SUPPLEMENTAL DATA

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

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Effect of TMEFF2 on the cell cycle of HEK293T cells. (A) Schematic representation of the constructs used. FS: follistatin; EGF: epidermal growh factor like; TM: transmembrane domain; GA: G-protein activating domain; myc-his: c-myc-his tag. (B) Cells expressing the TMEFF2-myc-his fusion or the empty vector (EV) were treated with Nocodazole for 12 hours to promote cell cycle arrest and the effect on cell cycle distribution was measured 4 and 8 hours after removing the Nocodazole from the growth medium. Overexpression of TMEFF2 resulted in a small increase in G1 accompanied by a proportional decrease in the number of cells in S phase and no change in the percent of cells in G2 phase, suggesting a possible G1-phase cell cycle checkpoint abnormality. (C) Fold up-regulation or down-regulation of cell-cycle involved genes as a result of TMEFF2 overexpression in HEK293T cells.

Figure S2. TMEFF2 inhibits invasion of RWPE cells. The effect of TMEFF2 overexpression on the invasion ability of RWPE1 cells was determined using a MTT-based modified Boyden chamber assay. RWPE1-TMEFF2i or RWPE1-tet cells were grown for 96 hours in the presence of 50 μM alanine or sarcosine and for 48 hours in the presence of doxycycline (250 ng/ml) and then added to the Boyden chambers and allowed to invade for 48 hours. The number of invading cells at the bottom side of the Matrigel chamber and the number of non-invading cells at the top of the matrigel were determined using a MTT assay and the percentage of the invading cells calculated from the total. Invasive cells from a random experimental repeat were visualized by fixing the cells adhering to the bottom of the membrane with 70% ethanol and staining with 0.1% crystal violet. Cells were then photographed (right).

Figure S3. Strategy to study co-immunoprecipitation of TMEFF2 and SARDH. (A). Schematic of the c-myc-his tagged TMEFF2 constructs used in this study. (B) Western blot of TMEFF2-myc-his (left) and ECTO-myc-his (right) 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 and bound proteins were eluted with 500-800 mM imidazole. The presence of TMEFF2 sequences in the eluate was detected with an anti-c-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 with anti-TMEFF2 antibody and immunoblotted (IBα) with anti-SARDH horseradish peroxidase (HRP) conjugated antibody. The size of the bands corresponding to SARDH (black arrows) after strong elution (50mM glycine pH2.8 and LDS buffer) lies between the 55-70 kDa and the 70-100 kDa markers. (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 (EV) as a control were immunoprecipitated (IPα) with anti-c-myc antibody coupled dynabeads (Invitrogen) and immunoblotted (IBα) with anti-TMEFF2 antibody. In addition, cell lysates from LNCaP or 22Rv1 cells were immunoprecipitated (IPα) with anti-TMEFF2 antibody coupled dynabeads and immunoblotted (IBα) with anti-TMEFF2 antibody. WCL: whole cell lysate; FT: flow through. Protein markers are in kD.

Figure S4. TMEFF2 and SARDH colocalize in HEK293T cells. (A) Confocal microscopy of HEK293T/SARDH-myc-his cells stained with anti-c-myc. Mitochondria were stained with MitoTracker Red. The merge image demonstrates the localization of SARDH in mitochondria (yellow signal). Scale bars represent 10 μm. (B) Confocal Z-stack sequential images (left to right) of the colocalization (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 colocalization (yellow) of TMEFF2-CFP (green) and SARDH-YFP (red) in HEK293T cells. (D) Immunocolocalization of TMEFF2 and SARDH in LNCaP cells. Cytospin preparations (upper panel) were methanol fixed, stained with anti-TMEFF2 (red) and a Dylight 488 conjugated anti-SARDH antibody (green) and mounted with medium containing DAPI (blue) for nuclear staining. LNCaP cells were also grown on chamber slides (lower panel), methanol fixed and stained with anti- SARDH (red) and a Dylight 488 conjugated anti-TMEFF2 antibody (green).

