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TRPM7 Regulates Polarized Cell Movements

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SYNOPSIS

TRPM7 is a Ca²⁺ and Mg²⁺ permeant ion channel in possession of its own kinase domain. As a kinase, the protein has been linked to the control of actomyosin contractility, whereas the channel has been found to regulate cell adhesion as well as cellular Mg²⁺ homeostasis. Here we show that depletion of TRPM7 by RNA interference in fibroblasts alters cell morphology, the cytoskeleton, and the ability of cells to form lamellipodia and to execute polarized cell movements. A pulldown purification assay revealed that knockdown of TRPM7 prevents cells from activating Rac and Cdc42 when stimulated to migrate into a cellular wound. Re-expression of TRPM7 reverses these phenotypic changes, as does, unexpectedly, expression of a kinase-inactive mutant of TRPM7. Surprisingly, expression of the Mg²⁺ transporter SLC41A2 is also effective restoring the change in cell morphology, disruption of the cytoskeleton, and directional cell motility caused by depletion of the channel-kinase. These data uncover an essential role for Mg²⁺ in TRPM7's control over the cytoskeleton and its ability to regulate polarized cell movements.

Keywords

magnesium; channel; transient receptor potential melastatin 7 (TRPM7)

INTRODUCTION

One of the earliest observations about TRPM7 was that chicken DT40 B cells made deficient in the channel-kinase via cre-lox-mediated ablation of the *TRPM7* gene go into growth arrest and die after a few days in culture [1]. Re-expression of human TRPM7 as well as a phosphotransferase-deficient mutant TRPM7-K1648R reverses the growth arrest phenotype [2]. Strikingly, supplementing the cells' growth media with 10-25 mM Mg²⁺ (but not Ca²⁺, Mn²⁺, or Zn²⁺) permits the knockout line to survive and grow in culture [2], as does overexpression of the plasma membrane Mg²⁺ transporter SLC41A2 [3]. This led Scharenberg and colleagues to conclude that TRPM7 is playing a pivotal role in controlling Mg²⁺ homeostasis in B cells [2].

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AUTHOR CONTRIBUTION

Li-Ting Su, Wei Lu, Hsiang-chin Chen, and Omayra González-Pagán performed the experiments. Li-Ting Su and Wei Lu were involved in the discovery of the ability of Mg²⁺ to rescue the TRPM7-depletion phenotype. Loren W. Runnels, Li-Ting Su, and Raymond Habas planned the experiments, analyzed the experimental data and wrote the manuscript.

While depletion of TRPM7 in DT40 cells linked the channel to the regulation of Mg^{2+} homeostasis, study of the channel-kinase's overexpression in other cell types has connected it to additional cellular roles, including the control of cell adhesion and actomyosin contractility [4, 5]. Nadler and colleagues were the first to report that overexpression of TRPM7 in HEK-293 cells elicits cell rounding, loss of adhesion and eventual cell death [1]. We investigated this phenomenon and found that overexpression of TRPM7 produces cell rounding by stimulating the activity of the Ca^{2+} -dependent protease m-calpain [5]. While overexpression of the channel causes cell rounding, knockdown of TRPM7 by RNA interference produces the opposite effect, increasing the adhesion, spreading and motility of HEK-293 cells [5]. More recently, we reported that cell rounding elicited by TRPM7 overexpression is initiated by a stress response brought on by the constitutive permeation of both Ca^{2+} and Mg^{2+} into cells [6]. The influx of divalent cations increases concentrations of reactive oxygen and nitrogen species, causing the activation of p38 MAPK and c-Jun N-Terminal Kinase (JNK) for the concomitant stimulation of m-calpain activity [6].

Further compelling evidence linking TRPM7 to the regulation of cell adhesion was provided by Clark and colleagues, who revealed that modest overexpression of TRPM7, as well as a kinase-inactive mutant, in N1E-115 neuroblastoma cells increases cell adhesion and cell spreading, the opposite effect of what occurs when the channel is overexpressed in HEK-293 cells [4]. Surprisingly, overexpression of TRPM7, but not the kinase-inactive mutant, in neuroblastoma cells treated with bradykinin (which has been shown to activate the channel and increase TRPM7-mediated Ca^{2+} influx [7]), stimulates the formation of adhesive structures reminiscent of podosomes [4]. Clark and colleagues hypothesized that because TRPM7 is a member of the alpha-kinase family, with notable homology to myosin heavy chain kinases from *Dictyostelium*, it may be affecting actomyosin remodeling and podosome assembly by directly coupling to and phosphorylating components within the actomyosin cytoskeleton [4]. Immunoprecipitation of TRPM7 from N1E-115 cell lysates copurified β -actin as well as the myosin IIA heavy chain. The association of myosin IIA with the channel is significantly enhanced by bradykinin stimulation and can be reversed by chelation of cellular Ca^{2+} by BAPTA or EDTA [4]. Interestingly, the association between TRPM7 and the myosin IIA heavy chain requires an active kinase domain [4], leading to a persuasive model in which bradykinin-stimulated Ca^{2+} influx, mediated by TRPM7, instigates massive autophosphorylation of the channel-kinase that is required for its association and subsequent phosphorylation of myosin IIA, causing local relaxation of the actomyosin cytoskeleton [8].

