2 cells treated with 2 μg/ml CT04 or control medium for 4 h (right). Scale bar, 25 μm.

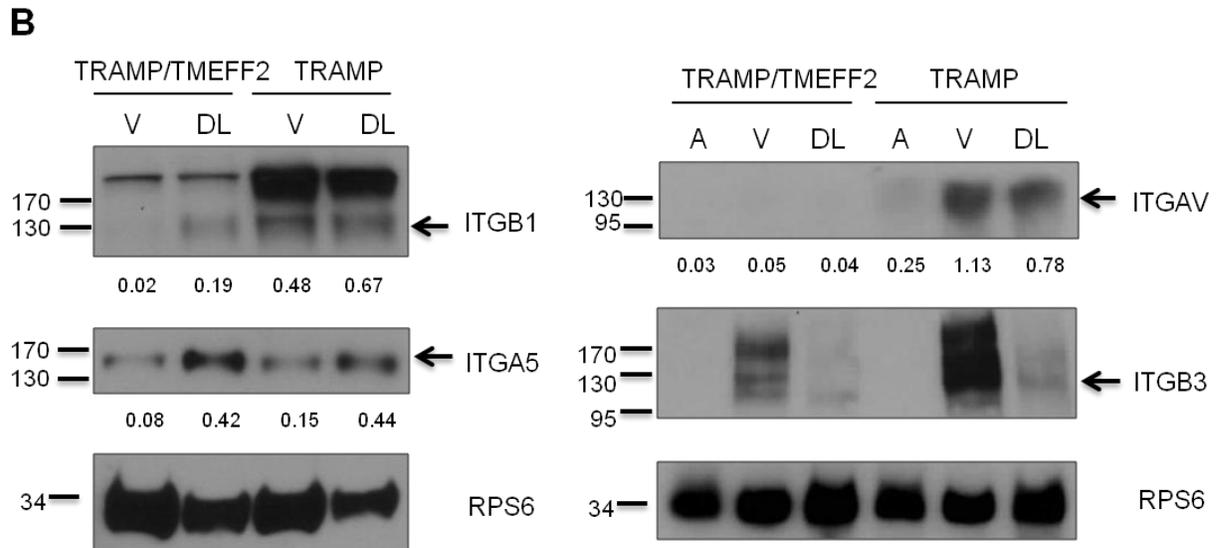
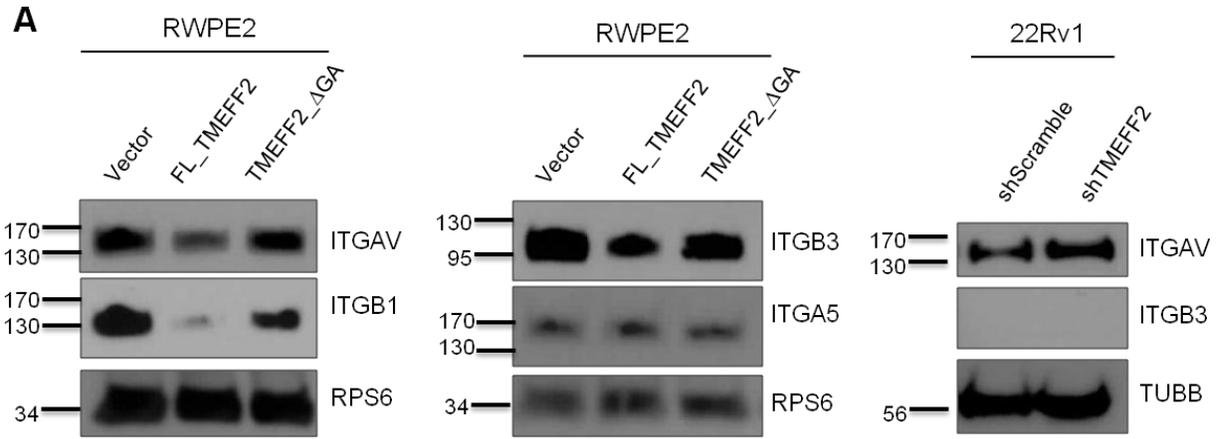


**Figure 20.** TMEFF2 decreases the expression of  $\alpha v$  and  $\beta 3$  integrin genes. The expression of key genes involved in cellular adhesion to the extracellular matrix (ECM) was determined by Human Focal Adhesions RT<sup>2</sup> Profiler PCR Array using the vector and TMEFF2-expressing RWPE2 cells. Arrows indicate *ITGAV* and *ITGB3* genes have over 2-fold reduction in TMEFF2-expressing cells compared with the vector cells. Fold change in expression of other genes is also indicated.

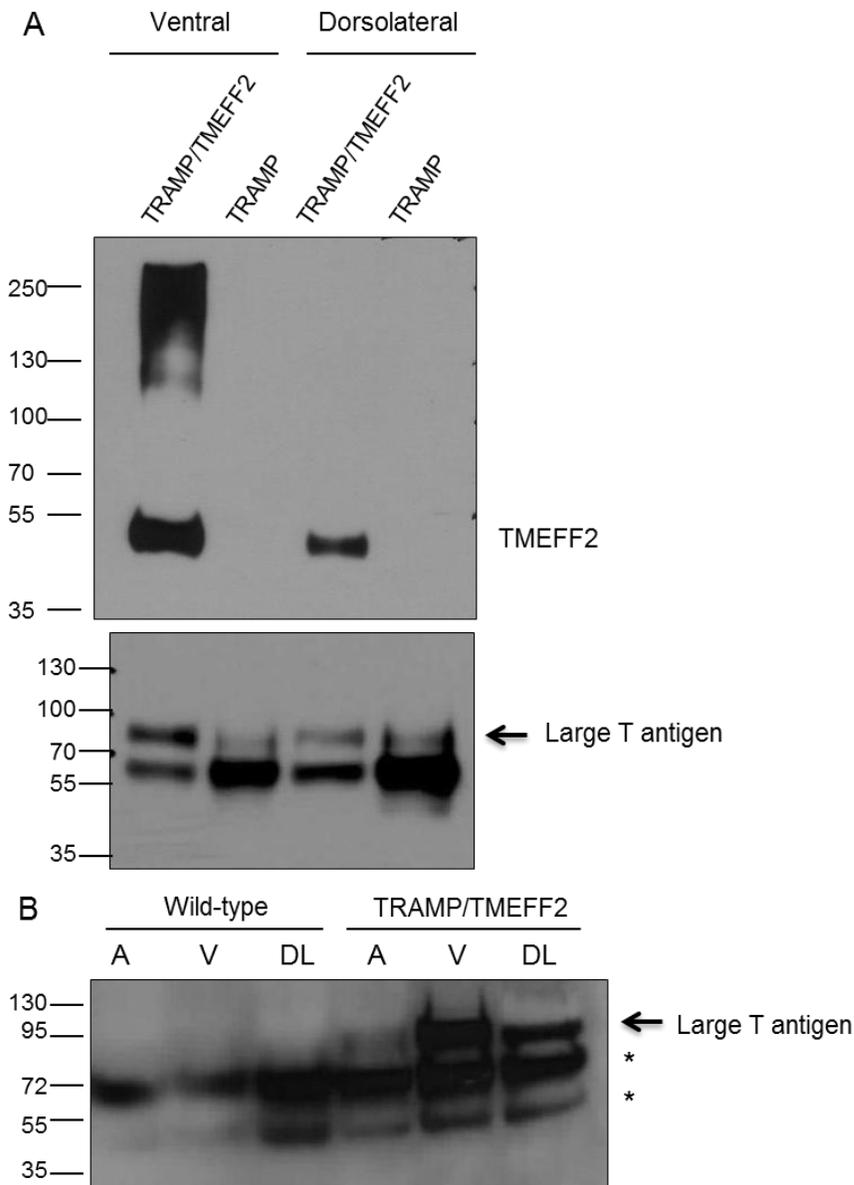
measured by western blot (Figure 21A). Expression of these integrins was also analyzed in 22Rv1/sh\_TMEFF2 cells, in which expression of endogenous TMEFF2 is reduced by shRNA, and in 22Rv1/sh\_scramble control cells (Green et al. 2013). As shown in Figure 21A, decreasing the level of TMEFF2 by shRNA promoted an increase in integrin  $\alpha_v$  protein levels. We did not detect expression of the  $\beta_1$  and  $\beta_3$  integrins in these cells (Figure 21A). These results demonstrate that FL\_TMEFF2 inhibits expression of the  $\alpha_v$ ,  $\beta_1$  and  $\beta_3$  integrins, and that the cytoplasmic tail is required for this inhibition.

