TRPM7 senses oxidative stress to release Zn\(^{2+}\) from unique intracellular vesicles

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Contributed by David E. Clapham, June 16, 2017 (sent for review May 3, 2017; reviewed by Michael D. Cahalan, Dejian Ren, and Haoxing Xu)

TRPM7 (transient receptor potential cation channel subfamily M member 7) regulates gene expression and stress-induced cytotoxicity and is required in early embryogenesis through organ development. Here, we show that the majority of TRPM7 is localized in abundant intracellular vesicles. These vesicles (M7Vs) are distinct from endosomes, lysosomes, and other familiar vesicles or organelles. M7Vs accumulate Zn\(^{2+}\) in a glutathione-enriched, reduced lumen when cytosolic Zn\(^{2+}\) concentrations are elevated. Treatments that increase reactive oxygen species (ROS) trigger TRPM7-dependent Zn\(^{2+}\) release from the vesicles, whereas reduced glutathione prevents TRPM7-dependent cytosolic Zn\(^{2+}\) influx. These observations strongly support the notion that ROS-mediated TRPM7 activation releases Zn\(^{2+}\) from intracellular vesicles after Zn\(^{2+}\) overload. Like the endoplasmic reticulum, these vesicles are a distributed system for divalent cation uptake and release, but in this case the primary divalent ion is Zn\(^{2+}\) rather than Ca\(^{2+}\).

TRPM7 | zinc | vesicles

TRPM7 (transient receptor potential cation channel subfamily M member 7), an ion channel and cytoplasmic kinase, is ubiquitously expressed and essential in early embryonic development (1–4) but also may mediate oxidative stress-induced apoptosis in neurons (5, 6). As an ion channel, TRPM7 conducts Zn\(^{2+}\) > Mg\(^{2+}\) > Ca\(^{2+}\) and monovalent cations (7–10) and contributes to labile cytosolic and nuclear Zn\(^{2+}\) concentrations (8). TRPM7’s C-terminal kinase can phosphorylate multiple substrates (11–13) and is cleaved to release a proapoptotic, chromatin-modifying enzyme (8, 14). Zn\(^{2+}\) regulates TRPM7’s kinase activity (11) and binding to transcription factors (8). It is a potent signal for many cellular processes previously related to TRPM7 function, including gene expression, mitosis, and cell survival, but is also proapoptotic at extreme concentrations (15–18).

TRPM7 has been proposed to regulate intracellular Mg\(^{2+}\) levels (19). However, as we have shown, Trpm7\(^{-/-}\) cells have normal total magnesium concentrations (3, 14), with the many abundant magnesium transporters (20) probably accounting for normal Mg\(^{2+}\) homeostasis. Under physiological conditions, plasma membrane TRPM7’s inward conductance is miniscule (<10 pA/pF at <100 mV), but it increases over minutes if the free cytosolic Mg\(^{2+}\) concentration falls below its normal level of ∼0.5–1 mM (21). Because in most cells the primary buffers for Mg\(^{2+}\) are phosphonucleotides (22), the metabolic state potentiates TRPM7 activity as ATP levels rise and fall around –10 mV, with 1 mM Ca\(^{2+}\) and 100 μM Zn\(^{2+}\) (23). TRPM7’s Zn\(^{2+}\) conductance suggests that it could support rapid changes in cytoplasmic Zn\(^{2+}\) levels under the right circumstances, either across the plasma membrane or from intracellular compartments.

Oxidative stress increases inward divalent ion permeation through TRPM7 (5, 9), whereas acute and chronic oxidative stress in culture (24–26) or during ischemia–reperfusion in vivo (27, 28) induces TRPM7 expression. The resulting increase in cytosolic Zn\(^{2+}\) could result in toxic cytosolic concentrations (9). Conversely, reduction of TRPM7 expression protects animals from posts ischemia reperfusion (5), a condition typically accompanied by increased reactive oxygen species (ROS) and Zn\(^{2+}\) release from intracellular stores (29). Physiological redox transitions required for stem cell differentiation and gene expression during embryonic development (30, 31) may also be sensed by TRPM7 and transduced via Zn\(^{2+}\)-dependent signals (32).

Because TRPM7 patch-clamp recording is limited to the plasma membrane, the assumption has been that TRPM7’s main function is on the plasma membrane. However, we have previously shown that TRPM7 is also on intracellular membranes (33–35). Indeed, ROS activation of divalent ion influx through TRPM7 (5, 9) is reminiscent of ROS activation of TRPM2, which releases Ca\(^{2+}\) and Zn\(^{2+}\) from lysosomes (36, 37). Here we show that TRPM7 is an intracellular Zn\(^{2+}\) release channel located on previously

Significance

TRPM7 (transient receptor potential cation channel subfamily M member 7) is required for normal organ development but also mediates anoxic neuronal death. TRPM7 contains a channel that conducts cations into the cytosol and C-terminal kinase that can phosphorylate multiple substrates. The kinase is cleaved to regulate gene expression and apoptosis. The link between TRPM7’s channel and its organisational function remains the least understood aspect of TRPM7. Here, we identify intracellular Zn\(^{2+}\) storage vesicles that contain the majority of TRPM7 protein. TRPM7 senses reactive oxygen species (ROS) to release Zn\(^{2+}\) from these vesicles. Just as the endoplasmic reticulum sequesters and releases Ca\(^{2+}\), we propose that these vesicles fulfill a similar function for Zn\(^{2+}\) and that TRPM7 coordinates fluctuations in cellular [Zn\(^{2+}\)] and ROS during development and injury.


Reviewers: M.D.C., University of California, Irvine; D.R., University of Pennsylvania; and H.K., University of Michigan. The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1707380114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1707380114

PNAS | Published online July 10, 2017 | E6079–E6088
uncharacterized vesicles (M7Vs) that are distinct from familiar organelles. These vesicles sequester Zn$^{2+}$ during cytosolic overload. We show that the intravesicular M7V environment is reducing and that oxidation induces TRPM7-dependent Zn$^{2+}$ release from these vesicles. We hypothesize that TRPM7-mediated Zn$^{2+}$ release from intracellular stores regulates ROS signaling events during embryonic development or postnatal stress/injury.

**Results**

**TRPM7 Is Most Abundant on Intracellular Vesicles.** Whole-cell patch-clamp measurements of endogenous and ectopically expressed TRPM7 establish that it is present in the plasma membrane (1, 2). As with many ion channels that are relatively sparse, ectopically expressed TRPM7 was undetected at the plasma membrane by immunocytochemistry (Fig. 1A). Instead TRPM7 was detected largely in intracellular vesicles (Fig. 1A–E) in multiple cell types. To rule out TRPM7 mislocalization artifacts caused by ectopic overexpression, we used a gene-trap strategy to insert GFP at the N terminus of endogenous TRPM7 in mouse ES cells. Expression of full-length GFP-TRPM7 was confirmed by immunoprecipitation of TRPM7 using either anti-GFP or anti-TRPM7, followed by Western blotting (Fig. S1A). Endogenous GFP-TRPM7, visualized by immunostaining with anti-GFP antibody, was abundant in intracellular vesicles similar in size and distribution to TRPM7-positive vesicles in cells ectopically expressing TRPM7 (Fig. 1F). We also coimmunostained cells for GFP (TRPM7) and β-actin, which lines the inner leaflet of plasma membrane (38), and imaged the localization of both proteins by stimulated emission depletion (STED) microscopy. β-Actin was concentrated at the cell periphery in a punctate distribution at the plasma membrane, whereas TRPM7 was detected in ~100-nm vesicles located only on the cytoplasmic side relative to β-actin (Fig. 1B).