Since this early work, additional evidence that TRPM7 is involved in the control of cell motility has come from a study by Wei and colleagues who found that knockdown of TRPM7 by RNA interference reduces the number of high-calcium microdomains (termed 'calcium flickers') elicited by platelet-derived growth factor (PDGF), which consequently disrupts the turning of migrating WI-38 fibroblasts [9]. Interestingly, a study by Abed and Moreau suggests the intriguing possibility that Mg^{2+} influx may also play a role in this process, as silencing of TRPM7 expression in osteoblasts by RNA interference prevents the induction of Mg^{2+} influx and cell migration by PDGF [10].

To better understand the role of TRPM7 in cell adhesion and actomyosin contractility we have generated stable cell lines in which TRPM7 expression in fibroblasts has been knocked down using vectors that express short hairpin RNAs (shRNAs) targeting the channel-kinase [5]. To determine the contribution of TRPM7's dual activities to the control of cell motility we have conducted gain-of-function experiments using TRPM7-knockdown cells and recombinant adenoviral vectors expressing TRPM7, a kinase-inactive mutant and the Mg^{2+} transporter SLC41A2. Here we demonstrate that knockdown of TRPM7 causes a dramatic disruption in cell morphology, cytoskeleton formation and directional cell motility which

can be recovered by expression the TRPM7 kinase-inactive mutant (TRPM7-G1618D) as well by expression of the Mg^{2+} transporter SLC41A2. Unexpectedly, these studies reveal a previously unappreciated role for Mg^{2+} in TRPM7's control of the cytoskeleton and polarized cell movements.

EXPERIMENTAL

Cells and plasmids

TRPM7-knockdown stable cell lines 3T3-M7shRNA2 and 3T3-M7shRNA6, and the control 3T3-shRNA-C originated from Swiss 3T3 cells (American Type Culture Collection number: CCL-92) and were made by standard approaches using previously characterized shRNAs that specifically target mouse TRPM7 and using a non-silencing control shRNA [5]. Briefly, pENTR/H1/TO (Invitrogen) containing TRPM7-specific and control shRNAs were linearized by PvuI digestion and then transiently transfected into Swiss 3T3 cells. The transfected cells were selected with Zeocin (100 μ g/ml) over 10 to 14 days. The 3T3-M7shRNA2 and 3T3-M7shRNA6 lines were selected from among 20 individual clones based on reduced TRPM7 expression. Native TRPM7 expression was assessed using immunoprecipitation and western blotting using the anti-C47 antibody and detected with the anti-CTERM antibody as previously described [5]. Briefly, fibroblasts were lysed using 2 ml of ice-cold RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, and 10 mM iodoacetamide). Proteins were immunoprecipitated overnight from cell lysates from a 150 mm dish using 2 μ g of the anti-C47 antibody bound to protein A agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were washed 3 times in TBST (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) and eluted by boiling in 2x SDS-PAGE sample buffer, and then resolved by SDS-PAGE and western blotting using the anti-CTERM antibody following standard protocols. The SuperSignal West Dura Maximum Sensitive Substrate (Pierce, Rockford, IL) was used for immunochemiluminescence detection. The FLAG-SLC41A2/pcDNA4/TO vector was generously donated by Dr. Andrew Scharenberg (University of Washington, Seattle, WA) [3].

Adenoviral transduction

To rescue the RNA interference phenotype caused by the shRNA targeted against mouse TRPM7, we transduced the 3T3-M7shRNA6 cells with a recombinant adenovirus expressing a silencing-resistant mouse TRPM7 (SR-TRPM7). To render the mouse TRPM7 coding sequence resistant to the TRPM7 shRNA6 (5'-GCA CCC CTC AGT TGC GAA AGA-3'), we altered the open reading frame to 5'-GCA CCC CTC AAC TCA GAA AGA-3'; these mutations, however, were silent in that they did not alter the translated amino acid sequence. SR-TRPM7 was generated using QuickChange (Stratagene, Santa Clara, CA) with pcDNA5/FRT/TO-TRPM7 as a template and the following primers: 5'-CCT CCA GCA GCA CCC CTC AAC TCA GAA AGA GTC ATG AAA CTT TTG GC-3' and 5'-GCC AAA AGT TTC ATG ACT CTT TCT GAG TTG AGG GGT GCT GCT GGA GG-3'. To create SR-TRPM7-G1618D a Sall/NotI fragment from SR-TRPM7 was exchanged with the Sall/NotI fragment from pcDNA5/FRT/TO-TRPM7-G1618D [5].

