Differential effects of two-pore channel protein 1 and 2 silencing in MDA-MB-468 breast cancer cells

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Abstract

Two-pore channel proteins, TPC1 and TPC2, are calcium permeable ion channels found localized to the membranes of endolysosomal calcium stores. There is increasing interest in the role of TPC-mediated intracellular signaling in various pathologies; however their role in breast cancer has not been extensively evaluated. TPC1 and TPC2 mRNA was present in all non-tumorigenic and tumorigenic breast cell lines assessed. Silencing of TPC2 but not TPC1 attenuated epidermal growth factor-induced vimentin expression in MDA-MB-468 breast cancer cells. This effect was not due to a general inhibition of epithelial to mesenchymal transition (EMT) as TPC2 silencing had no effect on epidermal growth factor (EGF)-induced changes on E-cadherin expression. TPC1 and TPC2 were also shown to differentially regulate cyclopiazonic acid (CPA)-mediated changes in cytosolic free Ca^{2+}. These findings indicate potential differential regulation of signaling processes by TPC1 and TPC2 in breast cancer cells.

Abbreviations: Ca^{2+}, calcium; $[Ca^{2+}]_{CYT}$, cytosolic free calcium; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; NAADP, nicotinic acid adenine dinucleotide phosphate; siNT, non-targeting siRNA; TPC1, two-pore channel protein 1; TPC2, two-pore channel protein 2; TRP, transient receptor potential

Keywords

Breast cancer, calcium, calcium signaling, epidermal growth factor, two-pore channel, vimentin

1. Introduction

The presence of specific Ca^{2+} channels is a feature of some cancer subtypes and in some cases silencing of proteins that constitute these channels can reduce cancer cell proliferation [1]. Examples include TRPV6 and Orai1, where silencing of these proteins alters Ca^{2+} signaling and attenuates the proliferation of breast cancer cells [2,3]. Studies of calcium channels in breast cancer cells have mostly focused on transient receptor potential (TRP), Orai, ligand-gated, and voltage-gated Ca^{2+} channels. Two-pore channel (TPC) proteins, TPC1 and TPC2, are recently identified ion channels that contribute to nicotinic acid adenine dinucleotide phosphate (NAADP)-mediated Ca^{2+} release from acidic intracellular endolysosomal stores [4]. TPC1 and TPC2 appear to have distinct gating mechanisms and have been reported to play differential roles in a variety of cell types and signaling pathways [4,5]. Despite the reported role of TPC1 in cell cycle regulation in a HEK293 overexpression model [6], and the role of TPC2, but not TPC1, in angiogenesis-associated signaling pathways [7], these channels have not been assessed in detail in breast cancer cell lines. However, evidence from studies in HER2 positive SKBR3 cells do suggest functional TPC channels in this breast cancer cell line [8].

Breast cancer is characterized by diversity at the prognostic, clinical and molecular level [9]. The triple negative breast cancer subtype, which overlaps largely with the basal molecular subtype, suffers from a lack of molecularly targeted therapies [9]. A number of triple negative breast cancer cell lines (such as MDA-MB-468 cells) have been used as *in vitro*

models for this subtype. MDA-MB-468 cells belong to the related basal A cell line subgroup [10] and with appropriate stimuli, such as epidermal growth factor (EGF), can undergo an epithelial-mesenchymal transition (EMT), which is associated with a loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as vimentin [11]. Recently, calcium signaling and specific Ca^{2+} permeable ion channels have been shown to be regulators of EGF-induced vimentin expression in MDA-MB-468 breast cancer cells [12].

In this study we sought to determine if TPC1 and TPC2 were present in tumorigenic and non-tumorigenic breast cell lines. We also assessed the consequences of TPC1 and TPC2 silencing on the proliferation of MDA-MB-468 breast cancer cells, EGF-induced changes in the expression of vimentin and E-cadherin, and phosphorylation of STAT3. Finally, in light of recent evidence suggesting differential selectivity of TPC1 and TPC2 for Ca^{2+} ions [4,13,14], we assessed the consequences of channel silencing on calcium influx in the MDA-MB-468 cell line.

