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Association of TM4SF4 with the human thiamine transporter-2 in intestinal epithelial cells

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Abstract

Background—The human thiamine transporter-2 (hTHTR-2) is involved in the intestinal absorption of thiamine. Recent studies with membrane transporters of other nutrients/substrates have shown that they have associated proteins that affect different aspects of their physiology and cell biology. Nothing is known about protein(s) that interact with hTHTR-2 in intestinal epithelial cells and influence its physiological function and/or its cell biology.

Aims—The aim of this study was to identify protein partner(s) that interacts with hTHTR-2 in human intestinal cells and determine the physiological/biological consequence of that interaction.

Methods—The yeast split-ubiquitin two-hybrid approach was used to screen a human intestinal cDNA library. GST-pull-down and cellular co-localization approaches were used to confirm the interaction between hTHTR-2 and the associated protein(s). The effect of such an interaction on hTHTR-2 function was examined by ^{3}H-thiamine uptake assays.

Results—Our screening results identified the human TransMembrane 4 SuperFamily 4 (TM4SF4) as a potential interactor with hTHTR-2. This interaction was confirmed by an *in vitro* GST-pull-down assay, and by live-cell confocal imaging of HuTu-80 cells co-expressing hTHTR-2-GFP and mCherry-TM4SF4 (the latter displayed a significant overlap of these two proteins in intracellular vesicles and at the cell membrane). Co-expression of hTHTR-2 with TM4SF4 in HuTu-80 cells led to a significant induction in thiamine uptake. In contrast, silencing TM4SF4 with gene-specific siRNA led to a significant decrease in thiamine uptake.

Conclusions—These results show for the first time that the accessory protein TM4SF4 interacts with hTHTR-2 and influences the physiological function of the thiamine transporter.

Keywords

yeast split-ubiquitin two-hybrid; hTHTR-2; protein-protein interaction; intestine; vitamin B_1

Introduction

Vitamin B_1 (thiamine) is a member of the water-soluble vitamin family of micronutrients. Thiamine plays an essential role in normal cellular functions, growth and development. In its coenzyme form, i.e., thiamine pyrophosphate, the vitamin plays a vital role in metabolism and energy production that includes the decarboxylation of pyruvic acid and alpha-ketoglutamic acid, and the utilization of pentose in the hexose monophosphate shunt [1]. Clinically, thiamine deficiency in humans leads to a variety of abnormalities mainly neurological and cardiovascular disorders [1–3]. Thiamine deficiency and suboptimal levels occurs in humans affected by diabetes mellitus, chronic alcoholism and celiac disease [4–8].

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Mammals cannot synthesize thiamine endogenously therefore they must obtain the vitamin from exogenous sources via intestinal absorption. Thus, the intestine plays an important role in maintaining and regulating normal thiamine body levels. Intestinal thiamine absorption occurs via a specific carrier-mediated mechanism [reviewed in 9], and involves both thiamine transporters-1 and -2 (THTR-1 and THTR-2; in humans they are referred to as hTHTR-1 and hTHTR-2; the proteins are product of the *SLC19A2* and *SLC19A3* genes, respectively) [9–11]. These transporters belong to the major facilitator superfamily of transport proteins and have a predicted 12 transmembrane domains [10, 11]. In polarized intestinal epithelial cells, these thiamine transporters are differentially distributed, with hTHTR-1 being expressed at both the apical and basolateral membrane domains (with a slightly higher expression at basolateral membrane), while hTHTR-2 is exclusively expressed at the apical membrane domain [12–14].

Recent studies with a variety of other membrane transporters (including those that are involved in transport of other water-soluble vitamins) have shown that these integral membrane proteins have associated proteins that interact with them and affect different aspects of their physiology and cell biology [15–21]. Nothing, however, is currently known about protein(s) that may interact with hTHTR-2 in human intestinal epithelial cells. Thus our aim in this investigation was to identify possible existence of interacting protein partner(s) with hTHTR-2 in human intestinal epithelial cells, and to study the physiological/cell biological consequence of that interaction. We used a yeast split-ubiquitin two-hybrid approach to screen a human intestinal cDNA library and identified the human TransMembrane 4 Super-Family 4 (TM4SF4) (also called the intestinal and liver Tetraspan Membrane Protein; il-TMP) as an associated protein partner for hTHTR-2. The interaction between hTHTR-2 and TM4SF4 was confirmed by an *in vitro* GST-pull-down assay and by live-cell confocal imaging of human intestinal epithelial HuTu-80 cells co-expressing hTHTR-2-GFP and mCherry-TM4SF4. Our studies also showed that co-expression of hTHTR-2 with TM4SF4 in intestinal HuTu-80 cells led to a significant induction in thiamine uptake, while silencing TM4SF4 (with gene specific siRNA) led to a significant decrease in the vitamin uptake.