To create the recombinant adenovirus, SR-TRPM7 and SR-TRPM7-G1618D were first subcloned into the Invitrogen Gateway® entry vector pENTR/D-TOPO and then transferred to the destination vector pAd/CMV/V5-DEST by recombination using the Gateway® system (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The recombinant adenoviruses expressing SR-TRPM7 (pAd/CMV/V5-SR-TRPM7) and SR-TRPM7-G1618D (pAd/CMV/V5-SR-TRPM7-G1618D) were made using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The

SR-TRPM7 and SR-TRPM7-G1618D recombinant adenoviruses were amplified and concentrated from two 150 mm plates of 293A cells (Invitrogen, Carlsbad, CA) using the Adenopack 100 kit (Sartorius Stedim Biotech, Aubagne, France) following manufacturer's instructions. The FLAG-SLC41A2 adenovirus was created by first cloning SLC41A2 from the pcDNA4/TO vector into pENTR/D-TOPO PCR using the following primers: 5'-CAC CTT GGC CGC CAC CAT GGG CGA CTA C-3' and 5'-TTA GTC TCC AAC ATC TCC ATC TCG ATC TCC AAT AAG-3'. FLAG-SLC41A2 was then transferred to the destination vector pAd/CMV/V5-DEST using the Gateway® system. The recombinant adenovirus expressing FLAG-SLC41A2 (pAd/CMV/V5-FLAG-SLC41A2) was made using the ViraPower Adenoviral Expression system as described above. The FLAG-SLC41A2 recombinant adenovirus was amplified, but was of sufficient titer that it did not need to be further purified. Viral titers were determined by a plaque-forming assay using 293A cells. The pAd/CMV/V5-GW/*lacZ* adenoviral construct (Invitrogen, Carlsbad, CA) was used as the negative control. 3T3-M7shRNA6 fibroblasts were transduced with recombinant adenoviruses at a multiplicity of infection (MOI) ranging from 150 to 180. At 5 days post-transduction, cells were harvested for analysis.

Assays and imaging

For cytoskeletal analysis fibroblasts were plated onto coverslips, allowed to adhere overnight, and fixed at room temperature for 10 min in phosphate-buffered saline (PBS) (pH 7.4) with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and permeabilized in PBS with 0.1% Saponin. A monoclonal antibody against vinculin (clone hVIN-1; Sigma, St. Louis, MO) was used to image focal adhesions. A polyclonal antibody against nonmuscle myosin IIA heavy chain (Covance, Emeryville, CA) was employed to detect myosin filaments, and Alexa Fluor 568 Phalloidin (Invitrogen, Carlsbad, CA) was used to stain actin filaments. A 1:2000 dilution of Alexa Fluor 488 or Alexa Fluor 568 goat anti-mouse or anti-rabbit IgG (Invitrogen, Carlsbad, CA) was used as the secondary antibody. Images were obtained using a Zeiss LSM 410 confocal microscope using a 488 nm excitation wavelength and a 512 nm band pass filter for detection of Alexa Fluor 488 fluorescence and 568 nm excitation wavelength and 610 nm band pass filter for detection of Alexa Fluor 568 fluorescence. The pinhole size used was 30, and the contrast/brightness settings were kept the same for each image. The cellular wound assay was performed as previously described [5]. Briefly, cells were plated onto the 35 mm culture dishes and allowed to grow overnight to create a confluent monolayer. A "cellular wound" was created in the monolayer of cells by manually scratching with a P200 pipet tip, washing once with D-MEM containing 2 % FBS to remove loosely attached cells, and then maintaining in the same medium during the imaging experiment. Time-lapse images of cell migration were taken over 16 hours using Olympus IX70 microscope with a 37°C and 5 % CO₂ environmental chamber using a 10 × objective. Images were collected with a MicroMax CCD camera (Princeton Instruments, Trenton, NJ) at 5 min intervals and saved as images stacks using IPLab software (BD Biosciences Bioimaging, Rockville, MD). Images were processed using ImageJ software (NIH, Bethesda, MD). Migration paths were followed for approximately 150 cells for each condition from three independent experiments using the "Manual Tracking plugin" in ImageJ. Progressive lines were generated to view cell trajectories. Cells that migrated into wound but turned or reversed course were scored as cells that moved "non-directionally," whereas cells which migrated in a straight path towards the center of the wound were scored as moving "directionally." Analysis of Golgi reorientation was performed as described [11]. Cells were fixed 6 hours after creation of a cellular wound and stained with β -COP polyclonal antibody (Affinity BioReagents, Golden, CO). The Alexa Fluor 488 goat anti-rabbit IgG was used as the secondary antibody and cells were visualized by confocal microscopy as described above to show the distribution of the Golgi apparatus. Cells in which the Golgi apparatus was within the 120° sector facing the

wound were scored positive, and for each condition at least 150 cells were examined. Cell surface area was analyzed using ImageJ software, and for each condition a minimum of 150 cells from three experiments was examined.

Rho, Rac and Cdc42 activity assays

Detection of activated Rho, Rac, and Cdc42 was accomplished using a modified GST-pulldown purification assay [12, 13]. To detect Rho, Rac and Cdc42 activity during cell migration, the confluent monolayer of cells with multiple cellular wounds was lysed in 500 μ l of Rho lysis buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1 % Triton-X 100, 0.5 % sodium deoxycholic acid, 0.1 % SDS, and 10 mM $MgCl_2$). Cell lysates were incubated with partially purified GST-RBD or GST-PBD bound to glutathione beads for 1 hour (Amersham-Pharmacia, Piscataway, NJ). The GTP bound Rho, Rac and Cdc42 were resolved by SDS-PAGE and western blot analyses using antibodies against Rho A (clone 119; Santa Cruz Biotechnology, Santa Cruz, CA), Rac1 (Cytoskeleton, Denver, CO) and Cdc42 (Cytoskeleton, Denver, CO). For the comparison of Rho activity in different samples, the amount of RBD-bound Rho was normalized to the total amount of Rho in cell lysates. For the comparison of Rac and Cdc42 activity in different samples, the amount of PBD-bound Rac and Cdc42 were normalized to the total amount of Rac and Cdc42 in cell lysates.