Figure S5. TMEFF2 affects sarcosine levels and activity of SARDH but not the expression of endogenous SARDH. (A) Expression of TMEFF2 does not affect the levels of endogenous SARDH. Western blot of SARDH from cells

overexpressing the different forms of TMEFF2-myc-his fusion proteins. Whole cell extracts of HEK293T cells expressing TMEFF2-myc-his, ECTO-myc-his or the empty vector (EV) were prepared and subjected to western blot analysis using a rabbit polyclonal anti-SARDH antibody (SIGMA). The membrane was subsequently washed and a β-tubulin antibody used to control for differences in protein loading (bottom). * Note that the ≈ 60 kD major band is the main form detected with commercially available SARDH antibodies. The longest form of the protein is ≈ 100 Kd. Both of this bands are indicated by arrows. Protein markers are in kD. (B) Overexpression of TMEFF2 reduces the levels of sarcosine in RWPE1 cells. The TMEFF2 inducible RWPE1 cell line (RWPE1-TMEFF2i) or the cell line carrying the transactivator expressing construct only (RWPE1-tet) were grown in the presence or absence of doxycycline (250 ng/ml) and the effect of TMEFF2 on sarcosine levels analyzed by comparing the sarcosine levels of the RWPE1-TMEFF2i cell line with those of the RWPE1-tet control cell line, both in the presence of doxycycline. (C) Effect of TMEFF2 on the activity of commercially available purified *Pseudomonas sp.* SARDH. Lysates from HEK293T cells stably transfected with the TMEFF2-myc-his and ECTO-myc-his expression constructs were prepared, dialyzed to remove sarcosine and mixed with 1 μU of the purified enzyme. This amount of enzyme was sufficient to keep the reaction within the limits of the colorimetric assay. The rate with which the enzyme metabolized sarcosine was then measured after addition of different amounts of sarcosine and the necessary cofactors. To calculate the Km and Vmax the values obtained in 5 different experiments were averaged and analyzed using a Lineweaver-Burk plot. The Km of the enzyme decreased in the presence of the TMEFF2 overexpressing lysates with respect to the Km observed when the ectodomain containing lysates were used.

Figure S6. The shed TMEFF2 ectodomain promotes cell growth. (A) TMEFF2 is shed from the membrane. Conditioned medium (CM) from cell cultures of HEK293T/TMEFF2-myc-his and HEK293T/ECTO-myc-his was concentrated 20X before being subjected to western blot analysis with the indicated antibodies. The c-myc moiety remains in the cytoplasm after shedding; however the c-myc antibody detects the ECTO-myc-his form in the CM since it does not have a transmembrane domain and is therefore secreted intact into the CM. TNFα induces shedding as previously described (6, 24). (B) Growth curves of RWPE1 and HEK293T cells was determined by MTT assay (OD_{562}) at different time intervals after replacing the growth medium with conditioned medium from cultures of HEK293T/EV, HEK293T/ECTO-myc-his and HEK293T/TMEFF2-myc-his.

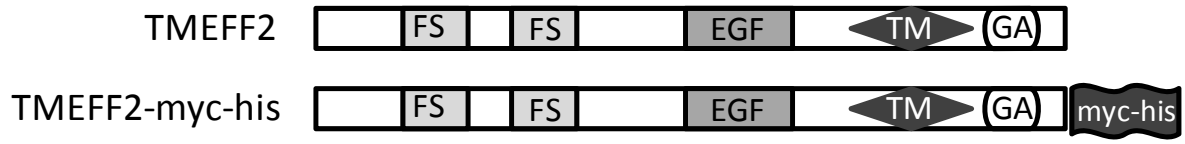
SUPPLEMENTARY TABLES

Table S1. Primers used for plasmid construction

	Construct name	Primer sequence (5' -> 3')
1	pCMV-SARDH	a) TAAGATCTCCACTGGGCAAGCCACAC b) TATCTAGACCTGTGAGAATGGATGG
2	SARDH-myc-his	a) TAGCTAGCCCCCATGGCCTCACTGAG b) TAGCGGCCGCGTAGATTCCCTTCACCC
3	pCMV-TMEFF2	a) TAGCTAGCAGTCATGGTGCTGTGGG b) TACTCGAGGTGTAGTCCAAGCTCTC
4	TMEFF2-myc-his	a) TAGCTAGCAGTCATGGTGCTGTGGG b) TACTCGAGAGATTAACCTCGTGGACG
5	ECTO-myc-his	a) TAGCTAGCAGTCATGGTGCTGTGGG b) TACTCGAGCATACTGAAATCGTACAGG
6	TMEFF2-CFP	a) TAGCTAGCAGTCATGGTGCTGTGGG b) TACTCGAGGATTAACCTCGTGGACG
7	SARDH-YFP	a) TAGCTAGCCCCCATGGCCTCACTGAG b) TAAAGCTTGTAGATTCCCTTCACCC
8	pRetroX-TMEFF2	a) TAGGATCCCTCCACCCTGCCTCCTCG b) TAACGCGTGTCTATAATACTGTATTGTGTAGTC