Materials and Methods

Materials

^{3}H-Thiamine (specific activity: > 20Ci/mmol; radiochemical purity: >98%) was obtained from American Radiolabeled Chemical (St. Louis, MO, USA). Human-derived duodenal intestinal epithelial HuTu-80 cells were purchased from ATCC (Manassas, VA, USA). DNA oligonucleotide primers were obtained from Sigma Genosys (Woodlands, TX, USA). All molecular biology grade chemicals and reagents were purchased from commercial vendors.

Cell culture, and transient and stable transfections

HuTu-80 cells were grown in MEM culture medium supplemented with 10% (v/v) fetal bovine serum (FBS), glutamine (0.29g/l), sodium bicarbonate (2.2g/l), penicillin (100,000 U/l), and streptomycin (10mg/l) in 75-cm^2 plastic flasks at 37 C in a 5% CO_2-95% air atmosphere. HuTu-80 cells were grown on sterile 12 well plates (Corning, NY, USA) or glass-bottomed petri-dishes (MatTek, MA, USA), and transiently transfected at 90% confluency with 4μg plasmid DNA using Lipofectamine 2000 (Invitrogen, CA, USA). After 24–48hrs of transfection, cells were used for uptake assay, protein, mRNA expression studies and live HuTu-80 cells were imaged using confocal microscopy. Stable HuTu-80 cells were selected using G418 (0.5mg/ml) for 6–8 weeks [14].

Yeast split-ubiquitin two-hybrid assay

To identify potential interacting protein partner(s) for the human thiamine transporter-2 (hTHTR-2), the yeast split-ubiquitin two-hybrid system (Hybrigenics, S.A., Paris, France) was used. The coding sequence of hTHTR-2 (1–496 amino acids) was PCR-amplified and cloned into vector pB101 in-frame with the C-terminus of ubiquitin (Cub) and is coupled to the artificial transcription factor LexA-VP16 (LexA-VP16-Cub-hTHTR-2) (the pB101 vector was derived from the original pBT3-N vector). The construct was verified by sequencing the entire insert before being used as a bait to screen a dT-primed human intestinal cDNA library constructed in pPR3-N. The prey proteins were expressed as a fusion to the N-terminal half of ubiquitin (NubG-x). One hundred and nine million clones were identified by this assay. After selection in medium lacking tryptophan, leucine and histidine, 29 His^+ colonies were identified [22–24]. Sequences of the identified positive clones were amplified by PCR and sequenced. The resulting sequences were verified for their identity using GenBank database. Based on bioinformatics analysis (prey identification and sequencing, and selected interacting domain determination), biological characteristics, and tissue distribution pattern of the identified putative proteins, we selected TM4SF4 as a potential candidate for further analysis.

GST pull-down assay

The full-length GST-TM4SF4 recombinant protein was purchased from Novus Biologicals (Littleton, CO, USA). The hTHTR-2 stably expressing HuTu-80 cells was lysed in CelLytic M cell lysis reagent (Sigma) and then centrifuged (14,000rpm for 10min at 4°C). The cleared post-nuclear extract was separated. The GST-TM4SF4 and GST proteins were pre-incubated with glutathione-Sepharose4B beads (GE Healthcare, Piscataway, NJ, USA) and washed with cell lysis buffer. The cleared post-nuclear extract of HuTu-80 cells (1mg of total soluble protein) was incubated either with GST-TM4SF4 or GST bound to glutathione-Sepharose4B beads in cell lysis buffer on rotating wheels for 2hrs at 4°C. The proteins bound to beads were then eluted with 10mM glutathione and subjected to western blotting using a rabbit polyclonal anti-hTHTR-2 antibodies (Abcam, MA, USA). The immunoreactive bands were visualized using anti-rabbit IRDye-800 secondary antibodies and an Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NB, USA) [18].