2. Materials and Methods

2.1 Cell culture

Human MDA-MB-468 and MDA-MB-231 breast cancer cells were cultured in high glucose DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and L-glutamine (4 mM; Life Technologies, Carlsbad, CA, USA), and maintained in a humidified incubator at 37°C with 5% CO_2. Cells consistently tested negative for mycoplasma infection (screened 6-monthly) using the MycoAlert Mycoplasma Detection Kit (Lonza Inc., Basel, Switzerland). For EMT studies, MDA-MB-468 cells were serum reduced (0.5% FBS) for 24 h prior to stimulation with EGF (50 ng/mL; E9644, Sigma-

Aldrich) for the indicated time, as previously described [12], with the following changes: for EGF-stimulation protocols lasting greater than 48 h, media was replaced with fresh EGF after 48 h.

2.2 siRNA transfection

siRNA-mediated gene silencing studies were performed using DharmaFECT4 Transfection Reagent and Dharmacon ON-TARGET*plus* SMARTpool siRNAs at a final concentration of 100 nM (Thermo Scientific, Waltham, MA, USA), following the manufacturer's protocols and as previously described [12]. The following siRNAs were used in this study: TPCN1 (siTPC1; L-010710-00-0005), TPCN2 (siTPC2; L-006508-00-0005) and non-targeting control (siNT; D-001810-10-05).

2.3 Real time RT-PCR

Real time RT-PCR was performed as previously described [12], with the following changes: for Figs. 1A & B mRNA was amplified using TaqMan Universal PCR Master Mix (4324018; Applied Biosystems, Carlsbad, CA, USA), while TaqMan Fast Universal PCR Master Mix (4352042; Applied Biosystems) was used for Figs. 2A & B. RNA was isolated 48 or 96 h post-siRNA transfection for gene silencing studies. The following TaqMan Gene Expression assays were used to assess mRNA levels: TPC1 (Hs00330542_m1), TPC2 (Hs01552063_m1), and 18S rRNA (4319413E; Applied Biosystems). Fold change (mRNA) was calculated using the comparative C_t method with 18S rRNA used as the endogenous control [15].

2.4 Immunoblotting

Immunoblotting was performed as previously described [12]. Briefly, protein was loaded into NuPage Novex 4-12% bis-tris gels (Invitrogen) and run under reducing and denaturing conditions. Following protein transfer, PVDF membranes were blocked and incubated with primary antibody. The following antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): antiphospho-STAT3 (9138), anti-STAT3 (9139) (both diluted 1:1000). Anti-vimentin antibody (V6389) was purchased from Sigma-Aldrich (diluted 1:750). Membranes were then washed and probed with anti-mouse HRP-conjugated secondary antibody (170–6516, Bio-Rad Hercules, CA, USA) (diluted 1:10 000). Chemiluminescence imaging was performed using a Versadoc Imaging System (Bio-Rad). *β*-actin (A5441; Sigma-Aldrich) served as a loading control, and phosphorylated protein expression was further normalized relative to total protein concentration. Protein volume analysis was performed using Quantity One Software (Bio-Rad) using the global background subtraction method as outlined in the user manual.

2.5 Cell enumeration and S-phase analysis

At 96 h post-siRNA transfection, cell enumeration and S-phase analysis were performed by 5-ethynyl-2'-deoxyuridine (EdU) incorporation using the Click-iT® EdU Alexa Fluor 555 Imaging Kit (Invitrogen, Carlsbad, CA, USA) as previously described [16]. Cells were imaged with a 10X objective using an ImageXpress Micro automated epifluorescence microscope (Molecular Devices, Sunnyvale, CA, USA) using the following excitation/emission wavelengths: DAPI 377/447 nm and EdU 531/593. Normalized cell count and percent EdU positivity were determined using the Multi-wavelength cell scoring application module (MetaXpress v3.1.0.83; Molecular Devices).

2.6 Intracellular calcium measurements

At 72 h post-siRNA transfection, measurement of cyclopiazonic acid (CPA)-mediated changes in store-operated calcium entry was performed using a fluorometric imaging plate reader (FLIPR^{TETRA}; Molecular Devices) and the BD PBX no-wash calcium assay kit (BD Biosciences, Franklin Lakes, NJ, USA) as previously described [12].