RESULTS

Depletion of TRPM7 alters cell morphology

To better understand the mechanism by which TRPM7 controls cell motility we created two TRPM7-knockdown fibroblast cell lines (M7shRNA2 & M7shRNA6) by stably reducing TRPM7 expression in Swiss 3T3 fibroblasts using two independent and previously characterized shRNA sequences that specifically target the channel-kinase (shRNA2 & 6) [5]. A non-silencing shRNA was employed to create a control cell line (shRNA-C). Expression of each independent shRNA reduced TRPM7 expression by 50 to 80 percent for the M7shRNA2 and M7shRNA6 lines, respectively (Supplementary Figure S1A).

Knockdown of TRPM7 produced fibroblasts with a constricted cell morphology characterized by exaggerated membrane extensions, whereas wildtype Swiss 3T3 (WT) and shRNA-C fibroblasts displayed a normal cell shape (Figures 1A and 1B, and see Supplementary Figure S2 for larger images of the cells). Since TRPM7 was significantly more reduced in M7shRNA6 cells and because these fibroblasts had a more severe change in cell morphology, we chose this line for further analysis. To evaluate whether re-expression of TRPM7 was able to restore the morphology of M7shRNA6 cells to a shape similar to that of WT fibroblasts, we created a recombinant adenovirus expressing a “silencing-resistant” version of TRPM7 (SR-TRPM7) engineered to evade silencing by RNA interference, without altering the channel-kinase's amino acid sequence (Supplementary Figures S1B and S1C). Transduction of M7shRNA6 cells with the SR-TRPM7 recombinant adenovirus caused M7shRNA6 cells to recover a spread morphology similar to that of the WT and shRNA-C fibroblast cell lines (Figures 1A and 1B, and Supplementary Figure S2). Interestingly, we found that the kinase activity of TRPM7 was not required for the reversal in cell morphology, as transduction of M7shRNA6 fibroblasts with the kinase-inactive SR-TRPM7-G1618D was similarly efficient in restoring M7shRNA6 cell morphology to a normal cell shape (Figures 1A and 1B, and Supplementary Figure S2). As a negative control, we observed that adenoviral transduction of M7shRNA6 cells with LacZ failed to change M7shRNA6 cell morphology (Figures 1A and 1B, and Supplementary Figure S2). These results demonstrate that the difference in cell shape between WT and M7shRNA6 fibroblasts is caused by the depletion of TRPM7 and that

TRPM7's kinase activity appears to be dispensable to reverse M7shRNA6 cell morphology back to a normal cell shape.

Expression of the Mg^{2+} transporter SLC41A2 rescues M7shRNA6 cell morphology

Given that supplementation of DT40 B cells with elevated extracellular Mg^{2+} and expression of the Mg^{2+} transporter SLC41A2 reversed the cell arrest caused by deletion of the *TRPM7* gene [2, 3], we examined whether supplementation of the growth media with elevated Mg^{2+} and expression of the Mg^{2+} transporter SLC41A2 were sufficient to cause M7shRNA6 cells to revert to its original shape. Elevation of extracellular Mg^{2+} levels to 10 mM failed to change M7shRNA6 cell morphology (Figures 1A and 1B, and Supplementary Figure S2). In addition, increasing extracellular Ca^{2+} to 5 mM also did not affect TRPM7 knockdown cell shape (Figures 1A and 1B, and Supplementary Figure S2). However, transduction of the Mg^{2+} transporter SLC41A2 into M7shRNA6 fibroblasts was able to restore M7shRNA6 cell morphology to a shape similar to that of WT and shRNA-C cell lines (Figures 1A and 1B, and Supplementary Figure S2). Functional characterization of SLC41A2's transport properties in *Xenopus laevis* oocytes has shown that the transporter selectively transports in rank order, Mg^{2+} , Ba^{2+} , Ni^{2+} , Co^{2+} , Fe^{2+} , and Mn^{2+} but not Zn^{2+} , Cu^{2+} , or Ca^{2+} [14]. Consistent with this selectivity profile, the addition of supplemental Mg^{2+} with SLC41A2 at a multiplicity of infection (MOI) five times lower than the titer that is normally effective when SLC41A2 is used alone, permits reversal of M7shRNA6 cell morphology to a WT shape. Combining supplemental Ca^{2+} with SLC41A2 at the same low MOI, however, was ineffective in producing a reversal in cell shape (Figures 2A and 2B, and see Supplementary Figure S3 for larger images of the cells). These results confirm that Mg^{2+} is required to restore the change in cell morphology caused by depletion of TRPM7 in fibroblasts.