Generation of hTHTR-2 and TM4SF4 fusion constructs

The full-length hTHTR-2-GFP construct generated previously [14] was used in this study. The pFLAG-CMV-2-TM4SF4 construct was obtained from Origene (Rockville, MD, USA). The mCherry-TM4SF4 construct was generated by PCR using the primer combinations (Table 1) and conditions described previously [14]. Both the TM4SF4 PCR products and the mCherry C-1 vector (Clontech, Mountain View, CA, USA) were digested with *Sal I* and *Bam HI*, and digested products were gel-separated and ligated to generate in-frame fusion proteins with the red fluorescent protein (mCherry C-1) fused to the amino terminus of TM4SF4 full-length protein. The nucleotide sequences of the TM4SF4 fusion construct was verified by DNA sequencing (Laragen, CA, USA).

Confocal microscopy

HuTu-80 cells were grown on glass-bottomed petri-dishes (MatTek) and transiently transfected with hTHTR-2-GFP and mCherry-TM4SF4 constructs (3μg) at 90% confluency using 3μl of Lipofectamine 2000 (Invitrogen). Fusion constructs co-expressing live HuTu-80 cell were imaged using a Nikon C-1 confocal scanner head attached to a Nikon inverted phase-contrast microscope after 24–48hrs of transfection. The fluorophores were excited by using 488nm (GFP) and 568nm laser lines, and emitted fluorescence was

monitored with a 515 ± 30-nm band-pass (GFP) or at 630 ± 60-nm long-pass (mCherry) filter.

Small interfering RNA (siRNA) analysis

siRNA for human TM4SF4 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HuTu-80 cells (90% confluent) were transiently transfected with siRNA [pool of three different siRNA duplexes (duplex-1, sense: GAAGCGGUGUCUUGAUGAUtt, antisense: AUCAUCAAGACACCGCUUCtt; duplex-2, sense: GAUGAGGCCUUAUGGAACAtt, antisense: UGUUCCAUAAGGCCUCAUCtt; duplex-3, sense: CUAGCUGAUAAAGCUUAGAtt, antisense: UCUAAGCUUUAUCAGCUAGtt specific for TM4SF4 and negative control siRNA (scrambled) using Lipofectamine 2000. After 48hrs of transient transfection, total RNA was isolated from HuTu-80 cells to determine hTHTR-1, hTHTR-2 and TM4SF4 mRNA expression levels and to determine the effect of TM4SF4 knockdown on ^{3}H-thiamine uptake in intestinal epithelial cells.

Western blotting

Cellular extracts prepared from control cells and TM4SF4 siRNA pretreated HuTu-80 cells were used to perform western blotting [16, 18]. Total proteins (60 μg) were resolved onto premade 4–12% Bis-Tris minigel (Invitrogen), and subjected to western blotting [16, 18]. Proteins were electro-blotted onto immobilon polyvinylidene difluoride membrane (Fisher Scientific, CA, USA). Along with blocking buffer (LI-COR Bioscience), the membranes were incubated overnight at 4°C with human TM4SF4 specific rabbit polyclonal antibodies (Sigma) together with the human β-actin monoclonal antibody (Santa Cruz Biotechnology). The immunoreactive bands were detected and the band intensity was quantified using LI-COR software [18].

Quantitative real-time PCR

Five micrograms of total RNA was isolated from HuTu-80 cells and treated with DNase I and subjected to reverse transcription (RT) using iScript cDNA Synthesis Kit (Bio Rad, CA, USA). The RT-PCR products were then used for real-time PCR amplification with hTHTR-1, hTHTR-2, TM4SF4, and β-actin primers (Table 1). Data were normalized to simultaneously amplified β-actin and Ct values were calculated using relative relationship method [25].

Uptake studies

^{3}H-Thiamine uptake assay was performed using confluent HuTu-80 cells incubated in Krebs-Ringer (K-R) buffer at 37°C for 3 min. The labeled ^{3}H-thiamine (15 nM) was added to the K-R buffer at the onset of incubation, and the reaction was stopped by the addition of ice-cold buffer. Radioactivity was determined using a scintillation counter, and protein content was determined in parallel using a Bio-Rad Dc Protein Assay kit (Bio-Rad).