To determine the intracellular localization of TRPM7 in detail, we carried out transmission electron microscopy (TEM) of anti-GFP–labeled HEK293 cells expressing TRPM7 with a pH-sensitive GFP (pHluorin) (39) inserted into the S1/2 loop. Insertion of tags into the S1/2 loop did not compromise TRPM7 channel function (Fig. S2). TEM revealed unilamellar vesicles with an average diameter of ~150 nm specifically labeled with anti-GFP (Fig. 1C and Fig. S1B and C). The luminal anti-GFP labeling demonstrates the expected membrane orientation of TRPM7 polypeptide with the S1/2 loop in the lumen and the N and C termini in the cytosol (Fig. 1C). Imaging of live cells...
expressing the pHluorin-tagged TRPM7 established that the vesicles are acidic (Fig. S1D). Total internal reflection (TIRF) microscopy of GFP-tagged TRPM7 in live SY40MES-13 cells also revealed TRPM7 localization in vesicles (Movie S1). Thus, under all conditions examined, TRPM7 channels are located primarily on abundant intracellular vesicles with comparatively fewer numbers on the plasma membrane.

**M7Vs Are Distinct from Well-Characterized, Membrane-Bound Intracellular Compartments.** To determine whether M7Vs are simply in route to the plasma membrane via classical secretory pathways, we assessed the colocalization of TRPM7 and GFP-tagged vesicular stomatitis virus protein (VSVG-GFP) (40). TRPM7 did not colocalize with VSVG-GFP in transport vesicles in the cytoplasm or at the plasma membrane (Fig. 2A). Moreover, the vesicles did not colocalize with markers of several known cellular compartments, including calnexin, D1ER, Sec61β (endoplasmic reticulum; ER), PEX19, RFP-PTS (immature and mature peroxisomes), lamp1 (lysosomes), clathrin (endosomes), edem1 [EDEMosomes, ER-derived ERAD-tuning vesicles (41)], ergic-53 (ER–Golgi intermediate compartments), and tyrosinase (melanosomes) (Fig. 2B). M7Vs are also smaller and more abundant than zincoxones, ∼1-µm-wide vesicles that fluorescence brightly upon cellular Zn²⁺ overload (42). From their morphology, lack of markers, and lack of fusion with the plasma membrane by various stimuli (34), we surmise that M7Vs are previously uncharacterized intracellular membrane compartments.

**Purification of M7Vs.** Sucrose gradient fractionation confirms that endogenous TRPM7 is predominantly intracellular because it migrates as a peak distinct from the plasma membrane marker Na⁺/K⁺ ATPase (Fig. 3A). To probe the cellular function of TRPM7 vesicles, we isolated TRPM7-containing vesicles from postnuclear supernatants (PNS). We first tagged the C terminus of TRPM7 with an HA epitope and pulled down M7Vs with anti-HA–conjugated magnetic nanoparticles. The pulldown appears specific, because excess HA peptide blocked vesicle binding, the vesicles colocalized with nanoparticles, and the vesicle preparation was depleted of contaminating mitochondrial and endoplasmic reticulum markers (Fig. 3). We performed a second affinity purification of vesicles by N-terminal FLAG tag after elution of anti-HA–bound vesicles for proteomic analysis (Fig. S3A). The affinity binding of the vesicles via tags located on the N and C terminals supports our earlier conclusion that the termini of TRPM7 face the cytoplasm. The substantial reduction of ER and mitochondrial morphology, lack of markers, and lack of fusion with the plasma membrane by various stimuli (34), we surmise that M7Vs are previously uncharacterized intracellular membrane compartments.

**The M7V Proteome.** TRPM7 was enriched in the vesicle proteome, with a SILAC (stable isotope labeling with amino acids in cell culture) ratio of 65 in HEK293 cells and 98 in SV40MES-13 cells (Dataset S1). Proteins in known cellular compartments such as mitochondria, Golgi, and ER were present in the vesicle proteome, but ∼60% of the proteome was not classified in the Panther System (Dataset S1). We then screened the unclassified candidate proteins as potential markers for M7Vs and observed colocalization of M7Vs and vesicular integral membrane protein-36 (VIP36, also known as “LMAN2”) (Fig. S3B). VIP36 is an intracellular lectin of unknown function and has been observed in the ER–Golgi intermediate compartment and post-Golgi vesicles (43–45). VIP36 contains a single transmembrane domain with a luminal N terminus (46) that is ideal for targeting sensors into M7Vs (see below).

**Purified M7Vs Accumulate Zn²⁺.** TRPM7’s extracellular domains face the lumen of the M7V (Figs. 1C and 3 B–D), and thus inward cationic current should flow from the vesicular lumen into the cytoplasm under ionic conditions identical to those at the plasma membrane. Purified M7Vs immobilized on Cell-Tak–coated coverslips were loaded with the fluorescent divalent indicators Fluo-4-AM (Kᵦ, Ca²⁺ = 335 nM), MagFluo-4-AM (Kᵦ, Mg²⁺ = 4.7 mM), or FluoZin-3-AM (Kᵦ, Zn²⁺ = 15 nM) (Methods). These vesicles were permeant to Ca²⁺, Mg²⁺, or Zn²⁺ applied in the bath, but only Zn²⁺ remained trapped in the vesicles after extravesicular divalent ions were washed away (Fig. 4 A–D and Fig. S4 A and B). Accumulated intravesicular Zn²⁺ then was chelated with membrane-permeant TPEN [N,N,N’-tetakis(2-pyridyldimethyl) ethylenediamine], decreasing indicator fluorescence (Fig. 4 C and D). To rule out the possibility that Zn²⁺ was trapped by FluoZin-3, we loaded the vesicles with Zn²⁺ in the absence of FluoZin-3, washed out extravesicular Zn²⁺, and loaded the vesicles with FluoZin-3-AM. FluoZin-3 fluorescence increased to saturation over 10 min and remained at these levels after the extravesicular fluorophore was washed out (Fig. 4 E and F). These data show that purified M7Vs are capable of selectively accumulating Zn²⁺.

**M7Vs Accumulate Zn²⁺ in Intact Cells.** To study vesicular Zn²⁺ in intact cells, we targeted two genetically encoded Zn²⁺ sensors to the M7V lumen. We created an intravesicular Zn²⁺ sensor by targeting eCALWY4 (47) to the TRPM7 vesicle lumen by fusion of VIP36’s N-terminal 36 amino acids (including the signal peptide) to eCALWY4’s N terminus (Fig. 5B). Resistance to proteinase-K digestion indicated that the sensor was inside the vesicle (Fig. 5D). The intravesicular sensor did not respond to membrane-permeable TPEN but was responsive to increased luminal [Zn²⁺] (Fig. 5E), indicating low intravesicular free [Zn²⁺]. The Zn²⁺-binding constant of the vesicular VIP36-eCALWY4 sensor (0.36 ± 0.06 nM) was determined simultaneously with that of the nuclear Zn²⁺ sensor, ZapCmR2-NLS (48) (0.41 ± 07 nM), as a reference. Both binding constants were comparable to the Zn²⁺-binding constants of eCALWY4 (0.63 nM) (47) and Zap1 (0.81 nM) (49) (Fig. S5A). The calculation of Zn²⁺ concentrations using the binding constant suggests that the vesicles contain <0.1 nM free Zn²⁺ under resting conditions. To rule out any possible effects of vesicular pH on the sensor’s Zn²⁺ affinity, we showed that neutralization of the vesicular lumen with NH₄Cl did not increase FRET (Fig. S5B). In contrast to M7Vs, ER-localized Sec61β-eCALWY4 (Fig. 5C) was saturated with Zn²⁺ under resting conditions (Fig. 5F), consistent with previously reported storage of Zn²⁺ in the ER (50). Vesicular Zn²⁺ entry continued after influx was stopped by extracellular Zn²⁺ chelation with EGTA (Fig. 5H). We also targeted a recently developed intramembranous Zn²⁺ sensor, ZnGreen1 (51), to the M7V lumen by inserting it into the S1/2 intraluminal loop of TRPM7. M7Vs containing ZnGreen1 did not contain TPEN-chelatable Zn²⁺ (Fig. S5C) but took up Zn²⁺ after cytosolic Zn²⁺ elevation (Fig. 5F and Fig. S5D); cytosolic and M7V-luminal ZnGreen1 have similar Zn²⁺ equilibrium binding constants of 2.1–2.9 nM (Fig. S5 E and F). ZnGreen1-tagged TRPM7 channels were also functional (Fig. S2A).