Fig. S1

A



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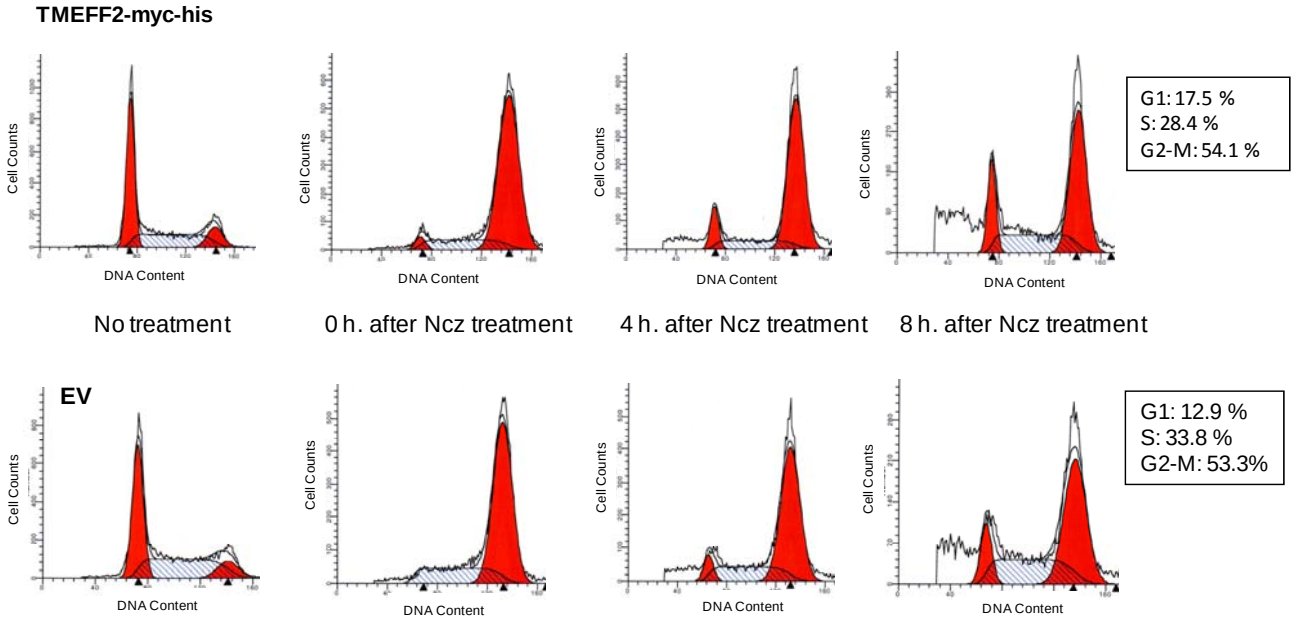


C

	up-regulation	down-regulation	Comments
P15	3-fold	NA	Inhibits CDK4 (important for G1 progression)
CCNE2	NA	2-fold	Cyclin E2. Role in G1/S transition
CDK2	NA	2-fold	Cyclin-dependent kinase 2. Essential for G1/S