Statistical analysis

All uptake data presented are mean SEM of multiple separate experiments and are expressed in percentage relative to simultaneously performed controls. Uptake of ^{3}H-thiamine by the carrier-mediated process was determined by subtracting uptake by passive diffusion [represented by residual uptake of ^{3}H-thiamine in the presence of a high pharmacological concentration of unlabeled thiamine (1 mM) from total ^{3}H-thiamine uptake]. Western analysis and real-time PCR experiments were performed using multiple sample preparations. Student’s t-test was used for statistical analysis and P 0.05 was considered as statistically significant.

Results

Identification of the TM4SF4 protein as an interacting partner with hTHTR-2 by yeast split-ubiquitin two-hybrid screening of the intestinal cDNA library

Studies with hTHTR-2 polypeptide truncation and mutational analysis have shown that the integrity of the hTHTR-2 polypeptide is important for cell surface expression and transporter function [14, 26]. To search for proteins that may interact with hTHTR-2, the full-length hTHTR-2 (1–496 amino acids) was used as a bait in our yeast split-ubiquitin two-hybrid screening. The prey sequences of the positive clones were amplified by PCR, sequenced and identified using GenBank database (NCBI). Based on bioinformatics analysis (prey identification and sequencing, and selected interacting domain determination), as well as biological characteristics and tissue distribution pattern of the identified putative proteins (i. e., abundant expression in the human intestine), we selected the human transmembrane 4 superfamily member 4 (TM4SF4) (also called the intestinal and liver tetraspan membrane protein, il-TMP) as possible interacting partner with the hTHTR-2 for further investigations [27, 28].

GST-TM4SF4 fusion protein interacts with hTHTR-2 in HuTu-80 cells

A GST pull-down assay was used to further confirm the interaction between hTHTR-2 and TM4SF4 in intestinal epithelial HuTu-80 cells. An affinity purified GST-TM4SF4 fusion construct, and GST alone, were compared in their ability to pull-down hTHTR-2 from cell extracts prepared from a HuTu-80 cell line stably expressing hTHTR-2-GFP. The results showed that the GST-TM4SF4 fusion protein, but not GST alone (negative control), can recover hTHTR-2 from the HuTu-80 cell extract (Fig. 1).

hTHTR-2 and TM4SF4 co-localize in HuTu-80 cells

In this study, we transiently co-expressed hTHTR-2-GFP and mCherry-TM4SF4 in HuTu-80 cells and performed live cell confocal imaging 24 to 48 hrs after transfection. As seen before [14] HuTu-80 cells displayed the expression of hTHTR-2-GFP at the plasma membrane as well as in intracellular trafficking vesicular compartments. mCherry-TM4SF4 has also expressed at the plasma membrane and in intracellular trafficking vesicles as expected [27, 29]. Upon co-expression of the two fusion proteins (hTHTR-2-GFP and mCherry-TM4SF4), a significant overlap in expression was observed both in intracellular vesicles and at the plasma membrane of HuTu-80 cells (Fig. 2).

Functional consequence of interaction between hTHTR-2 and TM4SF4 in HuTu-80 cells

To examine the consequence of the TM4SF4 interaction with hTHTR-2 on the functionality of the latter, we performed uptake experiment comparing ^{3}H-thiamine uptake (15 nM, pH 7.4) by HuTu-80 cells that were stably over-expressing hTHTR-2 alone to those co-expressing hTHTR-2 and transiently expressing TM4SF4. ^{3}H-Thiamine uptake was performed 48hrs after transfection with TM4SF4. The results showed that the co-expression of hTHTR-2 with TM4SF4 leads to a significant ($P < 0.01$) increase in ^{3}H-thiamine uptake compared to cells expressing hTHTR-2 alone (Fig. 3). To further confirm the contribution of TM4SF4 towards thiamine uptake by HuTu-80 cells, we examined the effect of silencing the endogenous TM4SF4 (using gene-specific siRNA) on ^{3}H-thiamine uptake by HuTu-80 cells. The results showed a significant ($P < 0.05$) inhibition in thiamine uptake by cells pretreated with TM4SF4 siRNA compared to those treated with scrambled siRNA (Fig. 4A). The effectiveness of the siRNA silencing of TM4SF4 was verified by establishing a significant ($P < 0.05$ for both) reduction in level of TM4SF4 protein and mRNA expression (Fig. 4B & C). It is relevant to mention here that the levels of expression of hTHTR-1 and hTHTR-2 in the TM4SF4 siRNA treated cells were not affected (data not shown).