To test whether vesicles in intact cells retain loaded Zn²⁺, we took advantage of the low Zn²⁺ affinity and wide dynamic range of ZnGreen1 (Fig. S5 E and F), enabling us to monitor changes in Zn²⁺ that otherwise would saturate eCALWY4 sensors (47). As shown in Fig. S5 D–F, elevation of extracellular [Zn²⁺] to 500 μM elevated cytosolic and M7V [Zn²⁺] to >40 nM, saturating ZnGreen1. When cells were allowed to recover for 15 min in culture medium at 37 °C (Fig. 5J), cytosolic ZnGreen1 fluorescence was no longer saturated, but the vesicular sensor remained saturated (Fig. 5 K and L). These results indicate that Zn²⁺ was enriched in M7Vs relative to the cytoplasm.

To examine whether divalent cation retention in M7Vs was selective for Zn²⁺, we also imaged Ca²⁺ uptake and release from...
M7Vs in situ using GCaMP6s (52). This probe was also inserted in the S1/2 loop of TRPM7 (Fig. S4C and D) and did not alter channel function (Fig. S2B). The addition of 100 μM ATP to intact cells triggered Ca$^{2+}$ spikes in M7Vs that were synchronous with ER Ca$^{2+}$ release (simultaneously monitored with the red fluorescent, ER-targeted Ca$^{2+}$ sensor, BCECF) (Fig. S4C) (53) or cytosolic spikes (simultaneously monitored with cytosolic red fluorescent R-GECO1) (Fig. S4D) (54). The equilibration of Ca$^{2+}$ between the cytosol and M7Vs is consistent with the lack of Ca$^{2+}$ trapping in vesicles in vitro (Fig. S4A) and supports our

Fig. 2. M7Vs are distinct from well-characterized intracellular vesicular compartments. (A) Immunocytochemistry of VSVG-GFP and TRPM7-HA vesicles in HEK293 cells. (B) Absence of colocalization of TRPM7-HA and markers of the ER (Calnexin), EDEMosomes (Edem1), the tyrosinase ER–Golgi intermediate compartment, peroxisomes (RFP-PTS and Pex19), lysosomes (Lamp1), melanosomes (Tyrosinase or Tyros), or clathrin-coated endosomes. See Table S1 for additional description of markers.
Percentage of oxidized roGFP in the cytosol or in mitochondria (Fig. 6).

TRPM7 tripled the rate of Zn$^{2+}$ measured using cytosolic eCALWY4 (Fig. 6).

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vesicles were affinity purified with anti-HA nanoparticles (Fig. S6).

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enriched sevenfold in M7Vs compared with total postnuclear mem-

ules: enrichment of vesicles and depletion of ER (Calnexin; Canx or PDI) and

ers were affinity purified with Alexa Fluor 546-conjugated

conjugated magnetic nanoparticles. Vesicles were visualized by incubation

B, Bound; NB. Not bound. See also Fig. S3.


to Calcium Green-1. Zn$^{2+}$ did not alter the binding of background Zn$^{2+}$ (~5 μM) or

were normalized to those in TPEN. Results are shown as the mean and SD from

 Peroxidation of M7Vs. (A) Gradient centrifugation of endogenous

were isolated using mouse anti-

traptin peptide. (C) Anti-HA Western blot: HA-peptide blocks nanoparticle binding to vesicles. (D) Western blot of isolated vesicles: enrichment of vesicles and depletion of ER (Calnexin; Canx or PDI) and mitochondria (cyt-C). B, Bound; NB. Not bound. See also Fig. S3A.

Reduced GSH Inhibits Zn$^{2+}$ Passage Through TRPM7. The observation of GSH enrichment in M7Vs motivated us to test whether GSH affects ion conductance through TRPM7. We measured plasma membrane TRPM7 because the extracellular medium can be altered readily to model the M7V lumen. First, Zn$^{2+}$ entry was measured using cytosolic eCALWY4 (Fig. 6D). Overexpression of TRPM7 tripled the rate of Zn$^{2+}$ influx from the extracellular medium, whereas a control, nonconducting TRPM7 pore mutant (33) did not enhance Zn$^{2+}$ influx (Fig. 6E and F). The addition of 1 mM GSH, but not glutathione disulfide (GSSG), blocked the TRPM7-dependent Zn$^{2+}$ influx (Fig. 6G and H) in a dose-dependent manner. To rule out the possibility that the GSH block was caused by Zn$^{2+}$ chelation, we assayed Zn$^{2+}$ binding to Calcium Green-1 ($K_d = 10 \mu M$ (59)). As shown in Fig. 6I, 1 mM GSH did not alter the binding of background Zn$^{2+}$ (~5 μM) or 50 μM Zn$^{2+}$ to Calcium Green-1. Zn$^{2+}$ not only permeates TRPM7 (Fig. 6E–H) but also blocks both inward and outward monovalent TRPM7 currents with an IC$_{50}$ of ~110 μM (Fig. S7 A–C). We conclude that GSH likely prevents Zn$^{2+}$ entry into the TRPM7 pore, an inference supported by patch-clamp experiments demonstrating that GSH, but not GSSG, enhances monovalent TRPM7 currents (Fig. S7E) even in the presence of Zn$^{2+}$ (Fig. S7E and F).

Oxidation Releases Zn$^{2+}$ from M7Vs in a TRPM7-Dependent Manner. GSH’s inhibition of TRPM7’s Zn$^{2+}$ conductance suggests that intravesicular reduced GSH enables Zn$^{2+}$ retention in vesicles and that changes in the intravesicular redox state, reflected in the degree of GSH oxidation (60), may regulate Zn$^{2+}$ release. To test oxidation-induced Zn$^{2+}$ release from M7Vs, we transfected WT and TRPM7$^{-/-}$ HEK293T cells with VIP36-eCALWY4, loaded the cells with Zn$^{2+}$ (as in Fig. 5), and monitored the increase in the mCitrine:mCerulean FRET ratio upon H$_2$O$_2$ treatment in Zn$^{2+}$-free extracellular medium. Exposure to 100 μM H$_2$O$_2$ resulted in a gradual increase of FRET (i.e., a reduction in Zn$^{2+}$) in the M7V lumen, which reached a plateau in ~30 min (Fig. 7A). The

earlier immunostaining observation that the vesicles are distinct from the ER. Thus, TRPM7 localizes to vesicles that selectively sequester Zn$^{2+}$ upon cytosolic Zn$^{2+}$ overload.