Fig. S2

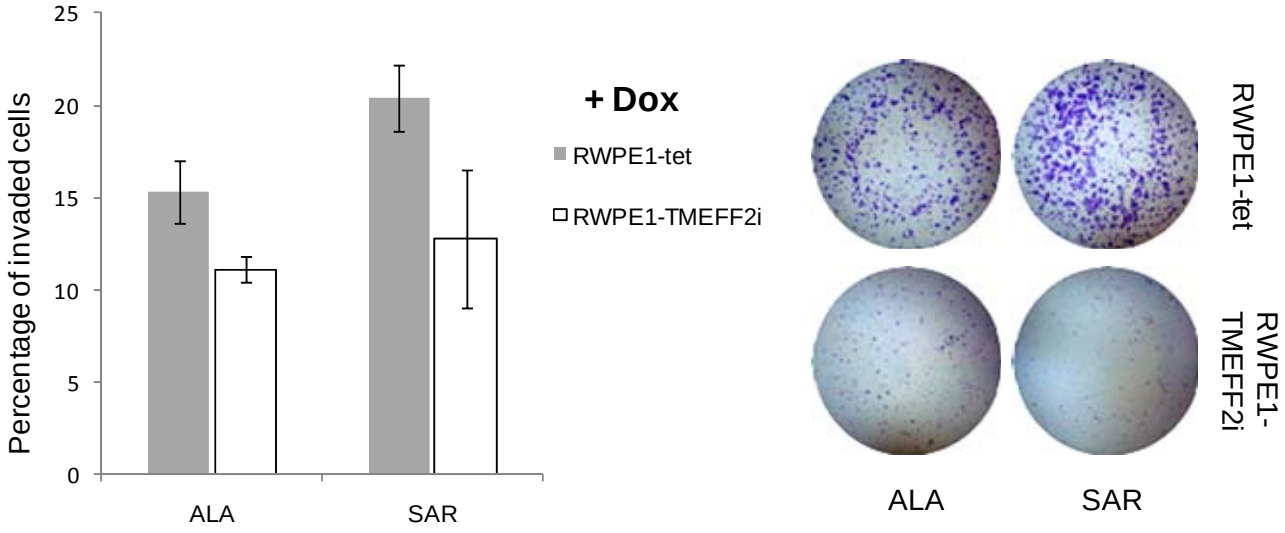
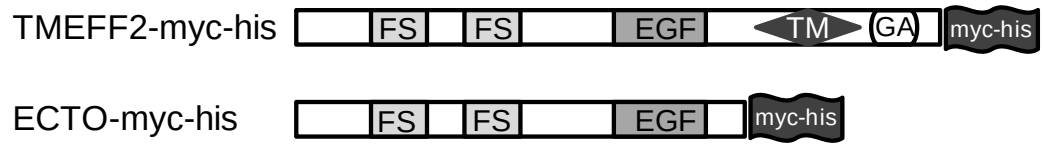
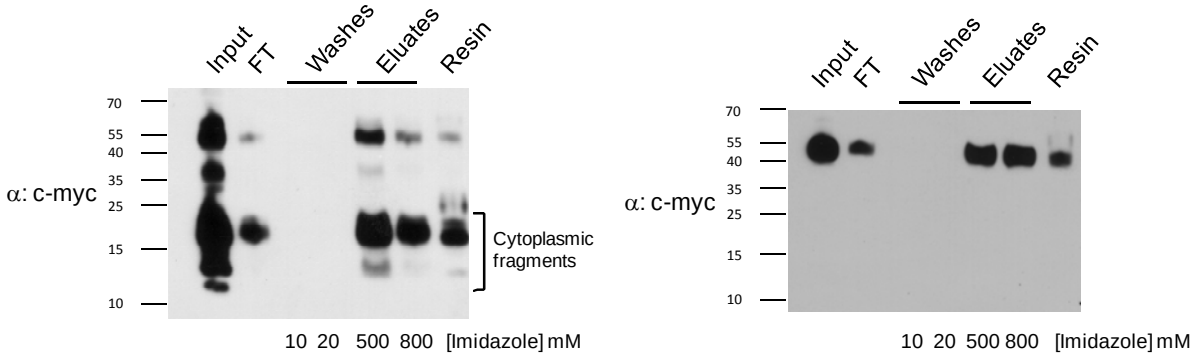

25
20
15
10
5
0
Percentage of invaded cells
ALA
SAR
+ Dox
RWPE1-tet
RWPE1-TMEFF2i
RWPE1-tet
RWPE1-TMEFF2i
ALA
SAR

Fig. S3

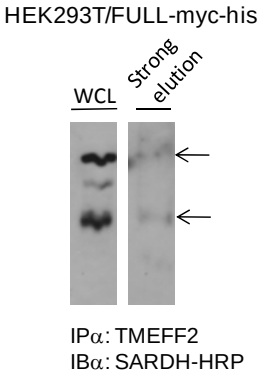
A


TMEFF2-myc-his
FS
FS
EGF
TM
GA
myc-his
ECTO-myc-his
FS
FS
EGF
myc-his

B


Input
FT
Washes
Eluates
Resin
70
55
40
35
25
15
10
α: c-myc
Cytoplasmic fragments
10 20 500 800 [Imidazole] mM
Input
FT
Washes
Eluates
Resin
70
55
40
35
25
15
10
α: c-myc
10 20 500 800 [Imidazole] mM