Discussion

The mammalian intestine plays a critical role in regulating body thiamine homeostasis as it represents the point of entry of this essential micro-nutrient (which cannot be synthesized endogenously) into the body compartment. Thus, understanding the details of the cellular and molecular mechanisms involved in the thiamine absorption process and its regulation are of clear physiological and biological relevance. Studies from our laboratory and others have previously characterized many different aspects of the intestinal thiamine absorption process [reviewed in 9, 30]. More, however, is needed especially with regards to possible involvement of accessory proteins that may interact with the thiamine uptake systems and influence their physiology/cell biology. The existence of such accessory proteins in the case of membrane transporters of other nutrients/substrates, and in a variety of cell types has been well documented in recent years [15–21]. Such interaction between accessory proteins and membrane transporter was found to affect the transport function, intracellular trafficking, and/or half-life of the membrane transporters [15–21]. In recent studies we used a bacterial two-hybrid system to screen a human intestinal cDNA library and showed that a member of the tetraspanin family of proteins, Tspan-1, interacts with hTHTR-1 and affects its stability [17]. There is nothing known on whether the hTHTR-2 also has an interacting partner(s), and whether the interaction between the two proteins affects the physiological function and cell biology of hTHTR-2 in intestinal epithelial cell. We addressed these issues in the current study using a yeast split-ubiquitin two-hybrid approach to screen a human intestinal cDNA library. We utilized (as bait) the entire coding region of hTHTR-2, and applied appropriate bioinformatics analysis (as well as biological/physiological knowledge and tissue pattern of expression of the identified putative proteins) to identify TM4SF4 as a potential interacting partner with hTHTR-2. The human TM4SF4 [202 amino acids (21.5 kDa)] is a member of tetraspanin protein family and has four transmembrane domains, two short cytoplasmic domains at the NH_2 and COOH termini, and two extracellular loops [27, 28, 31]. The protein is abundantly expressed in the human intestine (and liver) with little expression in many other tissues (e.g., stomach, kidney, lung, skeletal muscle, and heart) and plays a role in cell proliferation [27, 28]. At the cellular level, the human TM4SF4 is expressed at the apical membrane domain of the polarized human enterocytes [27].

Confirmation of the existence of the interaction between hTHTR-2 and TM4SF4 was done by mean of a pull-down assay and by actual demonstration of co-localization of the two proteins in HuTu-80 cells in studies utilizing live cell confocal imaging. In the latter study, co-localization of the two proteins was observed in both intracellular (trafficking) vesicles and the plasma membrane of the intestinal epithelial cells raising the possibility that TM4SF4 may be involved in trafficking of hTHTR-2 to the plasma membrane; further studies, however, are required to test this possibility. It is worth mentioning here that in intestinal epithelial cells hTHTR-2 was found to be in intracellular vesicles that display rapid bi-directional trafficking to and from cell membrane [14].

We also examined the functional consequence of the interaction between hTHTR-2 and TM4SF4. This was done using two approaches. In the first approach, we examined the effect of co-expression of these two proteins in HuTu-80 cells on the function of hTHTR-2 and found a significant induction in ^{3}H-thiamine uptake as a result of this co-expression. In the second approach, we examined the effect of silencing the endogenous TM4SF4 (using TM4SF4 specific siRNA) on carrier-mediated thiamine uptake by HuTu-80 cells and found a significant inhibition in thiamine uptake in TM4SF4 siRNA-pretreated cells compared to controls. These findings clearly suggest that TM4SF4 association with the hTHTR-2 affects the functionality of the latter.

In summary, our results showed for the first time that the accessory protein TM4SF4 is an interacting partner with hTHTR-2 in human intestinal epithelial cells, and that this interaction has physiological consequence on thiamine uptake.