#### *TMEFF2 expression inhibits integrin expression in vivo*

In humans TMEFF2 is expressed mainly in brain and prostate, however, TMEFF2 is not expressed in the adult mouse prostate (Afar et al. 2004). We have generated a transgenic TMEFF2 mice that expresses TMEFF2 from the probasin promoter and therefore transgene expression is restricted to the prostate (Figure 22). As described for the probasin promoter expression pattern (Greenberg et al. 1994), TMEFF2 is expressed more in the ventral (V) and dorsolateral (DL) prostate with almost no expression in the anterior lobe (A) of the transgenic mouse (Overcash and Ruiz-Echevarria, personal communication). The TMEFF2 transgenic animals were crossed to a mouse model of prostate cancer designated TRAMP (transgenic adenocarcinoma of mouse prostate) and 15 weeks TMEFF2/TRAMP and TRAMP siblings were selected for analysis of integrin expression. For this purpose, after euthanasia, the prostates were dissected and protein lysates from the anterior, ventral and dorsolateral lobes were prepared and analyzed for the expression of  $\alpha_v$ ,  $\beta_3$  and  $\beta_1$  integrin subunits. At least the  $\beta_1$  integrin has been shown to be upregulated in the TRAMP mouse (Goel et al. 2005). As shown in Figure 21B, expression of TMEFF2 in the TRAMP mouse significantly reduced expression of the  $\alpha_v$ ,  $\beta_1$  and  $\beta_3$  integrins indicating that expression of TMEFF2 reduces integrin expression in vivo. We did



**Figure 21.** TMEFF2 modulates the abundance of  $\alpha_v$ ,  $\beta_1$  and  $\beta_3$  integrins in cell lines and in mouse prostates. **A)** Total lysates of the indicated cells lines were subjected to immunoblotting with antibodies against the integrins as shown. Note that TMEFF2 did not promote changes in  $\alpha_5$  integrin levels and that 22Rv1 cells do not express  $\beta_3$  integrin. **B)** Tissue lysates from the anterior (A), ventral (V) or dorsolateral (DL) lobes of the mouse prostate were subjected to immunoblotting with antibodies against the specified integrins. Arrows indicate the sizes of the individual integrin subunits observed in cell lines. Additional bands may represent precursor proteins, which are also decreased by TMEFF2.  $\beta$ -tubulin (TUBB) or ribosomal protein S6 (RPS6) were used as a loading controls. Numbers under the western blots are the densitometry quantifications of the arrow-pointed bands normalized to the loading control using ImageJ. Note that the complexity of the banding pattern for the  $\beta_3$  integrin in mouse prostate lobes did not allow quantification; however, the decrease in the abundance as a result of TMEFF2 expression is clear.



**Figure 22.** Expression of TMEFF2 and large T antigen in the TRAMP/TMEFF2 and TRAMP animals. **A)** Western blot of the ventral (V) and dorsolateral (DL) lobes from the TRAMP and TRAMP/TMEFF2 transgenic animals demonstrating the presence of TMEFF2 only in the TRAMP/TMEFF2 mice and large T antigen in the TRAMP and TRAMP/TMEFF2 transgenic mice (arrow). **B)** The lower bands with the large T antigen antibody are contaminating bands since they also appear in the lobes of wild-type (C57BL/6) non-transgenic siblings (asterisks).

not observe changes in the level of the  $\alpha 5$  integrin subunit, as it was the case in the prostate cell lines tested.

## Discussion

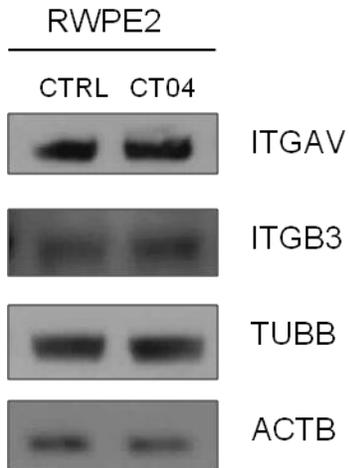
Cell migration is an essential step in embryonic development as well as in the metastatic process during cancer progression and it is usually associated with decreased survival rates and limited therapeutic options. While the metastatic process is not clearly understood, increased ability to migrate on extracellular matrix (ECM) substrates correlates with increased metastasis. Here we present data that indicate that TMEFF2, a transmembrane protein with limited expression to embryo and adult brain and prostate, plays a role in cellular adhesion and migration by modulating activation of the small GTPase RHOA, and/or integrin expression.

In this chapter we demonstrated that expression of TMEFF2 in prostate cancer cells, or in prostate lobes of a TRAMP/TMEFF2 transgenic mouse, significantly reduced the expression of at least three integrin subunits,  $\alpha v$ ,  $\beta 3$  and  $\beta 1$ . In agreement with this, we observe that TMEFF2 inhibits prostate cancer cell migration in vitronectin;  $\alpha v \beta 3$  is the major receptor for vitronectin. Importantly, *in vitro* and *in vivo* experiments have demonstrated that expression of  $\alpha v \beta 3$  integrin plays an essential role in the metastasis of prostate cancer to bone, where more than 80% of the advanced prostate cancers metastasize (Shah et al. 2004). The  $\alpha v \beta 3$  integrin plays numerous roles in prostate cancer metastasis not only by modulating engraftment and survival after bone colonization but also, due to the osteoclastic activity of the integrin, allowing bone resorption and the metastatic growth of the tumor in the bone (McCabe et al. 2007). Similar results have been observed in breast cancer where expression of  $\alpha v \beta 3$  in a mammary carcinoma line that

metastasizes to the lung, but not to bone, was sufficient to promote its spontaneous metastasis to bone (Sloan et al. 2006; Schneider et al. 2011). Moreover, in pancreatic cancer an additional ligand-independent role for  $\alpha v\beta 3$  has been described promoting anchorage-independent growth in a manner that is therefore also independent of tumor cell adhesion or the activation of FAK (Desgrosellier et al. 2009). Expression of  $\alpha v\beta 3$  has also been associated with metastasis to lungs (Duan et al. 2004). Interestingly, preliminary data from our laboratory indicates that formation of metastasis to lungs is reduced in the double TRAMP/TMEFF2 transgenic when compared with the TRAMP mice (not shown), suggesting that TMEFF2 inhibits metastasis by affecting integrin expression. Although the effect was less pronounced, the results presented here indicated that expression of TMEFF2 was also able to significantly inhibit attachment/migration to laminin. This result was expected since TMEFF2 affects expression of the  $\beta 1$  integrin, and at least four of the  $\beta 1$  containing integrins bind to laminin (Hynes 2002; Mecham 1991). In addition,  $\beta 1$  integrins have been shown to play a role in prostate cancer metastasis (Lee et al. 2013). Laminins and the integrin-laminin receptors  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  are expressed in normal prostate glands, while only the laminin binding receptors  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  are expressed in prostate cancer. These changes in laminin receptors together with modifications in the laminins during prostate cancer progression allow migration of the epithelial cells during prostate cancer progression (Sroka et al. 2010). The function of integrin-laminin receptors in prostate cancer metastasis is especially relevant since laminins are very abundant in the peripheral nervous system and play an essential role in perineural invasion, a process that contributes to prostate cancer metastasis (Sroka et al. 2010; Liebig et al. 2009). Interestingly, it has been reported that  $\beta 1$  integrin deletion in a TRAMP mouse increases prostate epithelial cell differentiation and more aggressive tumors while having no effect on metastases occurrence, as determined by visual inspection (Moran-

Jones et al. 2012). Conversely, in our TRAMP/TMEFF2 transgenic animals, in which expression of  $\beta 1$  and other integrins is reduced, we do not observe changes in the latency or grade of the tumors but in the occurrence and number of metastasis (Overcash and Ruiz-Echevarria, unpublished observations). It is possible that this reflects differences in the balance of integrin heterodimer formation.

Finally, in this chapter we have demonstrated that expression of TMEFF2 decreased RHOA activation and stress fiber formation and this effect also depended on the presence of an intact cytoplasmic domain in TMEFF2. Based on a predicted homology of this domain with G protein-coupled receptors (GPCR), we had initially envisioned that TMEFF2 could be modulating RHOA activation by, for example, restricting the function of GPCRs that are involved in  $G\alpha 12/13$  or  $G\alpha q$  activation which induce RHO (Dutt et al. 2002), or by promoting the activity of the RHO inhibitory  $G\alpha z$  signaling (Dutt et al. 2004; Mei et al. 2011). Although the ability of TMEFF2 to modulate any of the G protein  $\alpha$  subunit types ( $G\alpha s$ ,  $G\alpha i/o$ ,  $G\alpha q/11$ ,  $G\alpha 12/13$ ) has not been reported and we failed to observe changes in cAMP levels in response to TMEFF2 (not shown), an antibody targeting the follistatin domain of TMEFF1 increased cAMP levels in hematopoietic cells, suggesting TMEFF1 inhibits cAMP formation (Penning et al. 2006). At this point, TMEFF2 may have two independent mechanisms, i.e. independently regulating RHOA activation and integrin expression, to control migration. It is also possible that, TMEFF2 may downregulate integrin expression through suppression of RHOA activation. Several studies have provided evidence that changes in components of the  $G\alpha 12/13$ -RHO-SRF pathway lead to changes in integrin expression (Brandt et al. 2009; Kong et al. 2010; Leitner et al. 2011; Reymond et al. 2012). While our preliminary data indicate RHOA inactivation does not seem to affect integrin expression (Figure 23), we have not formally ruled out this possibility.