C


HEK293T/FULL-myc-his
WCL
Strong elution
IPα: TMEFF2
IBα: SARDH-HRP

D

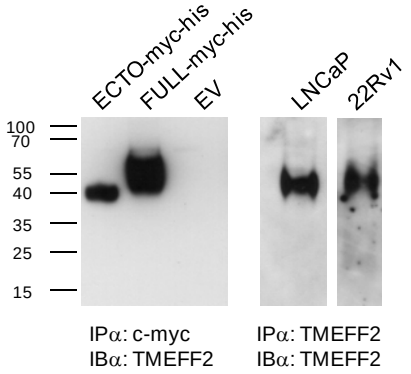

ECTO-myc-his
FULL-myc-his
EV
LNCaP
22Rv1
100
70
55
40
35
25
15
IPα: c-myc
IBα: TMEFF2
IPα: TMEFF2
IBα: TMEFF2

Fig. S4

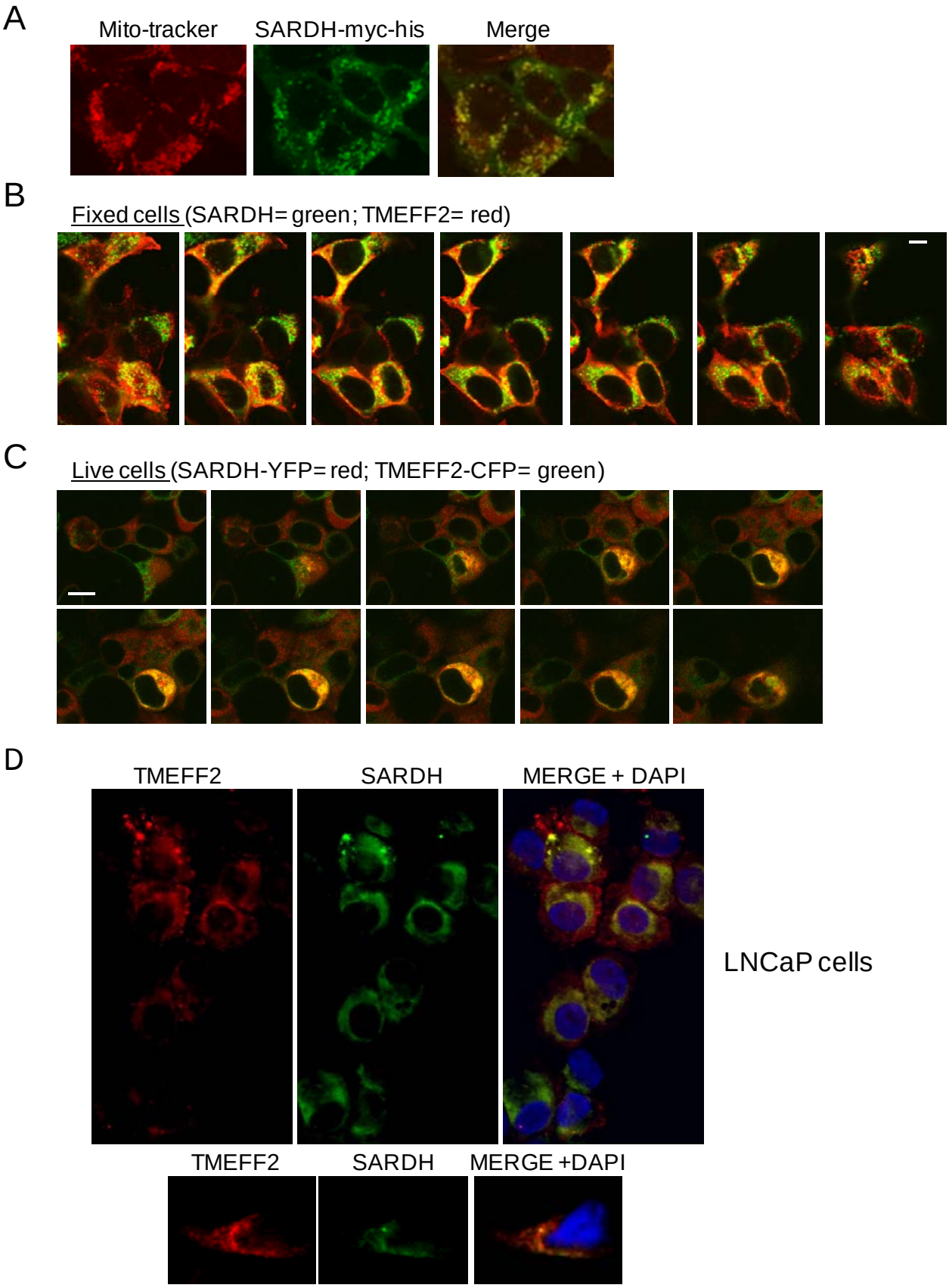
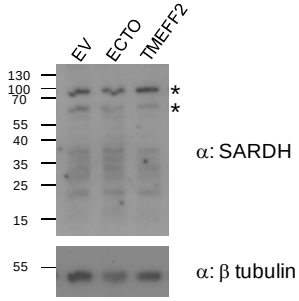
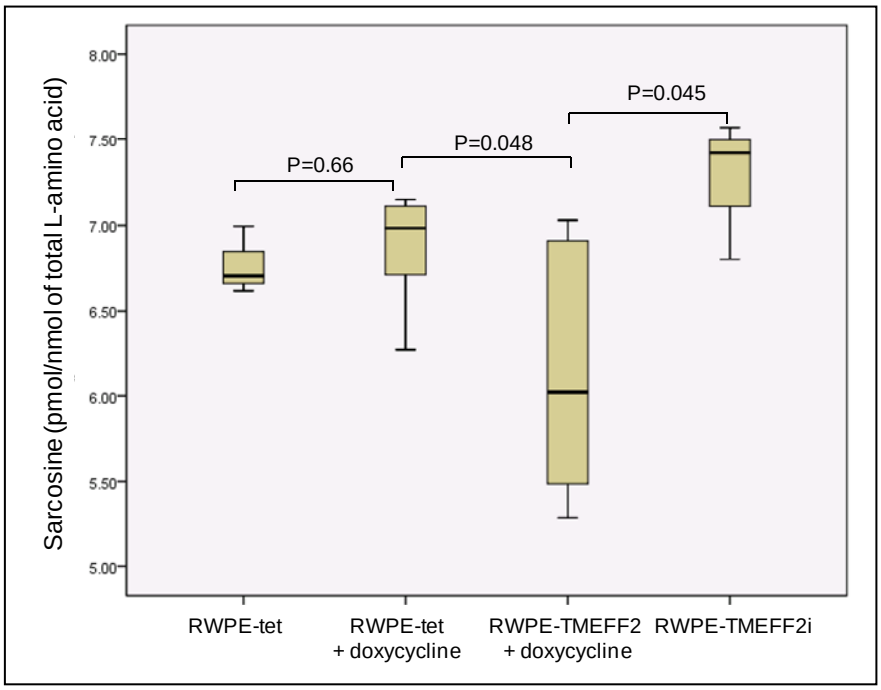