2.7 Statistical analysis

Details of statistical analyses are provided in figure legends and were performed using GraphPad Prism version 6.05 for Windows.

3. Results

3.1 TPC isoform mRNA levels in a panel of breast cell lines

TPC1 and TPC2 mRNA was detected in all tumorigenic and non-tumorigenic breast cell lines assessed. There was no clear trend for greater levels in tumorigenic breast cell lines compared to non-tumorigenic breast cell lines (184B5 and 184A1), or between subgroups such as luminal (MCF-7, T47D, ZR-75-1, BT-483), HER2 enriched (SKBR3) or basal/claudin low (MDA-MB-231, MDA-MB-468) [17,18] (Fig. 1). These results suggest that elevated TPC levels are not a defining characteristic of particular breast cancer cell line subgroups, which represent different breast cancer subtypes.

3.2 Effect of TPC silencing on MDA-MB-468 breast cancer cell number

To explore the potential role of TPC1 and TPC2 in a basal breast cancer cell line, TPC isoforms were selectively silenced in MDA-MB-468 breast cancer cells. TPC1 (Fig. 2A) and TPC2 (Fig. 2B) mRNA levels were effectively silenced at 48 h (>85%) and 96 h (>75%) post-siRNA transfection relative to the non-targeting siRNA control (siNT). At 96 h post

silencing, compensatory increases in the related isoform was observed (Fig. 2A and B). Silencing of TPC1 or TPC2 had no effect on cell number (Fig. 2C) or percentage of cells in S-phase (EdU positive) relative to the siNT control (Fig. 2D).

3.3 TPC2 silencing attenuates EGF-induced vimentin expression in MDA-MB-468 cells

The MDA-MB-468 breast cancer cell line is a commonly utilized *in vitro* model for the study of EMT in the context of defining responses to signals from the surrounding tumor microenvironment [11,19,20]. TPC2 but not TPC1 silencing significantly inhibited EGF-induced vimentin expression relative to siNT in MDA-MB-468 cells (Fig. 3A). This effect was not due to a general inhibition of EMT induction since the suppression of E-cadherin expression by EGF was unaffected by TPC2 silencing (Fig. 3C). Inhibition of vimentin expression was specific to an induction pathway since TPC2 (like TPC1) silencing had no effect on the endogenous basal expression of vimentin protein in MDA-MB-231 breast cancer cells (Fig. 3B). However, effects of TPC2 on EGF-induced vimentin expression were not due to global suppression of EGF-induced signaling since TPC2 (like TPC1) silencing had no effect on activation of STAT3 by EGF (Fig. 3D).

3.4 TPC isoform silencing differentially modulates calcium stores and/or store-operated calcium entry in MDA-MB-468 cells

To assess the role of TPC isoforms in store-operated calcium entry, an important calcium influx pathway in epithelial cells [21], calcium stores of siRNA-transfected MDA-MB-468 cells were depleted by CPA (10 μM) (an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase channels) in order to activate plasma membrane localized store-operated channels (i.e. Orai1) (Fig. 4A & E). TPC1 silencing had no effect on CPA-induced calcium store release (peak 1) measured as change in relative cytosolic calcium (Fig. 4B) whereas TPC2

silencing resulted in significantly reduced CPA-induced calcium store release (Fig. 4F). TPC1 and TPC2 silencing also had opposing effects on store-operated calcium influx (peak 2), observed through the re-addition of extracellular calcium (1.8 mM). TPC1 (Fig. 4C) modestly but significantly increased Ca^{2+} influx whereas TPC2 (Fig. 4G) silencing decreased Ca^{2+} influx associated with CPA-induced store depletion. These effects may be due in part to changes in CPA-induced depletion since the ratio of peak 2 to peak 1 (a measure of store-operated Ca^{2+} entry) were not significantly altered by TPC1 or TPC2 silencing (Fig. 4D & H).