M7Vs Are Enriched in Glutathione. To search for clues about M7Vs’ function, we identified metabolites in isolated M7Vs by HPLC-MS. Only glutathione (GSH) was significantly enriched in M7Vs affinity-purified from HEK293 or HEK293T cells, compared with total postnuclear membranes (Dataset S2). We verified the GSH enrichment using a GSH luciferase assay in endogenous vesicles tagged with an HA-epitope inserted at the N terminus of endogenous TRPM7 (Methods). After HA-TRPM7 expression in the ES cells was

Methods

Intensity (a.u.)

Intensity (a.u.)

Intensity (a.u.)

Fig. 4. Zn$^{2+}$ accumulation in purified vesicles. (A and B) M7Vs isolated from SV40MES-13 cells were loaded with FluoZin-3-AM and imaged in medium containing EGTA (A) or 15 nM Zn$^{2+}$ (B). (C and D) Vesicles were perfused first with 15 nM Zn$^{2+}$ and then with 1.4 nM Zn$^{2+}$ or 50 μM TPEN. The mean, SD, and ANOVA statistics from four experiments are shown. *P < 0.001 compared with initial fluorescence; **P < 0.001 after TPEN addition. (E and F) Zn$^{2+}$ trapping in vesicles is not caused by fluorophore accumulation: Zn$^{2+}$-loaded vesicles were incubated with FluoZin-3-AM without Zn$^{2+}$; then extravesicular dye was washed off, and remaining vesicular fluorescence was reduced by Zn$^{2+}$ chelation by membrane-permeant TPEN but not membrane-impermeant EGTA. Fluorescence intensities in F were normalized to those in TPEN. Results are shown as the mean and SD from three experiments are shown; *P < 0.05; ANOVA. See also Fig. S5 A and B.
**Fig. 5.** Zn\(^{2+}\) sequestration by M7Vs in intact cells. (A) Colocalization of VIP36-V5 and TRPM7-HA in HEK293 cells. (B) Colocalization of immunostained VIP36-eCALWY4 intravesicular Zn\(^{2+}\) sensor and TRPM7-HA in HEK293 cells. (C) Lack of colocalization of ER-targeted eCALWY4 and TRPM7-HA. (D) ER lumen-targeted RCEPIA1er was coexpressed with cytosol-facing GFP (located on the N terminus of TRPM7) or VIP36-eCALWY4. Then the plasma membrane was permeabilized with 20 μM digitonin, and proteinase K was added to digest cytosolic fluorescent proteins. ER lumen-targeted RCEPIA1er was used as a proteinase K-inaccessible control. (E) HEK293 cells expressing VIP36-eCALWY4 were incubated with 50 μM TPEN or 50 μM Zn\(^{2+}\)/20 μM pyrithione. Shown are means and SEMs of seven cells in more than four representative time-course experiments. (F) eCALWY4 was targeted into the ER lumen using Sec61β, and cells were imaged in 50 μM TPEN or 50 μM Zn\(^{2+}\)/20 μM pyrithione. Shown are the mean and SEM of seven cells in a representative time-course experiment. (G) HEK293 cells were transfected with either VIP36-eCALWY4 or eCALWY4 cDNA and then were incubated with 500 μM Zn\(^{2+}\), followed by 50 μM Zn\(^{2+}\)/20 μM pyrithione and TPEN. Shown are time constants of Zn\(^{2+}\) entry and 95% Cs of 10–12 cells representative of multiple similar experiments; *P < 0.0001 after an extra sum of squares F-test comparison of the two curves. (H) Vesicular Zn\(^{2+}\) loading was initiated with 500 μM extracellular Zn\(^{2+}\), which then was replaced with 250 μM EGTA to chelate Zn\(^{2+}\). The mean and SEM of data from 19 cells from three experiments are plotted. (I) Cells expressing TRPM7 (ZnGreen1 inserted between the S1 and S2 domains of TRPM7) were perfused with Zn\(^{2+}\) and EGTA as in H. Plots show means and SEMs of cells from one experiment representative of more than three; axis breaks are 5 min. (J) The method for loading and releasing Zn\(^{2+}\) from M7Vs with luminal ZnGreen1. (K and L) After cytosolic and M7V-luminal ZnGreen1 were saturated with Zn\(^{2+}\) and cells were allowed to extrude cytosolic Zn\(^{2+}\) as described in J, residual cytosolic and vesicular Zn\(^{2+}\) were imaged in 50 μM HBT-A or 50 μM TPEN Zn\(^{2+}\)/20 μM pyrithione. *P < 0.05, ANOVA after Tukey’s multiple comparison test of 24–36 cells from three experiments. See also Figs. S2–S5 and Dataset S1.
FRET increase was substantially reduced in TRPM7−/− cells compared with WT cells (Fig. 7B and C), indicating that TRPM7 mediates oxidation-induced Zn2+ release from vesicles.

The initial reduction of the FRET ratio of the Zn2+-saturated sensor by H2O2 (Fig. 7A and B) raised the possibility that eCALWY was modified by oxidation. We therefore used Fluo-Zin-3 in place of genetically encoded protein sensors to monitor cytosolic Zn2+ in subsequent experiments, because previous studies have shown that FluoZin-3 responses to Zn2+ are not altered by H2O2 or ROS (61, 62). H2O2 increased Zn2+ (Fluo-Zin-3 fluorescence) in Zn2+-loaded WT cells but not in TRPM7−/− cells (Fig. 7D–F). Because the extracellular solution did not contain significant Zn2+ (<0.2 nM), we conclude that the source of the TRPM7-dependent, H2O2-induced increase in cytosolic Zn2+ was the M7V.

Finally, we tested whether endogenous ROS generation triggered by oxygen–glucose deprivation (OGD) (63, 64) elicits Zn2+ release into the cytosol through TRPM7. OGD with glucose-free sodium dithionite resulted in a wave of Zn2+ release, as recently reported (62), in WT but not in TRPM7−/− HEK293T cells (Fig. 7G–I). The OGD-induced Zn2+ release was rescued by ectopic TRPM7 expression in TRPM7−/− cells (Fig. 7I). Again, because the extracellular solution did not contain significant Zn2+, we conclude that M7Vs were the source of TRPM7-dependent, OGD-induced increase in cytosolic Zn2+. In conclusion, TRPM7 forms functional intracellular channels that mediate Zn2+ release from the lumen of a previously unrecognized population of vesicles during oxidative stress.

Discussion

Our prior observations of intracellular TRPM7 on 50- to 250-nm vesicles led us to investigate the existence of an additional and perhaps more important function of TRPM7 as an intracellular channel. Here we show that most TRPM7 forms intracellular...
Zn$^{2+}$-release channels in a previously unrecognized type of Zn$^{2+}$ storage vesicle in multiple cell types. Oxidizing conditions activate intracellular Zn$^{2+}$ release via TRPM7, likely by converting GSH in M7Vs to GSSG. We anticipate these findings will provide insights into the cellular function of TRPM7.

Several transient receptor potential (TRP) channels function in various intracellular compartments. For example, TRPML1 and TRPM2 release lysosomal Ca$^{2+}$ and Zn$^{2+}$, whereas TRPM8 may release Ca$^{2+}$ from the ER in some cell types (65, 66). The Drosophila TRP channel, proposed to release Zn$^{2+}$ from intracellular stores during larval development, exhibits TRPM7-like currents (67). We now include TRPM7 in the class of functional intracellular TRP channels. However, the intracellular function of TRPM7 is unique in two ways. First, TRPM7 functions in vesicles that are distinct from known compartments. Second, unlike ER and lysosomes that store both Ca$^{2+}$ and Zn$^{2+}$, M7Vs store only Zn$^{2+}$.