A
Mito-tracker
SARDH-myc-his
Merge
B
Fixed cells (SARDH= green; TMEFF2= red)
C
Live cells (SARDH-YFP= red; TMEFF2-CFP= green)
D
TMEFF2
SARDH
MERGE + DAPI
LNCaP cells
TMEFF2
SARDH
MERGE +DAPI

Fig. S5

A



B

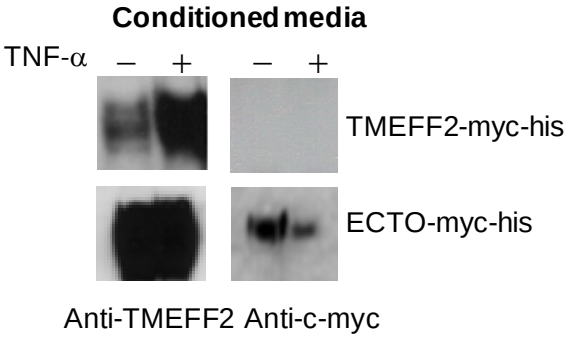


C

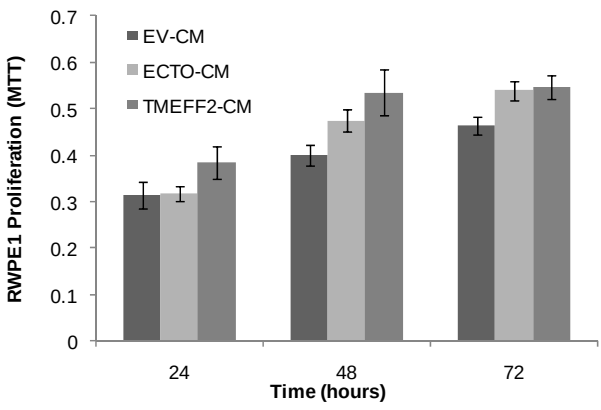
	Vmax (Diformazan formation/min)	Km (mM)
ECTO-myc-his	0.238	0.244
TMEFF2-myc-his	0.241	0.184