4. Discussion

These studies represent the first detailed assessment of TPC channels in breast cancer cells. Tumorigenic and non-tumorigenic breast cell lines had similar levels of TPC1 and TPC2 mRNA. Unlike other ion channels, such as Orai1 [22], elevated levels of TPC channel mRNA does not seem to be a feature of breast cancer cells. Likewise, alterations in TPC1 and TPC2 mRNA were not a feature of a particular breast cancer cell molecular subtype. The similar levels of TPC1 and TPC2 among breast cell lines may be reflective of the diverse tissue distribution of these channels [23,24] and of important roles in epithelial cells. Assessment of the consequences of selective TPC isoform silencing in MDA-MB-468 breast cancer cells suggest some degree of gradual compensation between these isoforms, with TPC1 up-regulation following TPC2 silencing and *vice versa* at 96 h post knock-down. Unlike previous studies that show a role for TPC isoforms in cell cycle regulation when overexpressed in HEK293 cells [6], silencing of endogenous TPC expression had no effect on MDA-MB-468 cell proliferation, highlighting differences between endogenous and overexpressing systems.

The results presented here provide further evidence for specific and differential roles for TPC isoforms in the same cell type. TPC2 but not TPC1 silencing reduced EGF-induced vimentin expression in MDA-MB-468 breast cancer cells. TPC1 and TPC2 silencing also had different effects on CPA-mediated changes in $[Ca^{2+}]_{CYT}$. The gating and permeability of TPC1 and TPC2 vary [4] and examples of differential roles are also reflected in the effects of TPC isoform silencing on endomembrane dynamics [5], effects of overexpression on multi-nucleation [6], and roles on local and global Ca^{2+} signals [25]. In light of previous studies indicating a role for TPC2 in store-operated calcium entry [26], future studies assessing the mechanism by which TPC2 remodels CPA-induced changes in $[Ca^{2+}]_{CYT}$ in this model are required, particularly in the context of calcium induced Ca^{2+} release [27].

The ability of TPC2 but not TPC1 to attenuate EGF-induced vimentin expression could have arisen from effects on EGF receptor trafficking given results of studies in TPC2 null mice [28]. However, this is unlikely given that EGF-mediated STAT3 activation was unaffected by TPC2 silencing in MDA-MB-468 breast cancer cells. Recent studies have identified the ability of JAK Inhibitor I to suppress EGF-induced vimentin expression in MDA-MB-468 cells at concentrations that do not reduce EGF-activation of STAT3. Indeed, like TPC2 silencing, JAK Inhibitor I does not reduce basal vimentin levels in MDA-MB-231 cells [29]. Future studies should now explore the potential association between TPC2 and the Janus kinase (JAK) signaling pathway in MDA-MB-468 cells. Despite the role of the Ca^{2+} signal in EMT induction and our report of effects of TPC2 silencing on the expression of the mesenchymal marker vimentin, TPC2 is clearly not a regulator of EMT induction, given that EGF-mediated changes in E-cadherin were not affected by TPC2 silencing [12].

In summary, these studies provide further evidence for the ability of TPC1 and TPC2 to differentially regulate pathways in the same cell type and begin to define the role of these recently identified ion channels in breast cancer cells, including shaping responses to tumor micro-environmental factors such as EGF.

Acknowledgements

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Figures/Figure legends

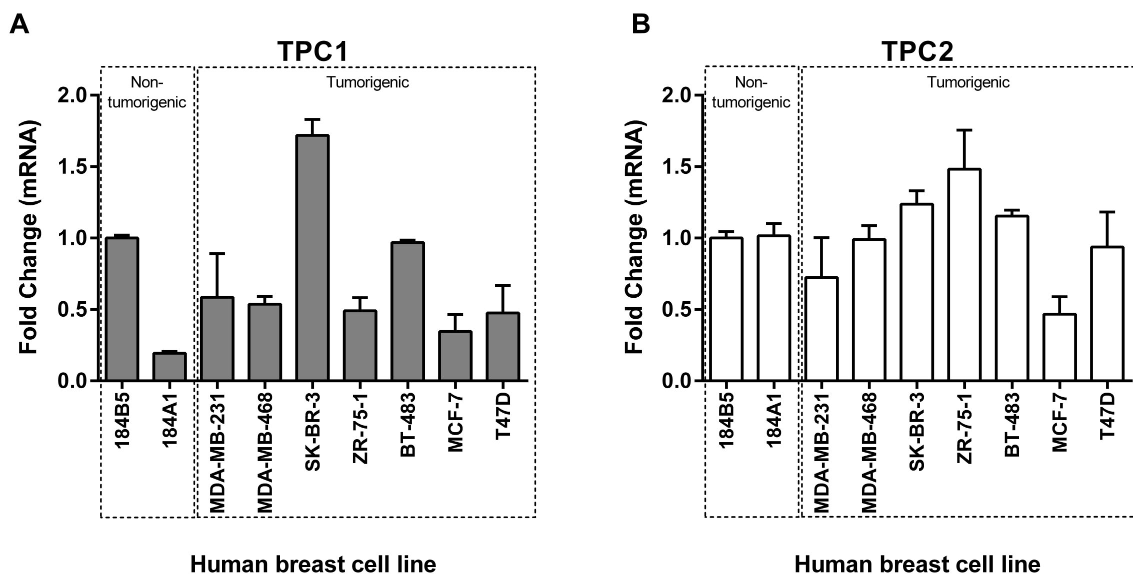
Fig. 1. Relative mRNA levels of TPC isoforms in a panel of human breast cell lines. (A) TPC1 and (B) TPC2 mRNA levels in non-tumorigenic and tumorigenic breast cell lines were assessed using real time RT-PCR. Data were normalized to 18S rRNA and are expressed as fold-change relative to 184B5 cells. Bar graphs represent the mean ± S.D. for six wells from two independent experiments.