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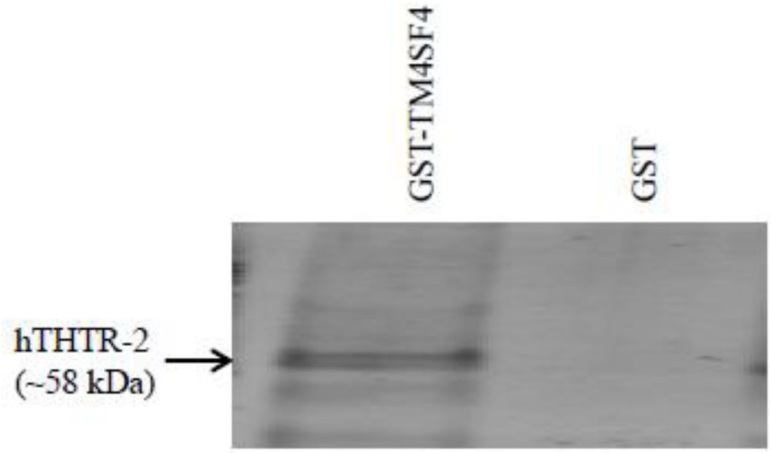

Figure 1. Confirmation of TM4SF4 interaction with hTHTR-2 in HuTu-80 cells by pull-down assay

HuTu-80 cell lysate (1mg of protein) was incubated with either 5μg of GST-TM4SF4 (1–202aa) or 100μg of GST alone bound to glutathione-Sepharose4B beads. The bound proteins were eluted with 10mM glutathione and separated in NuPAGE 4–12% Bis-Tris mini gels. The proteins were analyzed by western blotting using anti-hTHTR-2 polyclonal antibodies.

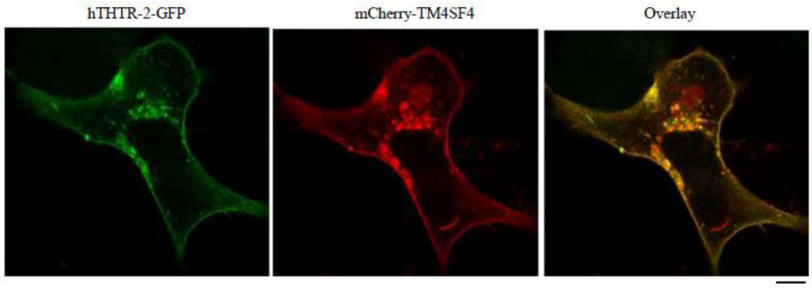

Figure 2. Co-expression of hTHTR-2-GFP and mCherry-TM4SF4 in HuTu-80 cells

HuTu-80 cells were transiently co-transfected with hTHTR-2-GFP and mCherry-TM4SF4 constructs. After 24–48hrs of transient transfection, live cell imaging was performed using confocal microscope as described in the “Methods”. Scale bar 10 μm.

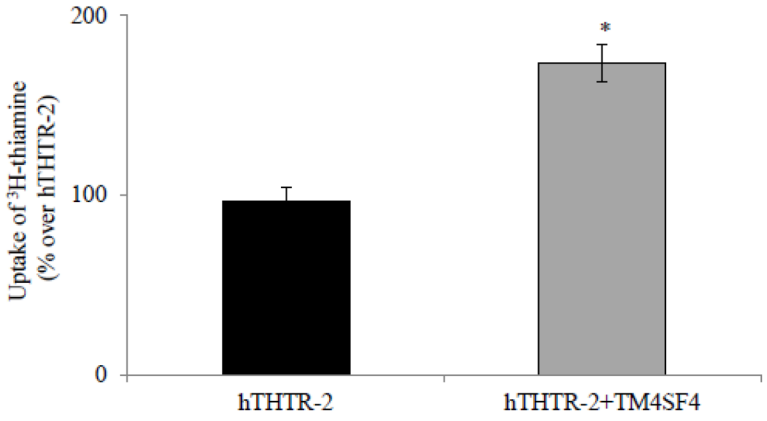

Figure 3. Co-expression of TM4SF4 with hTHTR-2 increases thiamine uptake in HuTu-80 cells

hTHTR-2-GFP stably expressing HuTu-80 cells were transiently co-transfected with the pFLAG-CMV-2-TM4SF4. Post transfection ^{3}H-thiamine uptake (15 nM; pH 7.4; 3 min) was determined. Data are mean ± SEM of at least 6 separate experiments. *P < 0.01.