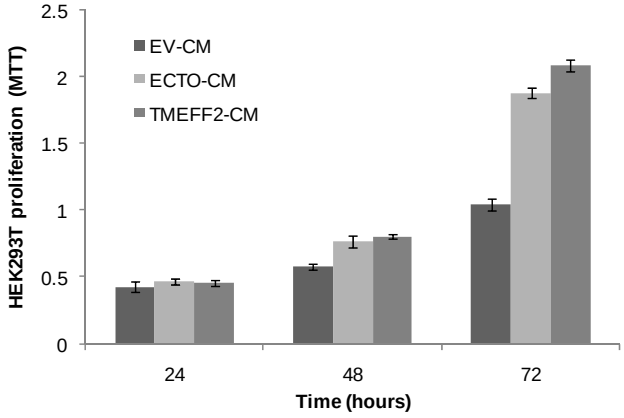
Fig. S6

A


Conditioned media
TNF-α – + – +
TMEFF2-myc-his
ECTO-myc-his
Anti-TMEFF2 Anti-c-myc

B


RWPE1 Proliferation (MTT)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EV-CM
ECTO-CM
TMEFF2-CM
24
48
72
Time (hours)


HEK293T proliferation (MTT)
2.5
2
1.5
1
0.5
0
EV-CM
ECTO-CM
TMEFF2-CM
24
48
72
Time (hours)

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Cell lines and culture conditions -- Cell lines used include: HEK293T (ATCC), FreeStyle 293-F cells (Invitrogen), LNCaP (ATCC), 22Rv1 (ATCC) and RWPE1 cell line. Cells were maintained in the appropriate medium with 10% heat-inactivated fetal bovine serum and 1% antibiotics at 37°C with 5% CO_2. HEK293T or RWPE1 stable cell lines expressing the protein of interest were generated by transfection or transduction, followed by drug resistance and clonal selection. To inducibly express TMEFF2 in RWPE1 cells, cells were grown in the presence of doxycycline (250 ng/ml).

Plasmid constructs and generation of stable cell lines -- C-terminal c-myc-his fusions to SARDH, TMEFF2 and ECTO, were made by inserting the relevant sequences into the eukaryotic expression plasmid pSec-Tag2A (InvitroGen). The untagged forms of TMEFF2 (TMEFF2-wt in Figure S1) and SARDH expressed under the CMV promoter were constructed using the pCMV-GLuc expression plasmid (New England Biolabs) and substituting the GLuc sequences for the TMEFF2 or SARDH sequences. The untagged form of the TMEFF2 ectodomain (ECTO) or TMEFF2 used for immnofluorescence 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 in RWPE1 cells, TMEFF2 sequences were cloned in the pRetroX-Tight-Pur vector (Clontech). All these constructs were made using PCR and standard cloning strategies (see primers table). HEK293T cells were transfected with the indicated expression plasmids using Lipofectamine 2000 transfection reagent (Invitrogen). 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 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 (rtTA) 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 (Sigma) dependent manner.

Cell cycle assay -- 100,000-300,000 cells/well were plated into 6-well plates and allowed to grow to 60% confluency. Cells were then treated with Nocodazole (200 mg/ml) for 12 hours and harvested at 4 and 8 hours after removing the Nocodazole from the growth medium. Sample cells were washed once with PBS, and fixed in cold 70% ethanol for at least 24 h at 4°C. Fixed cells were washed once with PBS, treated with RNase (1 mg/ml), and stained by propidium iodide (10 μg/ml). Flow cytometric analysis was performed with a FACSCan device (Becton Dickinson) and the cell cycle analyzed with Modfit 2.0 software.

Invasion assays -- Cell invasion was assayed using 24-well cell culture Boyden chambers containing a layer of matrigel (BD Biocoat; Becton Dickinson) and using NIH 3T3 conditioned medium as a chemoattractant. To analyze sarcosine induced invasion, RWPE1-TMEFF2i cells were grown in the presence of 50 μM sarcosine or alanine. Forty-eight hours later, doxycycline was added to induce TMEFF2 expression and the cells incubated an additional 48 hours. In total, the cells were grown 96 hours in the presence or sarcosine or alanine and 48 hours in the presence of doxycycline (250 ng/ml) to induce TMEFF2 expression before they were added to the Boyden chambers. Following 36-48 hr incubation, the number of invading cells at the bottom side of the Matrigel chamber and the number of non-invading cells at the top of the matrigel were determined using a MTT assay and the percentage of the invading cells calculated from the total. Alternatively, following 48-hr incubation, cells adhering to the bottom of the membrane were fixed with 70% ethanol and stained with 0.1% crystal violet, photographed and counted in several random fields of view. To analyze the effect of different forms of TMEFF2 on the invasion of HEK293T cells, the cells were allowed to invade for a period of 24 hours.