Under resting conditions, M7Vs do not contain free Zn$^{2+}$, unlike intracellular Zn$^{2+}$ stores such as mitochondria, ER, lysosomes, synaptic vesicles, and zincoxides (42). Although GSH is enriched in M7Vs and does not chelate micromolar [Zn$^{2+}$] in physiological solutions, it may still bind Zn$^{2+}$ under some conditions that may exist in M7Vs (68). The complex dynamics of cellular redox status and M7V GSH content, cytoplasmic free Mg$^{2+}$, and ATP (as well as other phosphonucleotides) will require careful examination in the future.

M7Vs are uniformly distributed in the cell and thus are an ideal ion storage system. We hypothesize that M7Vs sequester Zn$^{2+}$ to protect other organelles, but because Zn$^{2+}$ binds many proteins, DNA, and RNA, the vesicles also could release Zn$^{2+}$ to alter many cellular processes. We are exploring the possibility that Zn$^{2+}$ released from M7Vs regulates TRPM7 kinase cleavage, activity, or trafficking. Furthermore, the proteomics data in this study suggest that dozens of proteins are localized, at least partially, in M7Vs. In this study, we focused on iden-
Identifying probes for the lumens of M7Vs, but verification of the full complement of M7V proteins will elucidate their biogenesis and function. The presence of vesicle trafficking proteins (e.g., vesicle-associated membrane protein A, VAPA) and fusion proteins (e.g., vesicle-associated membrane protein 1, VAMP1, and extended syntaptogamin-1, ESYT1) in the M7V proteome is consistent with the previously reported M7V exocytosis during shear stress and cholinergic synaptic transmission (33, 34). Enrichment of ferritin light chain in vesicles isolated from SV40MES cells also suggests the possibility of iron storage by M7Vs. Our M7V proteome did not contain transporters for divalent cations (e.g., ZnTs, ZnPs, or other divalent metal transporters) or GSH, likely because these proteins are not highly expressed, but targeted proteomic analysis may reveal their identities.

Total Zn2+ increases by 50% during meiotic maturation of oocytes; about half of this increase is released by vesicular exocytosis within minutes of fertilization (69), and the rest persists into the regions flanking exon 1 of TRPM7. The cells then were electroporated with plasmids encoding GFP or HA-SNAP tags and flippase (FLP) recombinase. The cells then were vortexed in 0.9% NaCl and centrifuged; supernatants were used for LC-MS sample extractions.

**Imaging of Zn2+ Flux with Genetically Encoded Zn2+ Sensors.** HEK293 cells transfected with eCALWY4 or with eCALWY4 and mCherry-TRPM7 were imaged as described in ref. 47. To study Zn2+ flux in intracellular compartments, HEK293 cells expressing intravesicular Zn2+ sensors were imaged in HBT-A medium (20 mM Heps, 120 mM NaCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 10 mM glucose, pH 7.5) with or without extracellular Zn2+.

**Live Imaging of Vesicular Redox Environment.** A ratiometric redox sensor, roGFP2, was targeted to the lumen of M7Vs by insertion between the 51 and 52 domains of TRPM7. After baseline image acquisition, the sensor was maximally reduced with 10 mM DTT (Bio-Rad) and then maximally oxidized in 1 mM diamide (Sigma).

**Zn2+ Release from Intracellular Stores.** Untransfected (FluoZin-3–loaded) or VIP36-eCALWY4–transfected WT and TRPM7+/− HEK293T cells were incubated with 500 μM Zn2+ in Mg2+- and Ca2+-free solution for 10–20 min. The cells then were perfused with 10 mM sodium dithionite (in glucose-free) or 100 μM H2O2 (in normal) HBT-A medium containing 0.25 mM EGTA to chelate Zn2+ and 4 mM Mg2+ to block plasma membrane–localized TRPM7.

**TRPM7 Deletion in HEK293T Cells.** A variant of the CRISPR/Cas9 method (76) was used to insert puromycin- and blastidin-coding sequences into the second exon of Trpm7. The donor DNA contained the predicted coding sequence of Trpm7’s first 12 amino acids followed by an antibiotic resistance cassette, multiple stop codons, and SV40-polyA. Oligonucleotides encoding the genomic RNA (gRNA) sequence were annealed and cloned into BPK1520 (77). Donor DNA containing puromycin-SV40pA and blastidin-SV40pA cassettes were amplified from pPur (Clontech) and pcDNA6-V5-His (Invitrogen), respectively, and DNA containing puromycin-SV40pA and blasticidin-SV40pA cassettes were ligated into a target site on exon 2 of Trpm7. Dox-containing Puromycin-resistant transfectants were selected, and puromycin-resistant cells were cloned and genotyped, and knockout of Trpm7 was verified by immunoprecipitation, Western blotting, and electrophysiology.

**Statistical Analyses.** Data were plotted and analyzed in Prism 5 (GraphPad) by Student’s t tests, ANOVA with Tukey’s post hoc tests, or extra sums of squares F-tests (for nonlinear regressions).

**ACKNOWLEDGMENTS.** We thank Maria Ericsson for EM, Clary Clish for metabolomics, Eric Spooner for proteomics studies, Bayush Dinegde for DNA purification, Svetlana Gapon for cell culture, and Luba Krapivinsky for TRPM7 antibody purification. This work was supported in part by NIH Institutional Research Training (T32 Grants ST32HL007572-31 to S.A.A.) and K99/H124070 (to D.C.) at Boston Children’s Hospital.
Supporting Information

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SI Experimental Procedures

**Constructs.** GCaMP6s-TRPM7, pHluorin-TRPM7, and ZnGreen1-TRPM7 (all with sensors inserted into the S1/2 loop between I896 and P897 of mouse TRPM7) were made in the original pcDNA4TO-FLAG-mouse TRPM7 construct (1). GFP-TRPM7 and mCherry-TRPM7 (WT or with pore mutations N1090F, L1091A, and L1092P) were previously described (33–35). GCaMP6s and TRPM7 contained a C-terminal HRV3C protease cleavage site before an HA epitope. VIP36-eCALW4, VIP36-V5, and edem1-V5 were in pcDNA6V5HisB (Life Technologies) and Sec61β-eCALW4 was in pCMV (Clontech). Cell Light Peroxisomes-RFP BacMam (RFP-peroxisome targeting sequence or RFP-Pts vector) was purchased from Life Technologies/Thermo Scientific. The following constructs were from Addgene: pDTnLAPP2A (plasmid no. 28226), pCAG-Flpo (plasmid no. 60662), VSVG-GFP (plasmid no. 11912), R-GEICO1 (plasmid no. 32444), RCEPIa1er (plasmid no. 58216), GFP-Tyrosinase (plasmid no. 32781), ZapCmR-NLS (plasmid no. 59012), GCaMP6s (plasmid no. 40753), DIER (plasmid no. 36325), and roGFP2 (plasmid no. 49435).

**Antibodies.** The following antibodies were obtained commercially: mouse anti-FLAG and anti–FLAG-HRP (Sigma); rabbit anti-HA-HRP (Roche); rabbit anti-GFP (Life Technologies or Genetex); mouse anti-protein disulfide isomerase, chicken anti-GFP (Aves Labs); rabbit anti-Na+/K+ ATPase (Cell Signaling Technologies), mouse anti-protein disulfide isomerase, mouse anti-cytochrome C, rabbit anti-clathrin heavy chain, and mouse anti-calnexin (Abcam); mouse anti-TRPM7 (NeuroMab); and rabbit anti-lamp1, rabbit Ergc-53, rabbit anti-pex19, and rabbit anti-rab11 (Santa Cruz Biotechnologies). The rabbit anti-TRPM7 antibody developed in our laboratory was described previously (21).