Depletion of TRPM7 disrupts the actomyosin cytoskeleton and focal adhesions

As the cytoskeleton directly affects cell shape, we examined by confocal microscopy how knockdown of TRPM7 affected the cellular distribution of actin and myosin IIA, key components of the actomyosin cytoskeleton, as well as vinculin, a marker of focal adhesions. Phalloidin was employed to stain actin fibers and primary antibodies against myosin IIA and vinculin were used to visualize these proteins' localization by immunocytochemistry. In contrast to WT and shRNA-C fibroblasts, which displayed prominent actin stress fibers and myosin IIA staining with large focal adhesions distributed medially around the cells, reducing TRPM7 expression in fibroblasts decreased the amount of actin stress fibers while it increased cortical actin staining and the cortical localization of myosin IIA (Figure 3, see Supplementary Figure S4A for quantification of the results). In addition, vinculin was diffusely distributed in TRPM7-knockdown cells, with small weakly stained clusters of the protein at the cell's periphery, indicative of a disruption of focal adhesions. Re-expression of SR-TRPM7 and expression of the Mg^{2+} transporter SLC41A2 restored the distribution of actin, myosin IIA, and vinculin to a pattern similar to that found in WT and shRNA-C cells (Figure 3, see Supplementary Figures S4A and S4B for quantification of the results). Surprisingly, re-expression of the kinase-inactive SR-TRPM7-G1618D was just as efficient in restoring the distribution of these cytoskeletal structures, despite the fact that a previous study implicated TRPM7's kinase in the regulation of actomyosin contractility, with myosin IIA reported to be its substrate [4]. As expected, expression of lacZ and supplementation of the growth media with 10 mM Mg^{2+} were unable to restore the cytoskeleton and focal adhesions to a normal distribution (Figure 3 and see Supplementary Figures S4A and S4B for quantification of the results). Together, these data indicate that the change in cell morphology caused by depletion of TRPM7 was likely due to a collapse of the cytoskeleton and focal adhesions caused by the reduction of TRPM7

channel activity, however, the mechanism(s) by which depletion of TRPM7 produced these changes in cell morphology and the cytoskeleton still remained unclear.

TRPM7 is required for polarized cell movements

The Rho family GTPases, Rho, Rac and Cdc42 control actomyosin contractility, lamellipodial formation, as well as establishment of cell polarity for directional cell movements, respectively. This prompted us to investigate whether the regulation of these proteins was altered in response to depletion of TRPM7 in migrating cells. To simulate conditions for directed cell migration we employed a cellular wound healing assay in which a “wound” is created in a monolayer of cells by manually scratching with a pipet tip. After wounding, cells facing the wound polarize, orientate their Golgi apparatus toward the midline, and extend sheetlike membrane protrusions (lamellipodia) that directionally propel cells towards the midline of the wound region. A pulldown purification assay using the GST-RBD fusion protein (Rho binding domain fused to glutathione S-transferase) that recognizes GTP-bound Rho revealed diminished activation of Rho in TRPM7-knockdown cells in a cellular wound healing assay (Figures 4A and 4B). Strikingly, an assay employing the GST-PBD fusion protein (p21 binding domain fused to glutathione S-transferase), which recognizes GTP-bound Rac, demonstrated a nearly complete inability of M7shRNA6 cells to activate Rac during cell migration under the same conditions (Figures 4A and 4B). The cellular wound assays also revealed that nearly all of the WT and shRNA-C control cells exhibited broad lamellipodia (Figure 4C, see Supplementary Figure S5 for larger images of the cells, and Supplementary Videos 1 and 2), whereas TRPM7-knockdown cells showed a deficiency in their ability to form lamellipodia, and instead appeared to use pseudopodia-like protrusive membrane structures to migrate into the wound region (Figure 4C, Supplementary Figure S5, and Supplementary Video 3). Quantification of membrane protrusion in the wound healing assay revealed that 35 % of the M7shRNA6 cells migrated using lamellipodia and that 65 % of cells migrated using a single pseudopodium or two pseudopodia. By comparison, nearly 100% of WT and shRNA-C controls cells migrated using lamellipodia (Figure 4D). Re-expression of SR-TRPM7 and expression of the Mg^{2+} transporter SLC41A2, but not LacZ, restored M7shRNA6 cells’ ability to form lamellipodia, indicating Mg^{2+} is required for this process (Figures 4C and 4D, Supplementary Figure S5, and Supplementary Videos 4-6).