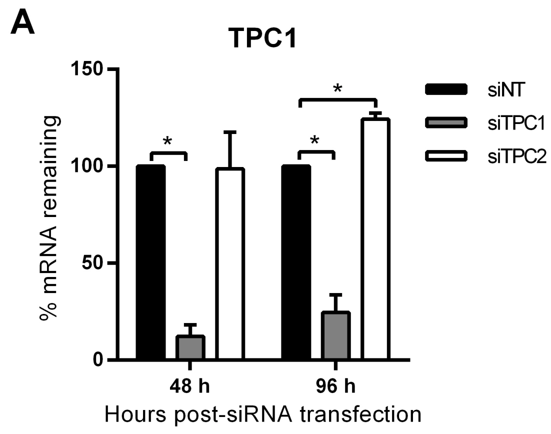
Fig. 2. Effect of TPC1 or TPC2 silencing on MDA-MB-468 cell proliferation. Efficient siRNA-mediated silencing of (A) TPC1 or (B) TPC2 was shown at both 48 h (>85 %) and 96 h (>75%). Bar graphs show percent TPC1 or TPC2 mRNA remaining relative to the non-targeting control (siNT), and represent the mean ±SD from three independent experiments. Statistical analysis was performed using two-way ANOVA with Dunnett's multiple comparisons test. The effect of TPC1 or TPC2 silencing on cell proliferation was assessed via (C) cell enumeration, shown relative to siNT, and (D) percentage S-phase (EdU positivity). Bar graphs represent the mean ±SD from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. $*P < 0.05$.

Fig. 3. TPC silencing in MDA-MB-468 and MDA-MB-231 breast cancer cells. The effect of TPC1 and TPC2 silencing on (A) Vimentin, (C) E-Cadherin and (D) STAT3 phosphorylation in MDA-MB-468 cells stimulated with EGF (50 ng/mL) for different times, and (B) basal vimentin protein expression in MDA-MB-231 cells at 72 h post knock down, was assessed using immunoblotting. (i) Representative blot and (ii) relative protein expression quantified from three independent experiments. MDA-MB-231 and MCF-7 protein lysates served as positive or negative controls in A(i) and C(i). Statistical analysis was performed using two-

way ANOVA (A), (C) and (D) or one-way ANOVA (B) with Tukey's multiple comparisons test. *$P < 0.05$, ns = not significant.

Fig. 4. Effect of TPC1 or TPC2 silencing on store-operated calcium entry in MDA-MB-468 cells. Calcium stores were depleted with cyclopiazonic acid (10 µM) in the presence of BAPTA (500 µM) (peak 1), resulting in activation of store-operated calcium entry channels and calcium influx upon addition of Ca^{2+} (1.8 mM) (peak 2). Calcium traces represent mean change (Δ) in relative $[Ca^{2+}]_{CYT}$ in the presence of (A) TPC1 or (E) TPC2 silencing. Bar graphs show the mean ±SD for (B) & (F) peak 1 and (C) & (G) peak 2 relative $[Ca^{2+}]_{CYT}$, and (D) & (H) the ratio of peak 1 to peak 2 in the presence of TPC1 or TPC2 silencing. Data is representative of three independent experiments. Statistical analysis was performed using unpaired, two-tailed *t* test. *$P < 0.05$.