**Figure 23.** Inhibiting RHOA activity does not decrease integrin expression. RWPE2 cells were treated with 2  $\mu\text{g/ml}$  CT04 or control medium overnight and total lysates were subjected to immunoblotting analysis with antibodies against the specified integrins.  $\beta$ -tubulin (TUBB) and  $\beta$ -actin (ACTB) were used as loading controls.

Alternatively, the fact that integrins, either by engaging SRC tyrosine kinases or crosstalk with growth factor receptor signaling, can control the activation of RHO GTPases, suggests a mechanism in which TMEFF2 by controlling integrin expression may also control RHOA activation and therefore affect cell spreading and migration. It is known that by regulating the balance between RAC1-mediated membrane protrusion and RHOA-mediated contractility, integrins control the actin-driven processes involved in cell adhesion and spreading and therefore cell movement and migration (Vicente-Manzanares et al. 2009; Huveneers and Danen 2009).

In summary, these results demonstrate that TMEFF2 negatively regulates cell adhesion and migration to the ECM by affecting integrin expression and RHOA activation, and suggest a potentially important role for TMEFF2 as a metastasis suppressor.

## CHAPTER FIVE: TMEFF2 Modulates the AKT and ERK Signaling Pathways<sup>3</sup>

### Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous cancer and the second leading cause of cancer death in men (Siegel et al. 2013). Despite recent advances in treatment of localized prostate cancer, effective therapies for the treatment of the advanced form of the disease are limited. The most common being disruption of androgen receptor (AR) signaling via hormone deprivation therapy, which although initially effective, ultimately leads to castration-resistant prostate cancer (CRPC), a highly lethal form of the disease (Karantanos et al. 2013).

Essential to the development of new therapies for prostate cancer is the understanding of the signaling pathways involved in the disease and the impact that these pathways have on each other during disease progression. The PTEN and MAPK pathways are often deregulated during prostate cancer progression leading to aberrant activation of the AKT and ERK kinase activity as well as their downstream effectors (Kinkade et al. 2008; Grant 2008). Activation of the AKT signaling pathway promotes cell survival by inhibiting apoptosis while activation of ERK increases cell proliferation and both pathways may function together to promote tumorigenesis (McCubrey et al. 2006). In fact, these pathways are known to regulate each other and co-regulate downstream functions (Mendoza et al. 2011; Moelling et al. 2002). Interestingly, although in some tumors phospho-ERK levels are very high (Bartholomeusz et al. 2012; Gee et al. 2001; Kress et al. 2010), it has been reported that advanced prostate cancer correlates with low phospho-ERK and high AKT levels (Malik et al. 2002), suggesting that the cross-talk between both pathways occurs during tumor progression.

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<sup>3</sup> This chapter contains previously published work. Xiaofei Chen and Maria J Ruiz-Echevarría. TMEFF2 modulates the AKT and ERK signaling pathways. *Int J Biochem Mol Biol*. 2013 Jul 29;4(2):83-94. Print 2013.

TMEFF2 is a single pass type I transmembrane protein expressed in the embryo (Uchida et al. 1999; Heanue and Pachnis 2006) and selectively in the adult brain and prostate (Afar et al. 2004; Gery et al. 2002; Glynne-Jones et al. 2001). TMEFF2 contains several potential biologically important features that suggest a role in signaling (Uchida et al. 1999; Horie et al. 2000; Glynne-Jones et al. 2001). The extracellular (ecto-) domain, which is cleaved from the membrane in an ADAM 17/ $\gamma$ -secretase dependent fashion (Ali and Knaüper 2007; Lin et al. 2003), consists of two follistatin modules and an EGF-like domain. The transmembrane domain and short cytoplasmic tail have features that resemble a potential G protein-coupled receptor (Uchida et al. 1999).

TMEFF2 is upregulated in a significant fraction of primary and metastatic prostate tumors suggesting a role in this disease (Afar et al. 2004; Glynne-Jones et al. 2001; Zhao et al. 2005). The full-length TMEFF2 protein functions as a tumor suppressor by inhibiting migration and invasion of prostate epithelial and prostate cancer cells (Chen et al. 2011; Green et al. 2013) and by modulating apoptosis and growth of HEK293T cells (Chen et al. 2011), prostate cancer cells (Gery et al. 2002) and colorectal cancer cells as examined in an anchorage independent growth assay and a xenograft model (Elahi et al. 2008). In contrast, a recombinant form of the TMEFF2 ectodomain promotes cellular proliferation of HEK293 cells and some type of neurons (Ali and Knaüper 2007; Horie et al. 2000). In addition, pharmacological inhibition of TMEFF2 shedding from the membrane or TMEFF2 siRNA knockdown reduces cell proliferation of the LNCaP prostate cancer cell line (Ali and Knaüper 2007). In support of the proliferative role of the ectodomain, we have demonstrated that ectodomain-containing conditioned medium from cells expressing the TMEFF2 protein promotes growth of prostate and HEK293T cells (Chen et al. 2011).

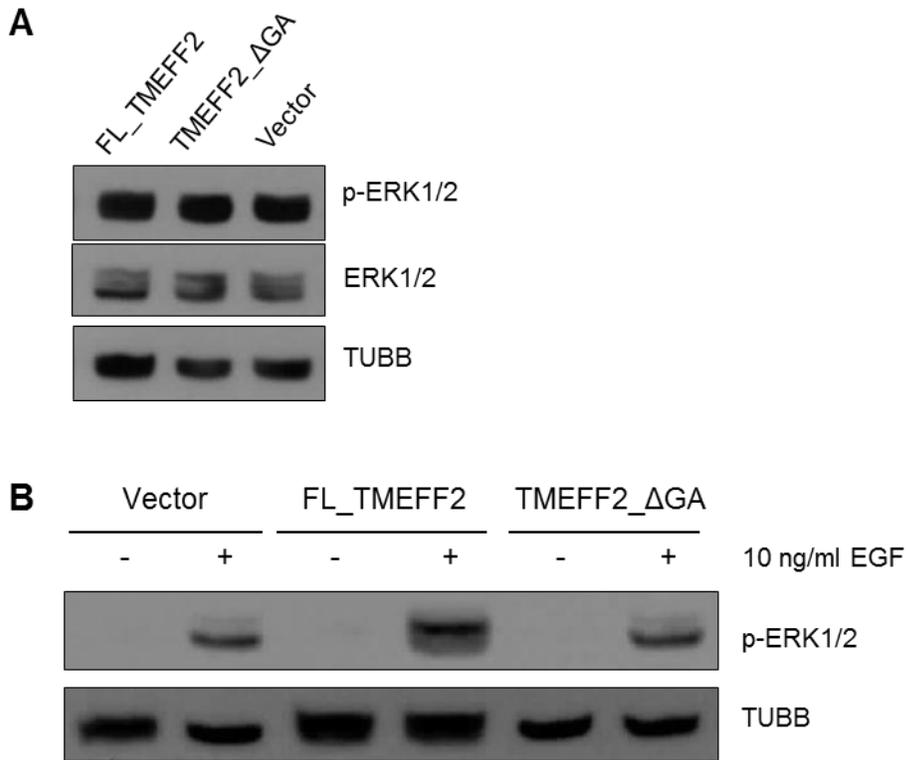
At the molecular level, recombinant TMEFF2 ectodomain has been shown to modulate ERK activation by promoting phosphorylation of erbB-4 and ERK1/2 and to interfere with platelet derived growth factor (PDGF) receptor signaling by binding and sequestering PDGF-AA from binding and signaling through its receptor (Uchida et al. 1999; Ali and Knaüper 2007; Lin et al. 2011). The full-length TMEFF2 protein also interacts with PDGF-AA (Lin et al. 2011), and with sarcosine-dehydrogenase (SARDH), the enzyme that catalyzes sarcosine conversion to glycine (Chen et al. 2011). The TMEFF2-SARDH interaction modulates sarcosine levels and one carbon metabolism leading to changes in cellular invasion, possibly due to changes in the methylation potential of the cell (Green et al. 2013). In colon cancer cell lines, TMEFF2 overexpression leads to STAT1 upregulation and this appears to be required for the TMEFF2-mediated growth suppression effect (Elahi et al. 2008).

The limited tissue distribution of TMEFF2, mainly expressed in brain and prostate (Afar et al. 2004; Glynne-Jones et al. 2001; Zhao et al. 2005), has drawn attention as a possible tool for conjugated antibody therapies. In addition, the occurrence of secreted forms of TMEFF2 (shed and spliced forms) suggests a possible role as a biomarker. The potential therapeutic use of TMEFF2 stresses the need for understanding the molecular mechanism of action. Here we explore the effect of TMEFF2 full-length and the ectodomain in the ERK and AKT signaling pathways. Our results indicate that these two different forms of the protein differentially regulate these pathways to either promote growth or to function as a tumor suppressor.