Cytospins –LNCaP cells were trypsinized and 100 μl of cell suspension (5×10^5/ml) was loaded into a cytospin chamber and centrifuged at 370 rpm for 5min. Cells were then fixed with methanol at -20ºC for 10min and blocked with 5% Normal Goat Serum (Invitrogen) in 0.1% PBST for 1hr.

Western blot analysis and detection of TMEFF2 sequences in the conditioned medium -- For Western blot analysis, cells were lysed in buffer containing 20 mM sodium phosphate, pH 7.4; 150 mM sodium chloride, 1% Triton X-100 and protease inhibitor cocktail (Roche or Sigma), unless otherwise indicated. Protein concentrations were determined with BCA protein assay (Pierce) and proteins resolved by SDS-PAGE following transfer to Immobilon transfer membrane (Millipore). Blots were blocked in 5% non-fat milk, and probed with the appropriate antibodies. Immunoreactive bands were visualized using ECL plus western blotting detection system (GE Healthcare). To detect the presence of TMEFF2 in the conditioned medium, HEK293T cells expressing the construct of interest were seeded, allowed growing to 80% confluency, washed with PBS and serum starved for 24 hours. The conditioned medium was then collected, concentrated 20-fold (Amicon Ultra-4 Centrifugal Filter Devices, Millipore) and analyzed by western blot analysis. When required, 100 ng/ml of recombinant TNFα (PeproTech) was added after the starvation period and cells incubated 16 hr in the presence of TNFα before the conditioned medium was collected.

Antibodies and other reagents -- Antibodies that recognize TMEFF2 were as follow: anti-mouse monoclonal for IP (Abcam or Santa Cruz), anti-rabbit for western blot (Abcam) and anti-rabbit (Sigma) for confocal microscopy. Other antibodies: anti- SARDH (Sigma-Aldrich), anti-SARDH (Abgent), anti-4E-BP1 (Santa Cruz), anti-eIF4E (Santa Cruz), anti-c-myc (Invitrogen), anti-β-actin (Abcam) and rabbit control IgG (Abcam). Secondary antibodies: goat anti-mouse IgG1-HRP(Santa Cruz), goat anti-rabbit IgG-HRP (Santa Cruz), goat anti-mouse IgG-FITC (Santa Cruz), Alexa Fluor 568 goat anti-rabbit IgG (H+L) (Invitrogen), monoclonal mouse anti-rabbit IgG light-chain specific and goat-anti mouse IgG light chain specific (Jackson ImmunoResearch). The Lightning-Link HRP Conjugation Kit (Novus & Innova) was used to prepare HRP-conjugated TMEFF2 and SARDH antibodies. Dynabeads Antibody Coupling Kit (Invitrogen) was used according to manufacturer's recomendations for the immobilization of the specified antibody on magnet beads. Staining of mitochondria was performed using MitoTracker Deep Red (Invitrogen). Doxycycline was from SIGMA. The DyLight 488 Microscale Antibody Labeling Kit (Pierce) was used to fluorescently label the TMEFF2 or SARDH antibodies used for colocalization studies in LNCaP cells. DAPI from Santa Cruz was used for nuclear staining.

Sarcosine dehydrogenase activity measurements – The activity of the enzyme was measured as described (Sigma) and is based on the following:

1) Sarcosine + H_2O + PMS → Glycine + HCHO + $PMSH_2$
2) 2PMSH2 + NBT → 2 PMS + Diformazan

(PMS = Phenazine Methosulfate; NTB = Nitro Blue Tetrazolium and PMSH2 = Phenazine Methosulfate (Reduced Form)).

Reaction 1) is catalyzed by SARDH and diformazan formation can be measured by determining absorbance at 540 nm. Cells were washed with PBS, resuspended in 0.5mL 50mM phosphate buffer (pH 7.5) containing protease inhibitors (Sigma), homogenized on ice with a Dounce glass homogenizer and dialyzed against PBS using 2500 MWCO G2 dialysis cassettes (Pierce). The dialyzed lysates (20 μg) were incubated at 37°C with 1μU of SARDH (Sigma). After 30 minutes different amounts of sarcosine and the PMS-NBT reagent were added to the lysate-enzyme mix and absorbance at 540 nm was recorded at different time points to determine the rate of sarcosine oxidation. A double reciprocal Lineweaver-Burk plot was used to calculate the Km of each sample.