Our pulldown purification assay also revealed a loss of the capacity of TRPM7-knockdown fibroblasts to regulate the activity of Cdc42, which controls the ability of cells to polarize and migrate directionally (Figures 5A and 5B). Compared to control cells, levels of activated Cdc42 were higher just after wounding and failed to increase after 10 minutes. Consistent with this finding, TRPM7-knockdown cells did not migrate directionally towards the midline of the cellular wound region, whereas nearly all WT and shRNA-C control cells migrated straight into the wound without turning (Figures 5C and 5D, and Supplementary Videos 1-3). Analysis of the trajectories of cell migration in the cellular wound assay revealed that 60 % of the TRPM7-knockdown cells did not migrate directionally into the wound region, indicating a defect in cell polarity (Figure 5D). Rather the TRPM7-knockdown cells migrated forward and turned, or reversed direction altogether. The expression of TRPM7 and Mg^{2+} transporter SLC41A2, but not LacZ, was able to partially reverse this trend (Figures 5C and 5D, and Supplementary Videos 4-6). In addition, an analysis of the ability of TRPM7-knockdown cells to orient their Golgi apparatus to face towards the wound region, a hallmark of the polarization of migrating cells, revealed a defect in Golgi reorientation when compared to WT and shRNA-C cells (Figures 5E and 5F). The loss of wound-induced Golgi reorientation was partially restored by re-expression of SR-TRPM7 and expression of the Mg^{2+} transporter SLC41A2 (Figures 5E and 5F). As expected, expression of LacZ did not increase wound-induced Golgi reorientation (Figures

5E and 5F). The above results clearly demonstrate that depletion of TRPM7 interferes with the signaling pathways required for lamellipodial formation, cell polarization and directional cell migration.

DISCUSSION

TRPM7 has been implicated in a number of physiological processes, including magnesium homeostasis, cell adhesion and motility, melanophore formation in zebrafish, and thymopoiesis [2, 4-6, 9, 15-18]. In addition, the channel-kinase has been revealed to be essential for the demise of neurons subjected to oxygen-glucose deprivation [19, 20]. The channel's inward current is divalent selective, allowing Mg^{2+} and Ca^{2+} as well as other divalent cations to pass through its pore [21]. Consequently, TRPM7's ability to permeate Mg^{2+} has linked the channel to the regulation of magnesium homeostasis. Whereas its ushering of Ca^{2+} into the cell appears to be controlling other functions to which the channel-kinase has been associated, including cell death during ischemia and cell migration [4-6, 9].

Ca^{2+} is vital to the control of cell migration, targeting numerous Ca^{2+} -dependent proteins to influence actin and focal adhesion remodeling among others processes. Unlike Ca^{2+} , Mg^{2+} has not been shown to play a vital role in cell motility and is generally not thought to play an active role in controlling the cytoskeleton and cell migration. The concentration of cytosolic Ca^{2+} is tightly regulated, varying from values of 10-100 nM in quiescent cells to high nanomolar/low micromolar concentrations in response to an array of different signaling events. By contrast, the concentration of free Mg^{2+} , the second most abundant cation within the cell after K^+ , varies within the submillimolar range, differing from cell type to cell type [22]. Mg^{2+} is an essential cofactor for the activity of numerous enzymes, participating in the regulation of basic biochemical activities ranging from nucleic acid metabolism and chromatin organization, to protein synthesis, to metabolic reactions involved in energy production within the cytoplasm or mitochondria [22]. However, relatively few reports have suggested a role for Mg^{2+} in the regulation of cell motility, despite the fact that more than 300 enzymes have been reported to be directly or indirectly regulated by Mg^{2+} [22]. Microinjection of Mg^{2+} into the cell to directly increase intracellular free Mg^{2+} by approximately 10 fold disrupts F-actin stress fibers [23]. Conversely, culturing endothelial cells in low Mg^{2+} has been shown to inhibit cell migration, which may be due in part to the down-regulated expression of a number of different genes, including c-src and zyxin [24]. How then are TRPM7 and Mg^{2+} controlling the cytoskeleton and cell polarity? Here we've shown that expression of the Mg^{2+} transporter SLC41A2 can reverse the change in cell morphology, disruption of the cytoskeleton, and the impairment of directional cell migration caused by depletion of TRPM7, indicating that Mg^{2+} is required for TRPM7's control of polarized cell movements. Strikingly, knockdown of TRPM7 interferes with the regulation of Rac and Cdc42 during directed cell migration. While Mg^{2+} is not required for high-affinity nucleotide binding to these GTPases, it acts as a "gatekeeper" to regulate nucleotide binding kinetics [25]. *In vivo*, the release of GDP from Rho proteins would be antagonized by intracellular free Mg^{2+} . Thus, a decrease in intracellular free Mg^{2+} could potentially interfere with the normal regulation of Rac and Cdc42 by their respective guanine nucleotide exchange factors. In addition, changes in intracellular free Mg^{2+} may alter expression of other proteins involved in the regulation of the cytoskeleton and cell polarity. Many kinases and phosphatases employ Mg^{2+} as a cofactor and could also be affected by a change in cellular Mg^{2+} status. Although the precise mechanism by which Mg^{2+} affects polarized cell movements is not yet clear, our results indicate that the TRPM7's permeation of the divalent cation is playing a critical role in the modulation of Rac and Cdc42 activity during directed cell migration.

Nevertheless, TRPM7 has also been implicated in the regulation of Ca^{2+} flickers [9]. Knockdown of TRPM7 decreased the frequency of Ca^{2+} flickers elicited by PDGF and impaired turning of fibroblasts in response to this growth factor. Therefore, permeation of both Mg^{2+} and Ca^{2+} through the channel may each be critical to the ability of fibroblasts to execute directional cell movements. Consistent with this premise that TRPM7 utilizes both Ca^{2+} and Mg^{2+} , we've previously shown that permeation of either of the two divalent cations by TRPM7 is sufficient to produce a loss of cell adhesion when the channel is overexpressed in HEK-293 cells [6]. Additional studies are required to tease apart the relative role that TRPM7-dependent permeation of these divalent cations have on polarized cell movements.