## **Results**

*The full-length TMEFF2 promotes ERK phosphorylation in response to epidermal growth factor*

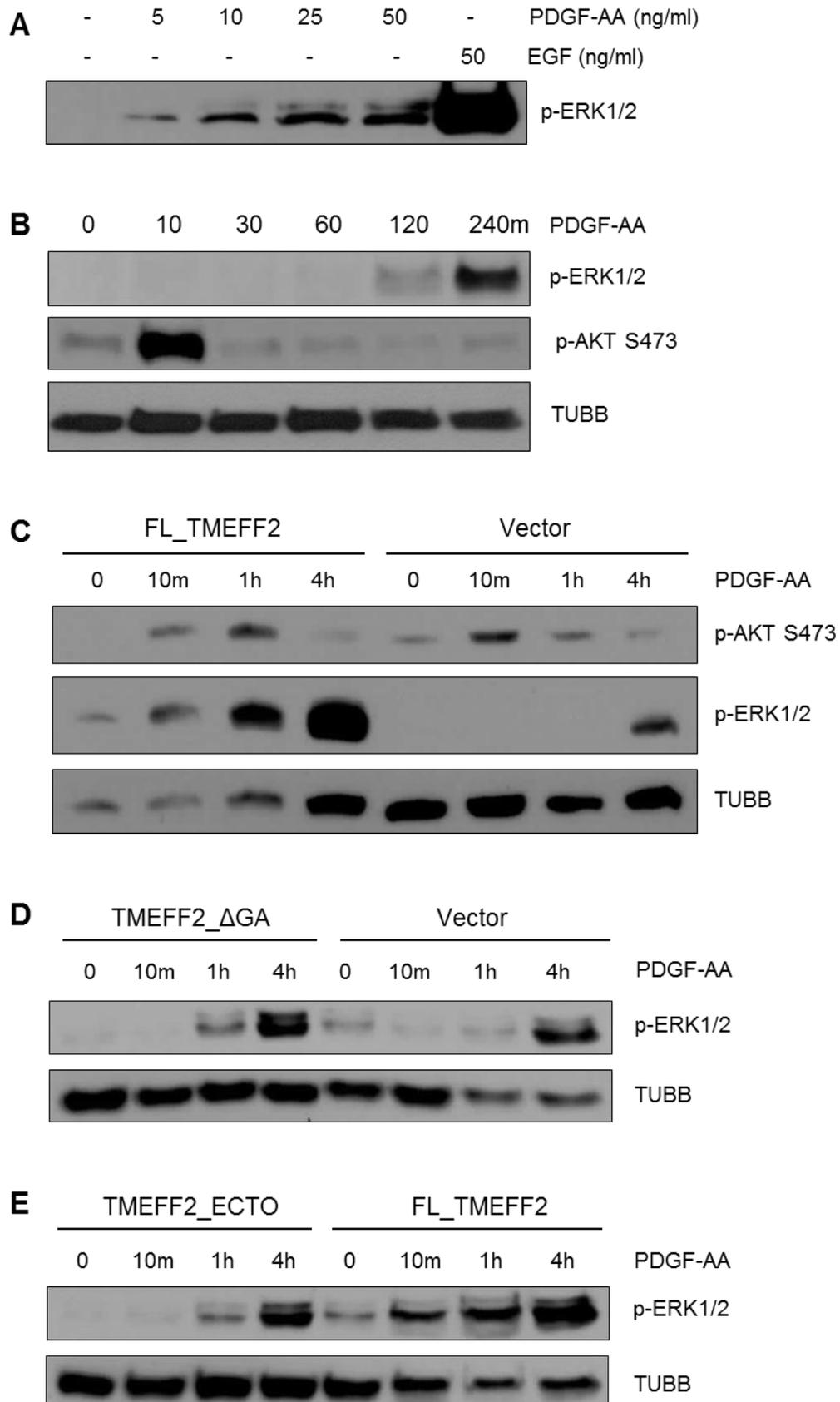
Expression of the full-length, membrane-bound TMEFF2 inhibits growth, invasion and migration of HEK293T cells and several prostate epithelial and prostate cancer cell lines (Chen et al. 2011; Green et al. 2013; Gery et al. 2002) indicating a tumor suppressor role for this protein. To further gain insight in the signaling pathways involved in TMEFF2 function, we analyzed the effect of the full-length TMEFF2 on RAS/RAF/MEK/ERK, one of the main pathways involved in Prostate cancer progression. Of note, a recombinant form of the TMEFF2 ectodomain has been shown to promote activation of several components of the EGF receptor/MEK/ERK signaling pathway, including ERK1/2 phosphorylation (Ali and Knaüper 2007). Prostate epithelial RWPE1 cells that are induced to express full-length TMEFF2 (FL\_TMEFF2) in response to addition of doxycycline, along with control cells transfected with the empty vector were used in these experiments. In addition, we created a deletion mutant, TMEFF2\_ΔGA, lacking 13 consecutive basic-rich amino acids in the cytoplasmic domain of the protein, to examine its potential role in signaling. As shown in Figure 24A, expression of either the FL\_TMEFF2 or the TMEFF2\_ΔGA did not affect ERK phosphorylation when grown under normal conditions (compared to cells expressing the empty vector). However, stimulation with EGF, a main EGFR/MEK/ERK activator, of cell cultures growing in KSF basal medium resulted in an increase in ERK phosphorylation in RWPE1 cells expressing full-length TMEFF2 as compared to cells expressing the empty vector or cells left untreated (Figure 24B). Interestingly, deleting the 13 basic-rich amino acids in the cytoplasmic domain (TMEFF2\_ΔGA) prevented TMEFF2 from promoting ERK activation suggesting that losing this region of the protein affects its signaling ability. These results indicate that in RWPE1 prostate epithelial cells, the TMEFF2 full-length protein promotes ERK phosphorylation in response to EGF and that it requires a functional cytoplasmic domain for this effect.



**Figure 24.** TMEFF2 promotes ERK1/2 phosphorylation in response to EGF. **A)** RWPE1 cells inducibly expressing FL\_TMEFF2, TMEFF2\_ΔGA, or the empty vector control were growing in complete K-SFM, lysed, and whole cell lysates were subjected to immunoblotting with anti-p-ERK1/2, anti-ERK1/2, and anti-β-tubulin (TUBB) antibodies. **B)** RWPE1 cells inducibly expressing FL\_TMEFF2, TMEFF2\_ΔGA, or the empty vector control were growing in basal K-SFM for 3 h and then stimulated with 10 ng/ml of EGF for 10 min. Whole cell lysates were then subjected to immunoblotting with anti-p-ERK1/2 and anti-β-tubulin antibodies. Representative examples of at least two independent experiments showing similar results are shown.

*PDGF-AA induces sustained phosphorylation of ERK in cells expressing TMEFF2*

As stimulation of PDGF receptors activates the RAS/RAF/MEK/ERK and the AKT/PI3K pathways in numerous cells and PDGF-AA has been shown to interact with TMEFF2 (Lin et al. 2011), we tested the effect of TMEFF2 on ERK and AKT activation using RWPE1 cells stimulated with PDGF-AA. RWPE1 cells are known to express the PDGF $\alpha$  and  $\beta$  receptors (Park et al. 2011). First, we determined that ERK phosphorylation reaches a limit at a concentration of 25 ng/ml of PDGF-AA in the medium since increasing concentrations did not result in increased ERK phosphorylation (Figure 25A), and established a concentration of 50 ng/ml PDGF-AA in subsequent experiments. The addition of PDGF-AA to RWPE1 cells growing in basal K-SFM without supplements led to an early and transient phosphorylation of AKT which peaked within 10 minutes and returned to baseline within an hour (Figure 25B). Increased phosphorylation of ERK was not apparent until 1 hour after the stimulation and was highest at the last time point, obtained 4 hours after PDGF-AA addition (Figure 25B; of note, it is possible that it is further increased). Therefore, these two signaling pathways do not seem to overlap in cells treated with PDGF-AA. Similar to the results observed with EGF stimulation, overexpression of TMEFF2, while having no significant effect on AKT phosphorylation, promoted an early and robust induction of ERK phosphorylation that was apparent 10 minutes after the addition of PDGF-AA to the culture and increased progressively throughout the duration of the experiment (up to 4 hours; Figure 25C). In addition, overexpression of the TMEFF2\_ $\Delta$ GA mutant protein nearly completely reversed the effect on ERK phosphorylation to the level observed with the empty vector-expressing RWPE1 cells suggesting that the presence of the cytoplasmic tail is required for the effect of TMEFF2 on ERK phosphorylation in response to PDGF-AA (Figure 25D). RWPE1 cells expressing the ectodomain protein (TMEFF2\_ECTO), did not demonstrate an