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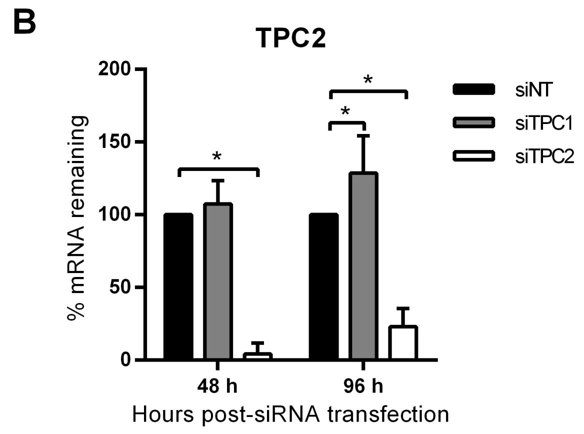
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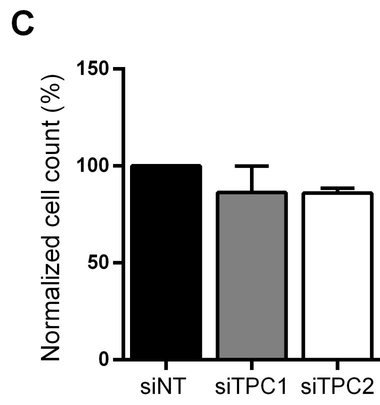
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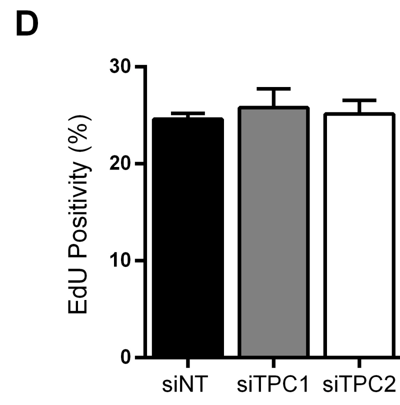
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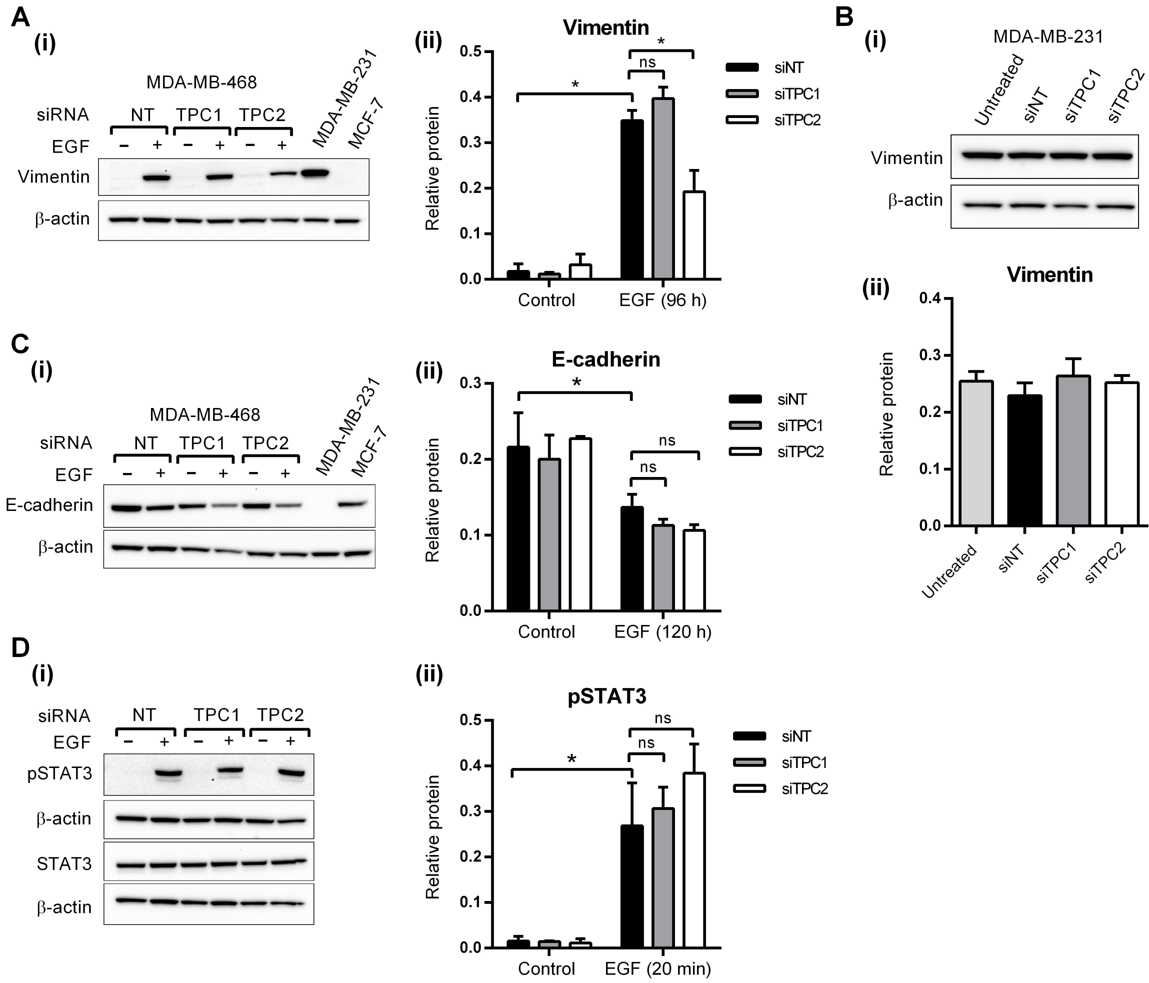
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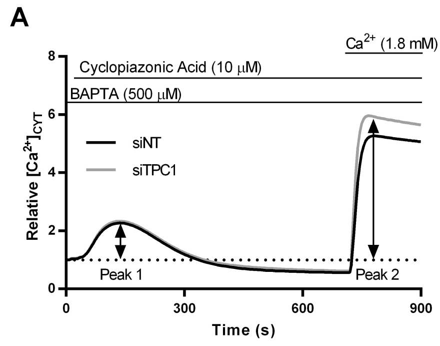