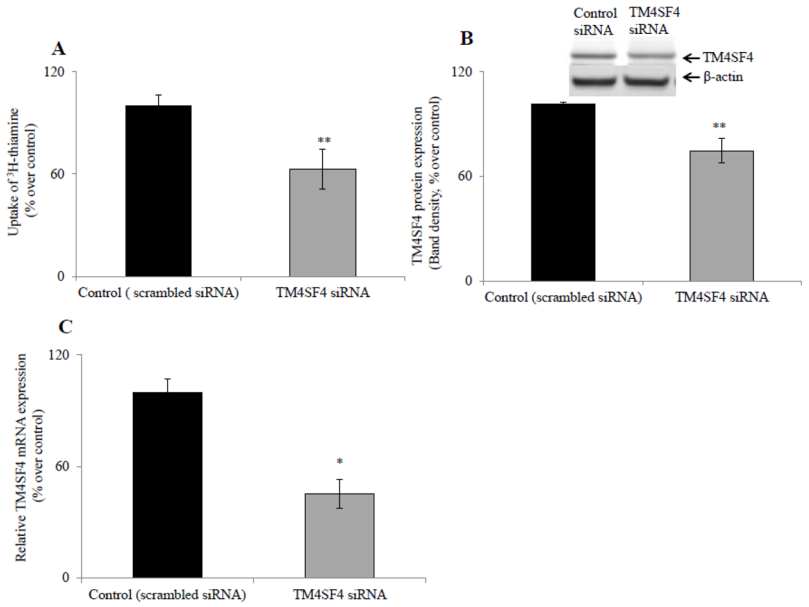

Figure 4. Effect of TM4SF4 silencing with siRNA on ^{3}H-thiamine uptake in HuTu-80 cells

A) HuTu-80 cells were transiently transfected with the TM4SF4 siRNA, or with the non-targeting fluorescein conjugated control siRNA (scrambled). Initial-rate (3min) of ^{3}H-thiamine (15 nM) uptake was determined after 48hrs of siRNA transfection. Data are mean ± SEM of at least 3 separate uptake determinations **P < 0.05. B) *Top:* cell extract (60 μg) isolated from control and TM4SF4 siRNA pretreated HuTu-80 cells was analyzed by western blotting as described in "Methods". Blots were incubated with rabbit polyclonal anti-human TM4SF4 specific antibodies (top) and monoclonal β-actin antibodies (bottom). *Bottom:* band density of the immunoreactive bands (in arbitrary units). Data are mean ± SEM of at least 3 independent sample preparations. **P < 0.05. C) Quantitative real-time PCR was performed using total RNA isolated from HuTu-80 cells and TM4SF4 specific primers (Table 1). Data are from at least three different experiments and expressed relative to β-actin as mean ± SEM. *P < 0.01.

Table 1

Primers used to prepare the hTHTR-2 and TM4SF4 fusion constructs by PCR and real-time PCR.

Gene name	Forward & Reverse Primers (5'–3')
Confocal studies	
hTHTR-2	CCG**CTCGAG**ATGGATTGTTACAGAACTTCACTAAG; CGGGATCCGAGTTTTGTTGACATGATGATATTAC
TM4SF4	GC*GTCGAC*ATGTGCACTGGGGGCTGT; CGGGATCCAACGGGTCCATCTCCCCC
Real-time PCR studies	
hTHTR-1	AGCCAGACCGTCTCCTTGTA; TAGAGAGGGCCCACCACAC
hTHTR-2	TTCCTGGATTTACCCCACTG; GTATGTCCAAACGGGGAAGA
TM4SF4	GGTGTTCTTGGGCCTGA; GATAATCCCCGTCGTGGA
hβ-actin	CATCCTGCGTCTGGACCT; TAATGTCACGCACGATTTCC

Restriction sites for Xho *I* (boldface text), *Bam HI* (underlined text) and *SalI* (italics underlined text) were added to the hTHTR-2 and TM4SF4 primers to allow subsequent sub-cloning into the GFP-N3 and mCherry-C1 vectors.