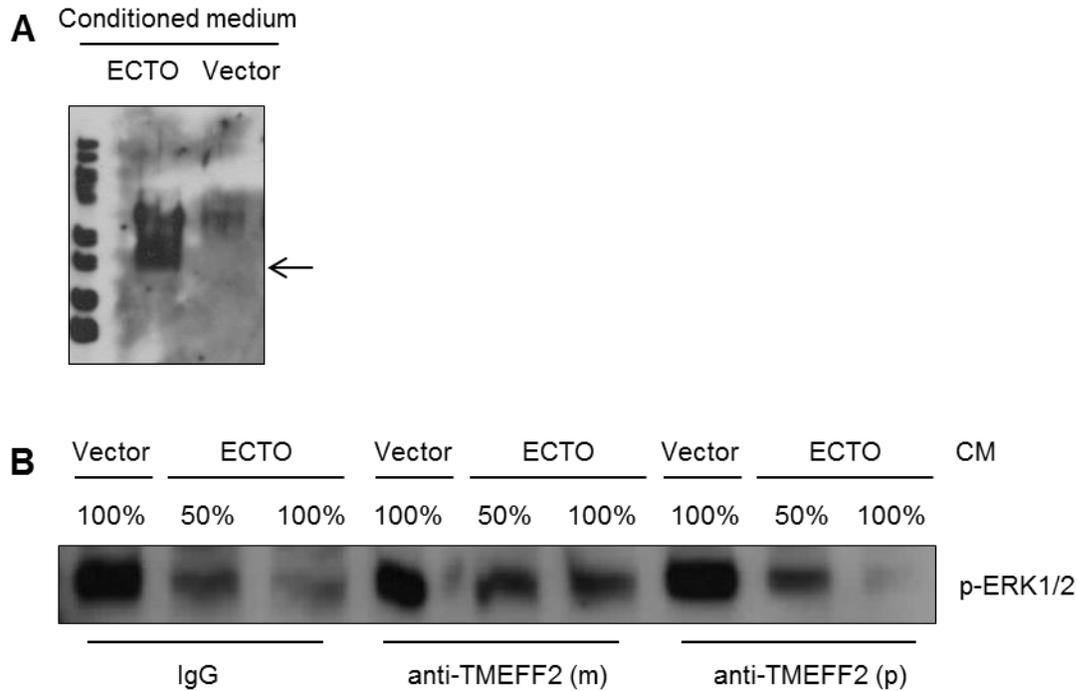


**Figure 25.** PDGF-AA induces sustained phosphorylation of ERK in cells expressing TMEFF2. **A)** RWPE1 cells were transferred to basal K-SFM for 30 min and then treated with various concentrations of PDGF-AA or 50 ng/ml of EGF. Whole cell lysates were then subjected to immunoblotting with anti-p-ERK1/2 antibody. **B)** RWPE1 cells were transferred to basal K-SFM for 30 min and then treated with 50 ng/ml of PDGF-AA for indicated times. Whole cell lysates were then subjected to immunoblotting with anti-p-ERK1/2, anti-p-AKT S473, and anti- $\beta$ -tubulin antibodies. **C,D,E)** RWPE1 cells inducibly expressing TMEFF2-ECTO, FL\_TMEFF2, TMEFF2\_ΔGA, or the empty vector control were transferred to basal K-SFM for 30 min and then stimulated with 50 ng/ml of PDGF-AA for indicated times. Whole cell lysates were then subjected to immunoblotting with anti-p-ERK1/2, anti-p-AKT S473, and anti- $\beta$ -tubulin antibodies. Representative examples of at least two independent experiments showing similar results are shown.

increase in ERK phosphorylation in response to PDGF-AA (Figure 25E). This result was expected since purified recombinant soluble TMEFF2 ectodomain has been shown to interact with PDGF-AA inhibiting its interaction with the PDGF receptor (Lin et al. 2011). Since the TMEFF2 ectodomain expressed in our cells lacks the transmembrane domain and is secreted into the medium, we hypothesized that it sequesters PDGF-AA from its interaction with the receptor inhibiting its signaling.

*The ectodomain region of TMEFF2 inhibits ERK phosphorylation*

The results presented above suggest that the TMEFF2 ectodomain may inhibit ERK phosphorylation in response to PDGF-AA. To further investigate the role of the ectodomain in ERK phosphorylation, we determined whether ectodomain-containing conditioned medium could directly modulate the activity of ERK in RWPE1 cells. Ectodomain-containing conditioned medium was collected from exponentially growing HEK293T cell cultures overexpressing the TMEFF2 ectodomain. The presence of the ectodomain in the conditioned medium was analyzed by western blot using antibodies against the TMEFF2 ectodomain (Figure 26A). The collected conditioned medium was used to replace the growth medium of the RWPE1 cells, and the ERK1/2 phosphorylation was examined by western blot analysis using a rabbit polyclonal antibody. Addition of increasing amounts of conditioned medium from the TMEFF2 ectodomain-overexpressing cultures resulted in a stepwise decrease in ERK phosphorylation in RWPE1 cells (Figure 26B, left lanes). This effect was partially reversed by the addition of a monoclonal antibody to TMEFF2 (Figure 26B, central lanes) but not by a polyclonal antibody against this protein (Figure 26B, right lanes). Immunoglobulin G (IgG) was used as a control (Figure 26B, left lanes). The differential effect of the two antibodies is likely due to different binding specificities. These results indicate that inhibition of ERK phosphorylation is, at least in

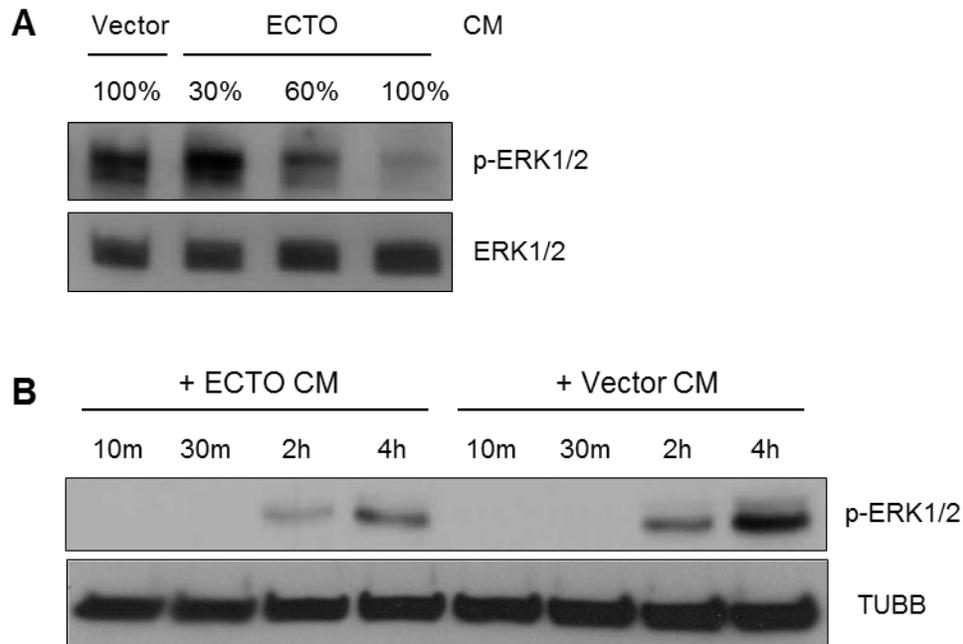


**Figure 26.** Conditioned medium from HEK293T cells expressing the ectodomain construct contains secreted TMEFF2 ectodomain and inhibits ERK phosphorylation. **A)** Exponentially growing HEK293T cells transfected with the TMEFF2 ectodomain construct or the empty vector as a control, were grown under serum starvation conditions for 24 h. A sample of the conditioned medium was then collected, concentrated and subjected to immunoblotting with an anti-TMEFF2 antibody targeting the ectodomain region of the protein. The presence of TMEFF2 ectodomain in the conditioned medium is indicated by the arrow. **B)** RWPE1 cells were transferred to basal K-SFM for 30 min before the medium was replaced with different amounts of TMEFF2 ectodomain-containing medium. Two different TMEFF2 antibodies were added to the conditioned medium (CM) to neutralize the TMEFF2 ectodomain. IgG was used as a control. Whole cell lysates were prepared and subjected to immunoblotting with anti-p-ERK1/2 antibody. Representative examples of at least two independent experiments showing similar results are shown.

part, due to the presence of the TMEFF2 ectodomain in the conditioned medium. The presence of IgG did not affect the TMEFF2 ectodomain mediated inhibition of ERK phosphorylation, as a similar dose-dependent inhibition was observed with increasing amounts of ectodomain-containing conditioned medium in the absence of IgG (Figure 27A). Finally, using a fixed amount of conditioned medium, we compared the effect of ectodomain-containing conditioned medium on ERK phosphorylation overtime. Conditioned medium collected from vector transfected HEK293T cultures was used as a control. The results (Figure 27B) show that although ERK phosphorylation occurred with similar kinetics in cells treated with TMEFF2 ectodomain-containing or empty vector control conditioned medium, the extent of ERK phosphorylation was decreased when RWPE1 cells were treated with TMEFF2 ectodomain-containing conditioned medium. These results indicate that in RWPE1 prostate epithelial cells, the effects of TMEFF2 ectodomain and the full-length protein on ERK activation are reversed and this could potentially explain the opposing functional roles of these two forms of the TMEFF2 protein.