**Immunofluorescence.** Cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton-X 100 at room temperature. Nonspecific binding was blocked with 10% goat serum in PBS, and cells were incubated in primary antibodies for 2 h at room temperature or overnight at 4 °C, followed by detection with fluorophore-conjugated secondary antibodies. Nuclei were stained with DAPI in PBS before the cells were mounted on slides using Prolong Gold (Life Technologies). Images were acquired on an Olympus Fluoview 1000 confocal microscope.

**STED Microscopy.** Immunocytochemistry for STED was performed as above but at higher concentrations of secondary antibodies (1:250). The mounting medium was cured for at least 48 h in Prolong-Gold before imaging. STED images were acquired using a 100×, 1.4 NA oil objective on a Leica SP8 X STED microscope. A white-light laser source was used to excite Alexa Fluor488 and Alexa Fluor 546 dyes; dyes were depleted with a 592-nm and 660-nm laser, respectively. Emitted photons were detected using a GaAsP/HyD hybrid detector.

**TIRF Microscopy.** Cells were plated on 3.5-cm glass-bottomed dishes (In Vitro Scientific) and imaged with a 60×, 1.45 NA oil objective (Olympus) excited with a 488-nm solid-state diode laser and a 532-nm diode laser. A dual band dichroic mirror (Z488/532RPC; Chroma Tech) reflected excitation light onto the specimen, and emitted light, filtered by HQ515/30(Chroma) or D620/60 (Chroma), was detected with a cooled CCD camera (ORCA II; Hamamatsu). SlideBook software (3i) was used for instrument control and data acquisition.

**Western blotting.** Cells then were electroporated with 30 μg of pDTnLAPP2A and 70 μg pCAG-Flpo plasmids at 240 V and 500 μF capacitance. After 48 h, the medium was replenished daily with fresh medium containing 200 μg/mL hygromycin for 10 d. Single clones were selected, expanded, and characterized by genotyping for GFP and Western blotting. For insertion of HA into the N terminus of TRPM7, the GFP DNA sequence was first excised from pDTnLAPP2A using BstXI and BsrGI. An HA-SNAP-V5 DNA sequence then was amplified with primers overprinting with the restriction sites and was ligated using a GeneArt cloning kit (Thermo Fisher). After sequencing, the construct was electroporated into ES cells with pCAG-Flpo, as above, and positive clones were selected with hygromycin. TRPM7 tagging was verified by anti-HA and anti-TRPM7 immunoprecipitation and Western blotting.

**Determination of Vesicular Acidity.** pHluorin-coding cDNA (a generous gift of G. Miesenbock, Sloan-Kettering Cancer Center, New York) was inserted into TRPM7 as described above and was transfected into HEK293 cells. Cells were first imaged in HBT-A, a modified Hapes-buffered Tyrode’s solution containing 20 mM Hapes, 120 mM NaCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose (pH 7.5), and then 50 mM NaCl was replaced with 50 mM NH₄Cl to alkalinize all intracellular compartments, thus increasing vesicular pHluorin fluorescence, as in ref. 35.

**Affinity Purification of TRPM7-Containing Vesicles.** SV40MES-13 cells stably expressing GFP-TRPM7-HA on HEK293 cells stably expressing GCaMP6s-TRPM7-HRV3C-HA were homogenized in a Dounce homogenizer and centrifuged at 1,000 × g for 5 min at 4 °C in PBS with protease inhibitors and 5 mM EDTA vesicle immunoprecipitation (VIP) buffer. The supernatants were centrifuged again at 1,300 × g for 5 min to yield postnuclear supernatants (PNS). The PNS was incubated with mouse anti-HA-conjugated magnetic nanoparticles (Miltenyi Biotec) for 2 h at 4 °C, with or without 20 μg/mL of HA-blocking control peptide. The mixture then was loaded onto LS columns (Miltenyi Biotec) and washed twice with PBS. Immobilized vesicles were eluted in 2 mL of VIP buffer. For Western blotting, the eluate was separated by SDS/PAGE, transferred to PVDF membranes, and detected with HRP-conjugated anti-HA antibodies. To visualize isolated but intact vesicles, Alexa Fluor 546-conjugated anti-mouse secondary antibodies were detected with a goat anti-mouse HRP conjugate. Immunogold-labeled vesicular structures were measured manually using Fiji ImageJ.

**Tagging of Endogenous TRPM7.** TRPM7 was tagged according to ref. 75. Briefly, ES cells with gene-trapped TRPM7 were purchased from EUMMCR and were verified by genotyping the regions flanking exon 1 of TRPM7. The cells were cultured in gelatin-coated dishes in knockout-DMEM supplemented with 10% FBS, glutamate, nonessential amino acids (all from Gibco/Thermo-Fisher Scientific), 2-mercaptoethanol, and 100 U/mL leukemia inhibitory factor (LIF) (Millipore/GE Healthcare). The cells then were electroporated with 30 μg of pDTnLAPP2A and 70 μg pCAG-Flpo plasmids at 240 V and 500 μF capacitance. After 48 h, the medium was replenished daily with fresh medium containing 200 μg/mL hygromycin for 10 d. Single clones were selected, expanded, and characterized by genotyping for GFP and Western blotting. For insertion of HA into the N terminus of TRPM7, the GFP DNA sequence was first excised from pDTnLAPP2A using BstXI and BsrGI. An HA-SNAP-V5 DNA sequence then was amplified with primers overprinting with the restriction sites and was ligated using a GeneArt cloning kit (Thermo Fisher). After sequencing, the construct was electroporated into ES cells with pCAG-Flpo, as above, and positive clones were selected with hygromycin. TRPM7 tagging was verified by anti-HA and anti-TRPM7 immunoprecipitation and Western blotting.
antibody (Life Technologies/Thermo Fisher Scientific) was added to the PNS during vesicle isolation, and vesicles were immobilized on Cell-Tak–coated 12-mm coverslips in PBS. The Alexa Fluor 546-labeled nanoparticles and bound vesicles then were imaged in 1 mM Ca\(^{2+}\) to enhance GCaMP6s fluorescence.

**Metabolomics.** HEK293 or HEK293T cells stably expressing GCaMP6s-TRPM7-HA were grown in 15-cm dishes and induced with 1 μg/mL doxycycline for 24 h. Vesicles were isolated using anti-HA nanoparticles as described above. The PNS and isolated vesicles were centrifuged at 35,000 × g for 1 h in weighed tubes, and the pellets were rinsed twice in PBS. After the PBS was aspirated, the tubes were weighed to determine the weight of the pellet, and the pellet was frozen at −80 °C. Thawed pellets were vortexed in 0.9% NaCl and centrifuged at 15,000 × g for 20 min. Supernatants were aliquoted for LC-MS sample extractions. LC-MS analyses were conducted using targeted measurement of polar metabolites in the negative-ion mode, nontargeted measurement of polar metabolites in the positive-ion mode, C18-negative, nontargeted analysis of metabolites of intermediate polarity in the negative-ion mode, and C8-positive nontargeted analysis of lipids in the positive-ion mode.