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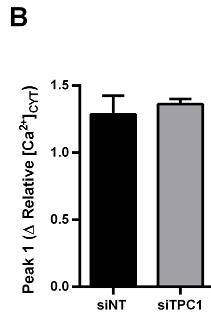

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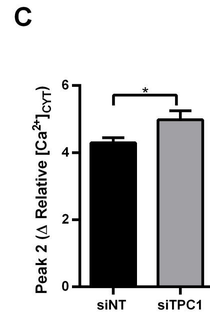

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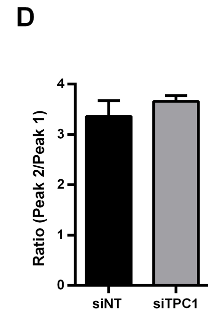

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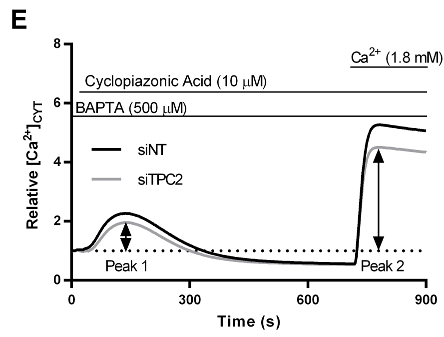

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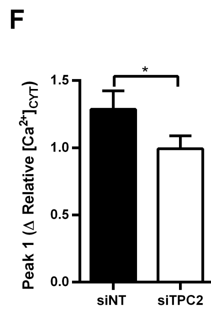

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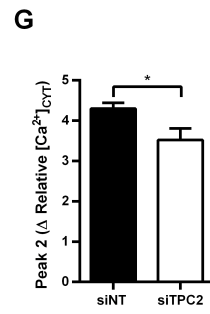

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