#### *TMEFF2 ectodomain promotes AKT activation*

The results presented above were unexpected since previous reports indicate increased ERK phosphorylation in response to purified recombinant TMEFF2 ectodomain. Since the RAS-RAF-MEK-ERK and the PI3K-AKT pathways are known to demonstrate crosstalk, impacting the outcome of the pathways (Mendoza et al. 2011), we analyzed whether treatment with ectodomain-containing conditioned medium was able to induce changes in AKT activation that could ultimately affect ERK phosphorylation. Following the same protocol as described above, conditioned medium collected from HEK293T cells transfected with the TMEFF2 full-length or the ectodomain constructs was used to replace the growth medium of cultures of RWPE1 cells,

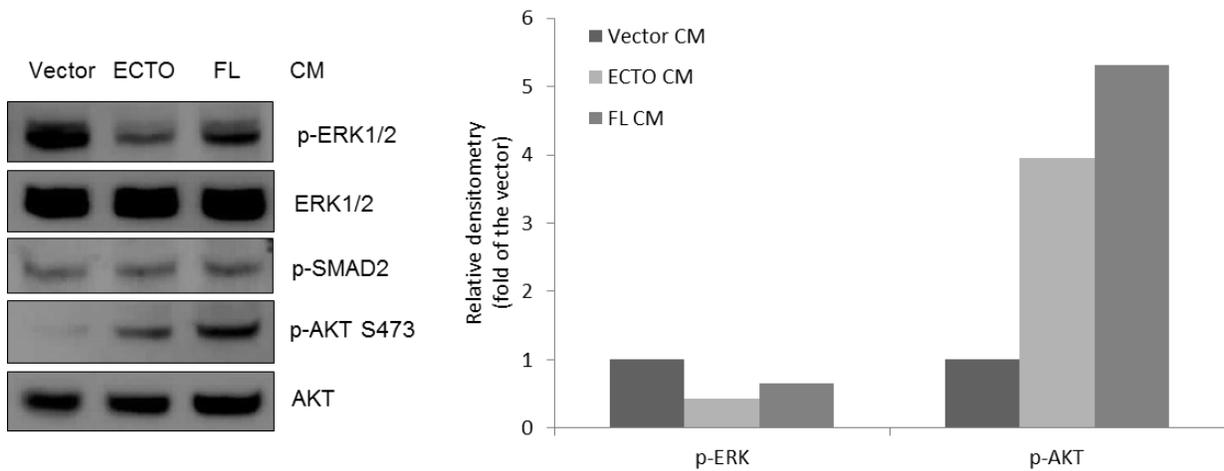


**Figure 27.** TMEFF2 ectodomain inhibits ERK1/2 phosphorylation in a dose-dependent manner but does not affect the activation kinetics. **A)** RWPE1 cells were transferred to basal K-SFM for 30 min before the medium was replaced with different amounts of ectodomain containing conditioned medium. Whole cell lysates were then subjected to immunoblotting with anti-p-ERK1/2 and anti-ERK1/2 antibodies. **B)** RWPE1 cells were transferred to basal K-SFM for 30 min before the medium was replaced with TMEFF2 ectodomain-containing conditioned medium for indicated times. Whole cell lysates were prepared and subjected to immunoblotting with anti-p-ERK1/2 and anti- $\beta$ -tubulin antibodies. Representative examples of at least two independent experiments showing similar results are shown.

and the phosphorylation of ERK and AKT was analyzed 30 minutes after the medium replacement. As a control, conditioned medium collected from vector transfected HEK293T cultures was used (Figure 28). As described above, ectodomain-containing conditioned medium - - from the full-length or ectodomain expressing cells -- decreased ERK phosphorylation. However, AKT phosphorylation was increased under these conditions, indicating an inverse correlation between ERK and AKT phosphorylation in response to the ectodomain in RWPE1 cells (Figure 28). These results are in agreement with data indicating that a proliferative stimulus can modulate the ERK pathway to prevent growth arrest by ERK-dependent upregulation of cell cycle inhibitors (Moelling et al. 2002; Reusch et al. 2001) and with the fact that AKT can play a positive or negative role in the regulation of ERK depending on several variables, such as growth condition, stage of differentiation, etc (Mendoza et al. 2011). Although TMEFF1, the only TMEFF2 homolog, has been shown to modulate Nodal signaling (Harms and Chang 2003; Chang et al. 2003), we did not observe differences in SMAD2 phosphorylation in response to TMEFF2 ectodomain conditioned medium (Figure 28). All together these results indicate that different forms of TMEFF2, full-length or ectodomain, distinctly modulate the ERK and/or AKT pathways to exert different roles.

## **Discussion**

In this study we report that TMEFF2, a protein with a role in prostate cancer, modulates the activity of the RAS/RAF/MEK/ERK and PI3K/AKT signaling pathways. Interestingly, while the full-length TMEFF2 protein promotes a strong ERK activation in response to growth factors but has no effect in AKT activation, a shed form of the protein, the ectodomain, inhibits ERK phosphorylation and promotes AKT activation. These opposing effects on ERK and AKT



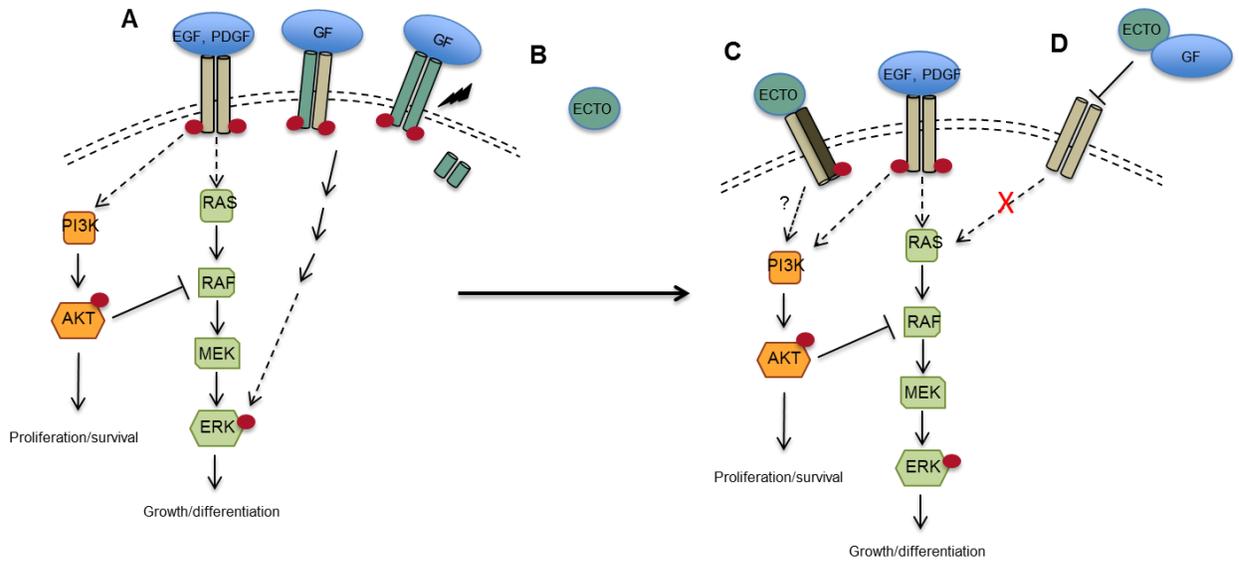
**Figure 28.** TMEFF2 ectodomain promotes AKT phosphorylation and that inversely correlates with its effect on ERK phosphorylation. RWPE1 cells transferred to basal K-SFM for 4 h before the medium was replaced with ectodomain-containing conditioned medium obtained from HEK293T cells that express the ectodomain (ECTO), the full-length (FL) or the empty vector constructs. Whole cell lysates were prepared and subjected to immunoblotting with anti-p-ERK1/2, anti-ERK1/2, anti-p-AKT S473, anti-AKT, and anti-p-SMAD2 antibodies (left). Densitometry quantification of the results is shown (right).

activation reflect a distinct role and effector site for each isoform; the full-length functions as a tumor suppressor (Gery et al. 2002; Chen et al. 2011; Green et al. 2013; Elahi et al. 2008) from the membrane or from inside the cell after internalization (Afar et al. 2004; Boswell et al. 2012; Zhao et al. 2005), while the shed ectodomain promotes growth (Ali and Knaüper 2007; Chen et al. 2011; Horie et al. 2000) and functions from outside the cell, potentially as a ligand.

The RAS/RAF/MEK/ERK and the PI3K/AKT signaling cascades play critical roles in control of cell survival, proliferation, differentiation, metabolism and cell motility and they are frequently activated during oncogenesis (Kinkade et al. 2008; Grant 2008; McCubrey et al. 2006). Accumulating evidence indicates that in addition to their independent roles, these pathways regulate each other (crosstalk) during normal growth and oncogenesis. For example, activated AKT inhibits ERK activation by phosphorylating and inhibiting RAF, upstream of ERK (Mendoza et al. 2011; Zimmermann and Moelling 1999), and by facilitating EGFR degradation to inhibit signaling to its downstream pathways (Er et al. 2013). This cross inhibition by activated AKT is especially relevant to prostate cancer since deregulated expression and/or mutations of the PTEN tumor suppressor gene, which lead to activation of AKT, occur with very high frequency in prostate cancer (Chow and Baker 2006). In fact, it has been reported that in advanced prostate cancer there are high levels of activated AKT, and this is inversely correlated with the level of ERK activation (low). In benign lesions, or low grade prostate cancer this relationship is reversed, demonstrating high phospho-ERK and low phospho-AKT levels (Malik et al. 2002; Graff et al. 2000; Kreisberg et al. 2004). These observations agree with a recently identified tumor suppressor role for ERK. High levels of ERK phosphorylation lead to an ERK-dependent protein degradation process and senescence (Deschênes-Simard et al. 2013).