Finally, despite TRPM7 kinase's reported role in controlling actomyosin contractility by phosphorylation of myosin IIA [4], re-expression of the kinase-inactive TRPM7-G1618D was by itself successful in restoring the disruption of cell morphology and of the cytoskeleton caused by reduced TRPM7 expression. Clark and colleagues had previously showed that TRPM7 physically associates with myosin IIA in a Ca^{2+} -dependent manner and that mild overexpression of TRPM7 in N1E-115 neuroblastoma cells stimulates the phosphorylation of myosin IIA, causing relaxation of the actomyosin cytoskeleton [4]. An *in vitro* kinase assay using a myosin tail fragment expressed as a GST-fusion protein in *Escherichia coli* as a substrate, demonstrated that immunoaffinity-purified wildtype, but not kinase-dead TRPM7, efficiently phosphorylated recombinant myosin IIA on Thr1800, Ser1803 and Ser1808. Mutation of these sites to alanine and aspartate respectively, led to an increase and a decrease in myosin IIA incorporation into the actomyosin cytoskeleton [26]. The association between TRPM7 and the myosin IIA heavy chain required an active kinase domain [4], which lead Clark and colleagues to propose a compelling model in which bradykinin-stimulated Ca^{2+} influx mediated by TRPM7 instigates massive autophosphorylation of the channel-kinase that is required for the association and subsequent phosphorylation of myosin IIA, promoting local relaxation of the actomyosin cytoskeleton [8]. However, we found that the disruption of actin filaments and myosin fibers as well as the decrease in the number of focal adhesions produced by depletion of TRPM7 could be completely reversed by the expression of the kinase-inactive TRPM7-G1618D as well as by the Mg^{2+} transporter SLC41A2. One possibility is that TRPM7's kinase may be regulating a small fraction of myosin within the immediate vicinity of the channel that was too low to be detected in our experiments. Alternatively, TRPM7's regulation of myosin IIA may be unimportant in fibroblasts. Nevertheless, our experiments clearly indicate that TRPM7's kinase is not required to control global actomyosin contractility in this cell type and that it may have a more limited role in controlling actomyosin contractility than previously thought. Rather it is the channel, acting through Rac and Cdc42, that is central to TRPM7's control of the cytoskeleton during polarized cell movements, with our results uncovering a previously undefined, yet critical role for the divalent cation Mg^{2+} in this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GST	glutathione S-transferase
MOI	multiplicity of infection
HEK-293	human embryonic kidney 293 cells
PBD	p21 binding domain
PDGF	platelet-derived growth factor
RBD	Rho binding domain
shRNA	short hairpin RNA
SLC41A2	solute carrier family 41 member 2
TRPM7	transient receptor potential melastatin 7

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Figure 1. Depletion of TRPM7 disrupts cell morphology

(A) Stable knockdown of TRPM7 in Swiss 3T3 fibroblasts (M7shRNA2 & M7shRNA6) by RNA interference produced cells with a constricted morphology with long membrane extensions (see arrows) compared to wildtype (WT) cells or fibroblasts expressing a non-silencing shRNA (shRNA-C). Adenoviral-mediated re-expression of SR-TRPM7 and the kinase-inactive SR-TRPM7-G1618D in TRPM7 knockdown M7shRNA6 cells restored cell morphology. Expression of the Mg^{2+} transporter SLC41A2 in M7shRNA6 cells also rescued the changes in cell shape caused by depletion of TRPM7, whereas supplementation of the growth media with excess Mg^{2+} (10 mM) and Ca^{2+} (5 mM) was ineffective. Bar, 100 μm . (B) Quantification of the change in cell morphology by measurement of cell surface area. Box-and-Whisker plots describe the distribution of cell surface area. Shown are the mean (square), 25th percentile (bottom line), median (middle line), 75th percentile (top line), 75th percentile + 1.5 interquartile range (upper whisker), the minimum measurement (lower

whisker) and the maximum measurement ($\times$). The asterisk indicates significant difference in cell surface area compared to WT, using Student *t* test ($P < 0.05$). For each condition, a minimum of 150 cells from three experiments was examined.

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Figure 2. Expression of SLC41A2 augments cell shape change by supplemental Mg²⁺
(A) Transduction of M7shRNA6 cells with the Mg²⁺ transporter SLC41A2 restored the morphology of M7shRNA6 cells to a shape similar to fibroblasts expressing a non-silencing shRNA (shRNA-C), whereas transduction using a multiplicity of infection (MOI) that is 5-times lower was significantly less effective (low MOI). Increasing the concentration of Mg²⁺ in the growth media to 10 mM increased the ability of transduction of SLC41A2 at low MOI to rescue cell morphology, indicating the expression of a Mg²⁺ transporter in Swiss 3T3 fibroblasts was required for supplemental Mg²⁺ to reverse the change in cell morphology caused by depletion of TRPM7. Bar, 100 μ m. NT (non-treated), cells grown in media with a normal concentration of Mg²⁺ (0.95 mM) and Ca²⁺ (2.14 mM). **(B)** Quantification of the change in cell morphology by measurement of cell surface area. Box-and-Whisker plots describe the distribution of cell surface area. Shown are the mean (squares), 25th percentile (bottom line), median (middle line), 75th percentile (top line), 75th percentile + 1.5 interquartile range (upper whisker), the minimum measurement (lower whisker) and the maximum measurement ($\times$). The asterisk indicates significant difference in

cell surface area compared to WT, using Student *t* test ($P < 0.05$). For each condition a minimum of 150 cells from three experiments was examined.