**Luciferase GSH Assay.** Vesicles were isolated from mouse ES cells expressing HA-tagged endogenous TRPM7 as above, except that the PNS and pellets were assayed for GSH with a GSH/GSSG-GLO kit (Promega), using the manufacturer’s protocol. The GSH concentration was normalized to the total protein concentration.

**Imaging of Zn\(^{2+}\) Flux with Genetically Encoded Zn\(^{2+}\) Sensors.** HEK293 cells were transfected with eCALWY4 or with mCherry-TRPM7 and then were split onto coverslips for 12 h before imaging in HBT-A solution. eCALWY4 FRET was imaged as described in ref. 47 on an Olympus IX70 microscope equipped with a DG-4 excitation light source (Sutter). Excitation light was filtered with a 440AF21 filter (Omega) and reflected onto the sample with a 455DRLP filter (Omega). 480AF30 (Omega) and HQ535/30m (Chroma) filters were alternated using a Lambda 10-3 filter changer (Sutter Instruments) to filter emissions from mCerulean and mCitrine, respectively. A cooled CCD camera (Hamamatsu Photonics) was used to detect emitted photons. Again, SlideBook software (3i) was used for instrument control and data acquisition. The time constants of Zn\(^{2+}\) entry were determined from single exponential decay fits of mCitrine/mCerulean fluorescence intensity ratios, with or without GSH. To study Zn\(^{2+}\) influx in intracellular compartments, HEK293 cells expressing intravesicular Zn\(^{2+}\) sensors were imaged with or without extracellular Zn\(^{2+}\). Cells expressing the intensitometric TRPM7-ZnGreen1 Zn\(^{2+}\) sensor were imaged using a GFP filter cube. Vesicles were first perfused with 15 nM Zn\(^{2+}\) and then with 50 μM TPEN. In an alternate protocol, we reversed the order of FluoZin-3 and Zn\(^{2+}\) loading: vesicles were first incubated with Zn\(^{2+}\), the excess Zn\(^{2+}\) was washed off, and FluoZin-3-AM then was added to the vesicles. To monitor Ca\(^{2+}\) and Mg\(^{2+}\) entry into TRPM7-containing vesicles, 40 μM Fluor4-AM or MagFluo4-AM, respectively, was loaded into vesicles at 37 °C for 20 min, followed by loading with CLM containing 1 μM Ca\(^{2+}\) or 5 mM Mg\(^{2+}\). Total and free ion concentrations were calculated using MaxChelator (Stanford University).

**Determination of Orientation of VIP36-eCALWY4.** RCEPIAer was coexpressed with GFP-TRPM7 or VIP36-eCALWY4. RCEPIAer and GFP fluorescence were imaged before and after the addition of 20 μM digitonin. Proteinase K (50 μg/mL) was subsequently added to digest cytoplasmic fluorescent proteins, as described in ref. 79.

**Zn\(^{2+}\) Release from Intracellular Stores.** Untransfected (FluoZin-3-AM–loaded) or VIP36-eCALWY4–transfected WT and TRPM7–HEK293T cells were plated on poly-L-lysine–coated coverslips for 12 h (TRPM7–/– cells were grown in 10 mM extra MgCl\(_2\)). The medium was replaced by medium containing normal Mg\(^{2+}\) (0.8 mM) for 6–12 h before imaging. To fill vesicles with Zn\(^{2+}\), transfected or Fluozin-3–loaded cells were rinsed once in HBT-A solution and then were incubated with 500 μM Zn\(^{2+}\) in Mg\(^{2+}\)-free HBT-A solution for 10–20 min. The cells then were perfused with 10 mM sodium dithionite (in glucose-free) or 100 μM H\(_2\)O\(_2\) (in normal) HBT-A medium containing 0.25 mM EGTA to chelate extracellular Zn\(^{2+}\) and 4 mM Mg\(^{2+}\) to block plasma membrane-localized TRPM7.

**Gradient Fractionation of Endogenous M7Vs.** PNSs from SV40MES-13 cells were prepared in Hapes-buffered saline [20 mM Hapes (pH 7.5), 150 mM NaCl, 2 mM EGTA, 2 mM MgCl\(_2\) and protease inhibitors] and were loaded on a 21–54% linear sucrose gradient buffered with 20 mM Hapes. The PNS then was centrifuged for 3 h at 40,000 × g in a Beckman L2 Ultracentrifuge, SW-41 rotor. One-milliliter fractions were collected and analyzed by SDS/PAGE and Western blotting.
Calcium Imaging. HEK293 cells were transfected to express GCaMP6s-TRPM7 either alone or with cytoplasmic R-GECO1 or ER-targeted RCEPIAer. The cells then were treated with 100 μM ATP in HBSS, and images were acquired at 0.2 Hz in epifluorescence mode on the previously described TIRF microscope.

Proteomic Analysis of M7Vs. HEK293 cells were stably transfected to express inducible FLAG-GCaMP6s-TRPM7-HRV3C-HA and were passaged at least five times in medium containing l-arginine-10 and l-lysine-6 heavy isotopes. After labeled cells were mixed with equal amounts of unlabeled, untransfected WT cells, vesicles were isolated from PNS using anti-HA-conjugated magnetic nanoparticles, as described above. Bound vesicles were incubated with HRV-3C protease (0.03 U/μL) in cleavage buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM DTT] for 12–18 h at 4 °C to elute nanoparticles. Eluates were immunoprecipitated again with anti-FLAG-conjugated magnetic nanoparticles. For proteomic analysis of vesicles isolated from SV40MES-13 cells stably expressing GFP-TRPM7-HA, all clones were clamped with an Axopatch 200B controlled by a Digidata 1440A patch-clamp system. The pipette electrode solution contained (in mM): 120 Cs-MeSO₃, 8 NaCl, 10 BAPTA [1,2-bis(2-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid], 2 CaCl₂, 2 ATP-Na₂, and 10 Hepes. The bath solution was a modified Hepes-buffered Tyrode’s solution, HBT-B, which contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 10 Hepes, 10 glucose, pH 7.4. In experiments with GSH, Cs-MeSO₃ was replaced with N-methyl-D-glucamine (NMDG), and the pH of the GSH-containing solution was adjusted to 7.4 before perfusion. Cells were held at 0 mV, and 200-ms ramps from −100 mV to +100 mV were applied every 2 s. Currents were digitized at 10 kHz and low-pass filtered at 2 kHz.

Electrophysiological Recordings. HEK293 cells were transiently transfected with Trpm7 constructs or stably incorporated doxycycline-inducible TRPM7 cDNA. The cells were voltage-clamped with an Axopatch 200B controlled by a Digidata 1440A (Molecular Devices). Pipettes with 3- to 5-MΩ resistance were used. The pipette electrode solution contained (in mM): 120 Cs-MeSO₃, 8 NaCl, 10 BAPTA [1,2-bis(2-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid], 2 CaCl₂, 2 ATP-Na₂, and 10 Hepes. The bath solution was a modified Hepes-buffered Tyrode’s solution, HBT-B, which contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 10 Hepes, 10 glucose, pH 7.4. In experiments with GSH, Cs-MeSO₃ was replaced with N-methyl-D-glucamine (NMDG), and the pH of the GSH-containing solution was adjusted to 7.4 before perfusion. Cells were held at 0 mV, and 200-ms ramps from −100 mV to +100 mV were applied every 2 s. Currents were digitized at 10 kHz and low-pass filtered at 2 kHz.