Our results are consistent with these observations. The tumor suppressor full-length TMEFF2 protein promotes strong ERK phosphorylation and requires the cytoplasmic tail for this effect, while the growth-promoting ectodomain activates AKT and inhibits ERK phosphorylation. TMEFF2 is cleaved from the membrane by ADAM17/ $\gamma$ -secretase-dependent cleavage that can be induced by inflammatory cytokines, i.e. TNF $\alpha$  (Ali and Knaüper 2007; Lin et al. 2011; Chen et al. 2011), which are characteristics of the tumor microenvironment. Therefore, regulated TMEFF2 cleavage can modulate the function of TMEFF2, as it plays a role during tumor establishment and progression. Based on the results presented here, the functional switch from a tumor suppressor to a growth-promoting role could be mediated by the activation of the AKT pathway and subsequent silencing of the ERK-mediated tumor suppressor function. In addition, the distinct functions of the membrane-bound and soluble forms of TMEFF2 suggest that TMEFF2 may signal either as a ligand, a membrane bound receptor and/or as a co-receptor. Based on these results we propose a model (Figure 29) in which the different TMEFF2 forms distinctly modulate AKT and/or ERK signaling to exert different functions: (1) Full-length TMEFF2 acting as a receptor or co-receptor promotes ERK phosphorylation. (2) Shedding of TMEFF2 leads to ectodomain accumulation that can (3) function as a ligand to an unknown receptor to promote AKT activation and subsequent RAF inhibition leading to low ERK phosphorylation and (4) interact with growth factors (i.e. PDGF) to prevent interaction with their receptor and ERK activation.

The data in Figure 26 indicate that the effect of the ectodomain-containing conditioned medium on ERK phosphorylation is partly reversed by a TMEFF2 blocking antibody suggesting that, at least in part, the effect on ERK phosphorylation is directly mediated by the ectodomain. While the non-complete reversion of ERK phosphorylation could be due to failure of the



**Figure 29.** Model of the role of TMEFF2 in AKT and ERK activation. **A)** Full-length TMEFF2 acting as a receptor (green bars) or co-receptor (grey and green) promotes ERK phosphorylation. **B)** Shedding of TMEFF2 leads to ectodomain accumulation (green circle) in the conditioned medium that can **C)** function as a ligand to an unknown receptor to promote AKT activation and subsequent RAF inhibition leading to low ERK phosphorylation and **D)** interact with growth factors (i.e. PDGF) to prevent interaction with their promoter and ERK activation.

antibody, a parallel explanation to the function of the TMEFF2 ectodomain, not depicted in our model (Figure 29), is that the cleaved TMEFF2 ectodomain may also have an indirect effect on ERK phosphorylation. In this role, expression/activity of the TMEFF2 ectodomain in HEK293T cells (utilized to collect the conditioned medium used in the experiments) would promote secretion of high levels of a factor (e.g. IGF) that can activate AKT in prostate epithelial RWPE1 cells. Whether direct or indirect, the ectodomain is functioning from the outside of the cell -- potentially as a ligand -- and therefore its effect may be cell specific depending on the repertoire of receptors present in the cell. In fact, several effects have been described for the ectodomain in different cell lines. Purified recombinant ectodomain, promotes growth of neurons (Horie et al. 2000) and non-transformed HEK293 cells (Ali and Knaüper 2007) and phosphorylation of some of the components of the ERK signaling pathway – erbB-4 in MNK28 gastric cancer cells and ERK1/2 in HEK293 cells (Uchida et al. 1999; Ali and Knaüper 2007) and corticotroph cells (Labeur et al. 2010). Interestingly, in corticotroph cells, ERK activation has been reported to occur as a consequence of AKT inhibition (Labeur et al. 2010). In contrast, ectodomain containing conditioned medium, although it promotes growth of HEK293T and prostate epithelial RWPE1 cells (Chen et al. 2011), this effect, as demonstrated here, correlates with a decrease in ERK phosphorylation and an increase in AKT activation in prostate epithelial cells. Finally, using NR6 fibroblast, Lin et al. (2011) demonstrated that recombinant ectodomain binds to and competes with PDGF-AA from binding to its receptor, inhibiting PDGF-AA promoted growth.

AKT and the androgen receptor (AR) have been shown to cooperate in cancer progression (Xin et al. 2006) and it has been suggested that AKT may directly phosphorylate AR inhibiting AR transactivation and blocking AR-induced apoptosis (Lin et al. 2001). Based on our results the

TMEFF2 ectodomain promotes AKT phosphorylation and thus negatively regulates AR activation, which in turn regulates TMEFF2 expression (Gery et al. 2002; Overcash et al. 2013), providing an additional link between AKT and TMEFF2 mediated by the AR.

In conclusion, our results provide evidence of a role for TMEFF2 on AKT and ERK signaling pathways that may be relevant to prostate cancer tumorigenesis.

## CHAPTER SIX: Discussion

The oncogenesis of prostate cancer and its progression are thought to occur due to an accumulation of mutations in genes related to cell growth, adhesion/migration, and death (Shen and Abate-Shen 2010). Because the molecular mechanisms leading to prostate cancer progression are poorly understood, searching for the responsible genes is essential for the development of novel therapies and disease prevention. In this study, we investigated the molecular mechanisms underlying a tumor suppressor of prostate cancer – TMEFF2. We uncovered an interaction between TMEFF2 and SARDH, and a correlation between the tumor suppressor activity of TMEFF2 and its ability to decrease cellular sarcosine levels as an outcome of the TMEFF2-SARDH interaction. With a particular focus on its inhibitory effect on cell migration and the knowledge that integrins play a central role in this process, we found that TMEFF2 suppresses the expression of several integrin subunits including  $\alpha_v$ ,  $\beta_1$ , and  $\beta_3$ . Consistent with this finding, TMEFF2-expressing cells show defects in cell spreading on migration towards vitronectin, whose major receptor is integrin  $\alpha_v\beta_3$ , with a concomitant reduction in focal adhesion and stress fiber formation, and RHOA activation. Finally, we demonstrated that different forms of TMEFF2 differentially modulate ERK and AKT pathways and this may contribute to distinct cellular responses of proliferation or tumor suppression. While full-length TMEFF2 promotes ERK phosphorylation in response to growth factors EGF and PDGF-AA, the ectodomain activates AKT and inhibits ERK phosphorylation.

At first, the tumor suppressor function of TMEFF2 seems hard to reconcile with the fact that it is overexpressed in prostate cancer. The molecular bases described in this study provide some plausible explanations. It is interesting to note that the effects that full-length TMEFF2 exerts on sarcosine concentration, integrin expression, RHOA activation, and ERK phosphorylation are all

dependent on the presence of the GA domain; deletion of the GA domain abolishes these effects. Further deletion of the transmembrane domain even has the opposite effect on ERK phosphorylation – the ectodomain inhibits ERK phosphorylation possibly due to activation of the AKT pathway. Accordingly, full-length and the ectodomain of TMEFF2 have opposing oncogenic properties. While the full-length TMEFF2 functions as a tumor suppressor (Chen et al. 2011; Green et al. 2013; Elahi et al. 2008; Gery et al. 2002), the ectodomain promotes growth and survival (Chen et al. 2011; Ali and Knaüper 2007; Horie et al. 2000). Under physiological conditions *in vivo*, the production of TMEFF2 ectodomain is the result of ADAM17/ $\gamma$ -secretase cleavage that can be induced by inflammatory cytokines, which is typical of tumor microenvironment. It is possible that the function of TMEFF2 changes during prostate cancer progression and is regulated by ectodomain shedding. For example, TMEFF2 expression increases as prostate cancer progresses, but the predominant form shifts from the full-length to the ectodomain by virtue of the inflammatory tumor microenvironment, therefore promoting tumor growth. Moreover, the existence of several alternative splice TMEFF2 isoforms, one of them (isoform 2; Figure 3) very similar to the TMEFF2 $\Delta$ GA mutant used in this study, further complicates the scenario. Alternative splicing of mRNA precursors is a neatly regulated gene expression mechanism that allows cells to create protein isoforms with differing or even opposing functions from a single gene. It is a ubiquitous phenomenon in normal cells and is often taken advantage of by cancer cells to promote their growth and survival. The function and expression of TMEFF2 alternative splice isoforms throughout prostate cancer progression are not known. Interestingly, although we did not observe any effect from the TMEFF2 $\Delta$ GA mutant on monolayer growth of RWPE1 or RWPE2 cells, it increased monolayer and anchorage-

independent growth of HEK293T cells (Chen et al. 2013, submitted manuscript), suggesting this mutant can present a gain of function.