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Figure 3. TRPM7 is required for cytoskeleton and focal adhesion formation

(A) Representative confocal images of actin staining of WT, shRNA-C, and M7shRNA cells cultured in media with normal concentration of Mg²⁺. Shown also are M7shRNA6 cells cultured in 10 mM Mg²⁺ and M7shRNA6 cells transduced with recombinant adenoviruses expressing LacZ, SR-TRPM7, the kinase-inactive SR-TRPM7-G1618D, and the Mg²⁺ transporter SLC41A2. A minimum of 80 cells for each condition was examined from two experiments. (B) Representative confocal images of myosin IIA staining. (C) Representative confocal images of vinculin staining. Bar, 100 μ m. See Supplementary Figures S4A and S4B for quantification of the results.

Figure 4. TRPM7 is required for lamellipodial formation

(A) Western blot analyses of levels of activated Rho and Rac, and the total amount of each protein in cell lysates during cellular wound assay at 0 and 10 minutes. Knockdown of TRPM7 decreased activation of Rho and Rac. (B) Quantification of GTP-bound protein was normalized to the total amount of each protein. Results are presented as fold changes compared to shRNA-C cells at 0 minute during the cellular wound assay. Values are presented as mean $\pm$ standard deviation of at least three experiments. The asterisk indicates that the change in Rho activity ($P = 0.0235$) and the change in Rac activity ($P = 0.0073$) in M7shRNA6 cells from 0 to 10 minutes compared to shRNA-C cells, using Student t test ($P < 0.05$), are significantly different. (C) Representative images of membrane protrusions at the wound edge 4 hours after wounding. Arrows indicate broad lamellipodium, whereas arrowheads indicate pseudopodium. Bar, 100 μ m. (D) Quantification of the percentage of cells that migrated using lamellipodia versus those that migrated with pseudopodia at the wound edge in the cellular wound healing assay. Approximately 150 cells for each condition were examined. Values are presented as mean $\pm$ standard deviation from three experiments. The asterisk indicates that the percentage of cells moving with lamellipodia for each condition examined when compared to M7shRNA6 cells, using Student t test ($P < 0.05$), is significantly different.

Figure 5. TRPM7 is required for cell polarization and directed cell migration

(A) Western blot analyses of levels of GTP-Cdc42, and of the total amount of Cdc42 during cellular wound assay at 0 and 10 minutes. Knockdown of TRPM7 altered the levels of activated Cdc42, with the amount of activated Cdc42 in knockdown cells being abnormally high at 0 minute, compared to control cells. Whereas the activity of Cdc42 in control cells increased from 0 to 10 minutes following wound formation, levels of activated Cdc42 in TRPM7 knockdown cells did not increase under the same conditions. (B) Quantification of GTP-Cdc42 was normalized to the total amount of Cdc42 protein. Results are presented as fold changes compared to shRNA-C cells at 0 minute. Values are presented as mean $\pm$

standard deviation of four experiments. The change in Cdc42 activity ($P = 0.0267$) in M7shRNA6 cells from 0 to 10 minutes compared to shRNA-C cells, using Student t test ($P < 0.05$), is significantly different. **(C)** Representative examples of the migratory paths of individual cells in the cellular wound assay. The migratory path of an individual cell from the wound edge over 6 hours is shown as a black path, with the image from the initiation of imaging at 0 minute overlayed. **(D)** Quantification of the percentage of cells that move directionally into the wound region towards the midline versus those that moved non-directionally into the wound region towards the midline. Cells that moved “non-directionally” were defined as cells that turned or reversed direction on their course towards the midline. Approximately 150 cells for each condition were examined. Values are presented as mean $\pm$ standard deviation from three experiments. The asterisk indicates that the percentage of cells that migrate directionally for each condition examined when compared to M7shRNA6 cells, using Student t test ($P < 0.05$), is significantly different. **(E)** Golgi reorientation. Cells were fixed and stained with β -COP antibody to show the distribution of the Golgi apparatus 6 hours after wounding. The arrow indicates the direction of the wound. Bar, 100 μ m. **(F)** The percentage of cells, having Golgi apparatus in the forward-facing 120 degree sector, was measured. Values are presented as mean $\pm$ standard deviation of at least three experiments. A minimum of 150 cells for each condition was examined. The asterisk indicates significant difference in the percentage of polarized Golgi apparatus compared to WT cells, using Student t test ($P < 0.01$). Bar, 100 μ m.