Statistical Analyses. Data were plotted and analyzed in Prism 5 (GraphPad) by Student’s t tests, ANOVA with Tukey’s post hoc tests, or extra sum of squares F tests (for nonlinear regressions).
**Fig. S1.** (Related to Fig. 1) TRPM7 is in acidic intracellular vesicles. (A) Confirmation of genetic tagging of endogenous TRPM7 with GFP. Reciprocal anti-GFP (Left) and anti-TRPM7 (Right) immunoprecipitations (IP) of TRPM7 from ES clones expressing endogenous WT (B05 parental ES cell line) or GFP-TRPM7 (clones 29 and 33). Immunoprecipitates were analyzed by Western blotting for GFP or TRPM7. (B and C) HEK293 cells expressing TRPM7 tagged with pHluorin (placed in the S1/2 loop) were fixed and immunolabeled with anti-GFP and 5 mM gold-conjugated protein A. Quantification of immunogold labeling in untransfected control cells or cells expressing pHluorin-TRPM7 (vesicular vs. mitochondrial) (B) and M7V size distributions (C). Diameters of vesicular structures were quantified from 35 images. (D) HEK293 cells expressing pHluorin-TRPM7 were treated with 50 mM NH₄Cl (pH 7.0) to alkalinize intracellular compartments, resulting in an increase in vesicular pHluorin fluorescence.

**Fig. S2.** (Related to Figs. 3 and 4) Electrophysiological characterization of TRPM7 function after insertion of fluorescent protein sensors in the S1/S2 loop. (A) TRPM7 currents recorded in HEK293 cells expressing ZnGreen1 in the S1/S2 linker with or without extracellular Mg²⁺. (B) A TRPM7 DNA construct containing GCaMP6s in the S1/S2 linker was transfected in HEK293 cells, and TRPM7 currents were recorded in Mg²⁺-free intracellular solutions. Current/voltage (I/V) plots at break-in and the current run-up after Mg²⁺ chelation are shown. Traces are representative of three to five cells.
Fig. S3. (Related to Figs. 2A and 3 and Dataset S1) Tandem affinity purification (TAP) and proteomic analysis of M7Vs. (A) Proteins of HEK293 cells expressing TRPM7 with an N-terminal FLAG-tag and cleavable C-terminal HA-tag were labeled with heavy isotopes of L-arginine and L-lysine (SILAC). After labeled cells were mixed with equal amounts of unlabeled, nontransfected wild-type (NT) cells, vesicles were isolated from the PNS using anti-HA–conjugated magnetic nanoparticles. Bound [immunoprecipitation (IP): HA, B] vesicles were eluted (IP: HA, EL) from nanoparticles using HRV-3C protease and were immunoprecipitated again with anti-FLAG–conjugated magnetic nanoparticles (IP FLAG: B). NB, not bound. TAP quality was monitored by Western blot of equal amounts of fractions for Na+/K+ ATPase, protein disulfide isomerase (PDI), cytochrome C (cyt-C), and Coomassie Blue protein staining. TAP vesicles then were analyzed by LC-MS-MS. (B) Images in Fig. 2A are presented separately to depict colocalization of VIP36-V5 and TRPM7-HA in HEK293 cells.

Fig. S4. (Related to Fig. 5) M7Vs do not sequester Ca²⁺ or Mg²⁺. (A) M7Vs isolated from SV40MES-13 cells were loaded with Fluo4-AM (A) or MagFluo4 (B) and were perfused with the indicated concentrations of Ca²⁺ or Mg²⁺. Then, 5 μM Bromo-A23187 in 1 mM EDTA was used to deplete cations at the end of the time course. (C and D) HEK293 cells coexpressing GCaMP6s in the intravesicular loop of TRPM7 (green) and ER-targeted (RCEPIAer, red, C) or cytosolic (R-GECO1, red, D) Ca²⁺ sensors were stimulated with 100 μM ATP in HBSS. ATP-induced vesicular Ca²⁺ spikes (C and D, green) and synchronous Ca²⁺ efflux from the ER (C, red) or influx into the cytosol (D, red) were monitored simultaneously. Traces are representative of at least three experiments.
Fig. S5. (Related to Fig. 5) Characterization of genetically encoded Zn$^{2+}$ sensors. (A) Vesicular and nuclear Zn$^{2+}$ were monitored concurrently in a mixture of HEK293 cells expressing VIP36-eCALWY4 or ZapCmR2-NLS and titrated with increasing Zn$^{2+}$ concentrations in 20 μM pyrithione. (B) VIP36-eCALWY4 FRET was monitored in HEK293 cells before and after neutralization of vesicular pH with NH$_4$Cl. (C) HEK293 cells expressing intravesicular ZnGreen1 (in the TRPM7 S1/2 loop) were incubated with 50 μM TPEN or 50 μM Zn$^{2+}$/20 μM pyrithione. (D) Cytosolic and vesicular ZnGreen1 sensors were saturated (indicated by the axis break) 20 min after loading with 500 μM extracellular Zn$^{2+}$ at room temperature. (E and F) Cytosolic (E) and vesicular (F) ZnGreen1 were titrated with increasing concentrations of Zn$^{2+}$ in 20 μM pyrithione to determine Zn$^{2+}$ affinities of the sensors. The $K_D$ of the TRPM7-conjugated sensors (21–2.9 nM) indicate higher affinity than that reported for the naked sensor (633 nM) (51). Note: We have inverted eCALWY-4 FRET changes to overlay the calibration curve of eCALWY-4 and that of a conventional FRET-based sensor (ZapCmR2) in Fig. S5A. To keep the panels consistent, intensity changes of ZnGreen1 were also inverted.
Fig. S6. Isolation of vesicles containing endogenous TRPM7. (A) Mouse ES cells containing a gene-trapped TRPM7 exon 1 (BO5) or their derivatives expressing HA-tagged endogenous TRPM7 were solubilized for anti-HA immunoprecipitation (IP) and Western blotting (WB) with anti-HA (Upper) or anti-TRPM7 (Lower). (B) Endogenous M7Vs were isolated from PNSs and were analyzed by Western blotting for HA and Na⁺/K⁺ ATPase or by Coomassie Blue (total protein) staining. B, bound; NB, not bound.
Fig. S7. Relief of Zn\(^{2+}\) block of TRPM7 currents by glutathione. (A and B) The current/voltage (I/V) relationship of monovalent TRPM7 currents (A) and time course of currents (B) in HEK293 cells stably overexpressing GFP-TRPM7, with or without 1 mM Zn\(^{2+}\). (C) Dose-dependence of TRPM7 current inhibition by Zn\(^{2+}\). (D) Compositions of intracellular and extracellular recording solutions for E and F. (E) Enhancement of monovalent TRPM7 currents by reduced, but not oxidized, glutathione. (F) Enhancement of monovalent TRPM7 currents by GSH despite 20 mM Zn\(^{2+}\). Time elapsed just after (0 min) and 1 min after break-in with 140 mM NMDG intracellular solution is indicated. Traces are representative of 5–14 cells.
Table S1. Probes used to label various compartments

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Indicators

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Sources of reagents not made in this study are described in Methods.
Movie S1. SV40MES-13 cells stably overexpressing GFP-TRPM7 were imaged in HBT-A solution at 1 Hz, 50 fps playback. (Scale bar: 1 μm.)

Dataset S1. M7Vs isolated from HEK293 or SV40MES-13 cells after SILAC labeling (see Methods)

Proteins with enrichment ratios of at least 1.3 are listed.

Dataset S2. HPLC analysis of vesicles isolated from HEK293 or HEK293T cells overexpressing HA-tagged TRPM7

The concentrations of metabolites in vesicles were divided by those in total postnuclear membranes to determine the fold enrichment.