In Chapter 3, we showed the tumor suppressor activity of TMEFF2 correlates with its ability to bind SARDH and reduce sarcosine levels. Recently proposed as a prostate cancer biomarker, sarcosine is a differential metabolite that is highly increased during prostate cancer progression (Sreekumar et al. 2009). The mere addition of exogenous sarcosine imparted an invasive phenotype to benign prostate epithelial cells (Chen et al. 2011; Sreekumar et al. 2009). It remains unclear how sarcosine promotes cell invasion, but sarcosine is involved in one-carbon metabolism essential for DNA synthesis, repair, and methylation. One-carbon metabolism is comprised of several connected metabolic pathways that promote the folate-mediated transfer of one-carbon units necessary for DNA synthesis and repair. Folate is also essential in its 5-methyl-tetrahydrofolate (THF) form as a methyl donor in the remethylation of homocysteine to methionine, which is then converted to S-adenosyl methionine (SAM), the universal methyl donor (Fox and Stover 2008). Sarcosine participates in both the folate cycle and the methionine cycle (Figure 30): in the folate cycle, sarcosine contributes to the generation of 5,10-methylene-THF by either donating a methyl group to THF and becoming glycine in a reaction catalyzed by SARDH, or being converted from dimethylglycine by dimethylglycine dehydrogenase (DMGDH); in the methionine cycle, excess SAM remethylates glycine into sarcosine in a reaction catalyzed by glycine N-methyltransferase (GNMT). This leads to our hypothesis that by modulating sarcosine levels, TMEFF2, in a bigger picture, perturbs one-carbon metabolism, thus affecting tumor progression. In fact, in our subsequent studies, we demonstrated that sarcosine metabolism, not merely its concentration, and thus one-carbon availability are responsible for the changes in cell invasion mediated by the function of TMEFF2 and SARDH (Green et al. 2013).



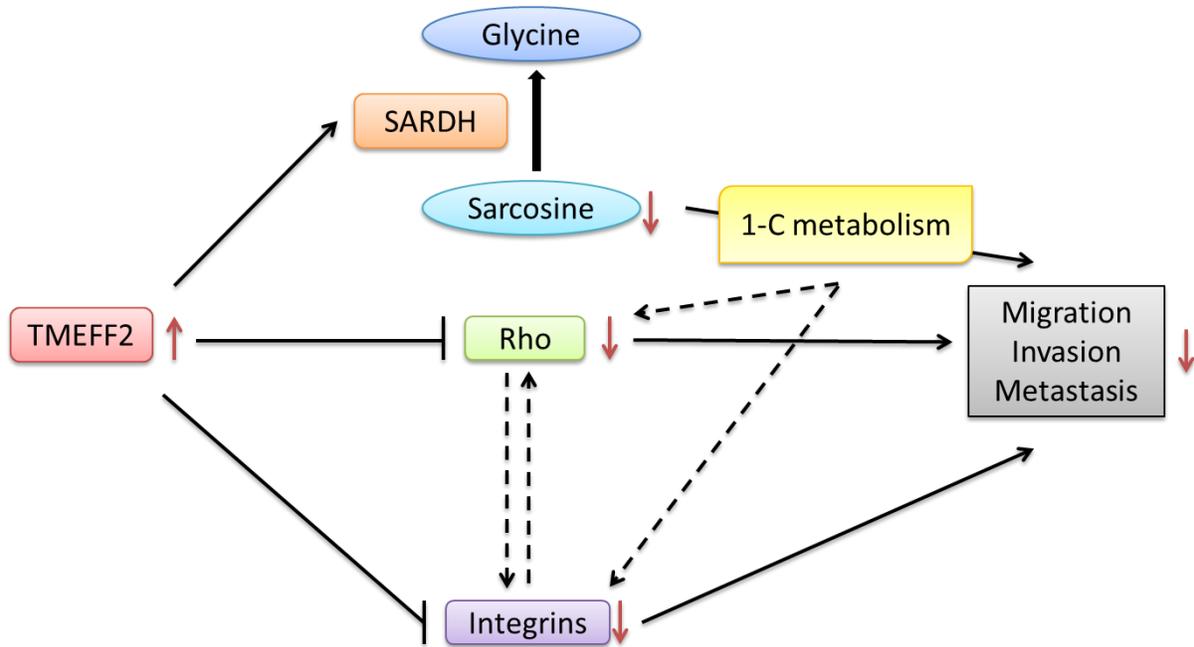
In Chapter 4, we presented data indicating an important role of TMEFF2 in regulating prostate cancer cell migration that involves integrin expression and RHOA activation. The data also support the involvement of sarcosine metabolism in the migratory behavior of the cells and a link to integrin expression and RHOA activation. While expression of the full-length TMEFF2 affects sarcosine metabolism and blocks cellular adhesion and migration, TMEFF2\_ΔGA does not affect sarcosine levels and does not inhibit adhesion and migration of prostate cancer cells. Moreover, the ability to inhibit adhesion and migration correlates with the ability of TMEFF2 to reduce integrin expression and RHOA activation, which is lost in the TMEFF2\_ΔGA mutant. Based on these results, and since sarcosine and one-carbon metabolism are responsible for the methylation reactions, it is reasonable to speculate that TMEFF2, by affecting one-carbon metabolism, may affect expression of integrin genes epigenetically, via methylation. Although we have not directly tested this hypothesis, several studies have described epigenetic alterations – DNA methylation and histone modifications – that affect integrin expression during tumor progression (Park et al. 2004; Uhm et al. 2010; Yang et al. 2009). Similarly, since methylation of RHOA impacts its activation (Cushman and Casey 2011), it is also possible that TMEFF2 inhibits RHOA activation by modulating its methylation status.

In Chapter 5 we demonstrated that different forms of TMEFF2 distinctly affect AKT and ERK activation and this may contribute to a different cellular response of either proliferation or tumor suppression. While full-length TMEFF2 activates ERK but has no effect on AKT phosphorylation, the ectodomain inhibits ERK phosphorylation concomitantly with AKT activation. Since integrins have been shown to induce AKT (Velling et al. 2008; Zeller et al. 2010) and ERK phosphorylation (Lai et al. 2001; Stupack and Cheresch 2002), it is possible that TMEFF2 modulates MAPK and PI3K pathways via its effects on integrin expression.

Alternatively, TMEFF2 may modulate the crosstalk between integrins and growth factor receptors to control these responses. In this respect, it is known that signaling through receptor tyrosine kinases (RTKs) is not only regulated by growth factors but also by functional collaboration with integrins, and integrins may activate RTKs in the absence of growth factors (Eliceiri 2001; Ross 2004; Streuli and Akhtar 2009). For example, integrins  $\alpha v\beta 3$  or  $\alpha 5\beta 1$  have been shown to influence and potentiate PDGF receptor  $\beta$  signaling in cell migration (Schneller et al. 1997; Woodard et al. 1998; Veevers-Lowe et al. 2011). Interestingly, TMEFF2 has been shown to interact with PDGF-AA and to interfere with PDGF receptor signaling (Lin et al. 2011). It is possible that by affecting integrin expression and PDGF interaction with its receptor, TMEFF2 can modulate the integrin - RTK crosstalk and migration. This mechanism could potentially be important regarding prostate cancer metastasis to bone. Although interaction of TMEFF2 with PDGF-D has not been documented, PDGF-D is expressed primarily by prostate cancer cells in the bone, and inhibition of PDGF receptor autophosphorylation reduces tumor formation and metastasis in mice (Uehara et al. 2003). TMEFF2 could inhibit migration and therefore metastasis by affecting PDGF-dependent and PDGF-independent/integrin-dependent signaling.

Finally, the role of TMEFF2 in one-carbon metabolism may suggest an additional link to AKT and ERK activation. By modulating one-carbon metabolism, TMEFF2 has the potential to impact homocysteine levels, which have been reported to modulate the AKT and ERK signaling pathways (Lee et al. 2012).

In summary, our studies added substantial knowledge about the biological function of TMEFF2 and its molecular mechanisms. The full-length TMEFF2 serves as a tumor suppressor (Figure 31), possibly through (1) modulating sarcosine metabolism and therefore one-carbon



**Figure 31.** Proposed model of TMEFF2 action in prostate cancer. As a tumor suppressor, full-length TMEFF2 expression leads to inhibition of cell migration, invasion, and cancer metastasis. In this dissertation, we described at least three underlying molecular mechanisms. First, TMEFF2 reduces Rho activity, leading to decreased migration. Second, TMEFF2 interacts with SARDH and reduces sarcosine levels, resulting in decreased migration. Third, TMEFF2 downregulates integrin levels and therefore reduces migration. Potential links between these three mechanisms are described in the text of Chapter 4 and this chapter.

metabolism, (2) inhibiting integrin expression, (3) inhibiting RHOA activation, and (4) promoting ERK phosphorylation, or a combination of those. Deletion of the GA domain abolishes all those effects, highlighting the importance of this domain in the function of TMEFF2. The ectodomain has a growth-promoting effect, consistent with its inability to reduce sarcosine levels and to promote ERK phosphorylation.

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