Journal of Cellular Physiology

Functional Multimerization of Mucolipin Channel Proteins

CYNTIA CURCIO-MORELLI,[1] PENG ZHANG,[2] BHUVARAHAMURTHY VENUGOPAL,[1] FLORIE A. CHARLES,[1] MARSHA F. BROWNING,[1] HORACIO F. CANTIELLO,[2] AND SUSAN A. SLAUGENHAUPT[1]*

[1]*Center for Human Genetic Research, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114*

[2]*Nephrology Division and Electrophysiology Core, Massachusetts General Hospital East, Charlestown, Massachusetts 02129*

MCOLN1 encodes mucolipin-1 (TRPML1), a member of the transient receptor potential TRPML subfamily of channel proteins. Mutations in *MCOLN1* cause mucolipidosis-type IV (MLIV), a lysosomal storage disorder characterized by severe neurologic, ophthalmologic, and gastrointestinal abnormalities. Along with TRPML1, there are two other TRPML family members, mucolipin-2 (TRPML2) and mucolipin-3 (TRPML3). In this study, we used immunocytochemical analysis to determine that TRPML1, TRPML2, and TRPML3 co-localize in cells. The multimerization of TRPML proteins was confirmed by co-immunoprecipitation and Western blot analysis, which demonstrated that TRPML1 homo-multimerizes as well as hetero-multimerizes with TRPML2 and TRPML3. MLIV-causing mutants of TRPML1 also interacted with wild-type TRPML1. Lipid bilayer re-constitution of in vitro translated TRPML2 and TRPML3 confirmed their cation channel properties with lower single channel conductance and higher partial permeability to anions as compared to TRPML1. We further analyzed the electrophysiological properties of single channel TRPML hetero-multimers, which displayed functional differences when compared to individual TRPMLs. Our data shows for the first time that TRPMLs form distinct functional channel complexes. Homo- and hetero-multimerization of TRPMLs may modulate channel function and biophysical properties, thereby increasing TRPML functional diversity.

J. Cell. Physiol. 222: 328–335, 2010.

Mutations in the *MCOLN1* gene result in mucolipidosis-type IV (MLIV), an autosomal recessive lysosomal storage disorder characterized by severe psychomotor retardation and visual impairment (Slaugenhaupt et al., 1999; Bargal et al., 2000; Sun et al., 2000; Altarescu et al., 2002). *MCOLN1* encodes mucolipin-1 (TRPML1), a transmembrane protein with topological homology to other members of the TRP superfamily of ion channels. TRPML1 was initially reported as a novel nonselective cation channel with permeability to Ca^{2+} and regulation by changes in Ca^{2+} concentration (LaPlante et al., 2002). Studies performed in endosomal vesicles from MLIV fibroblasts, as well as in liposomes containing the in vitro translated protein, indicate that the TRPML1 channel can be inhibited by a decrease in pH and Ca^{2+} transport (Raychowdhury et al., 2004; Cantiello et al., 2005). Recent studies have suggested that TRPML1 channel activity is regulated by protein kinase A phosphorylation and might function to regulate lysosomal pH and to maintain the required acidity for lysosomal acid lipase function (Kiselyov et al., 2005; Soyombo et al., 2006; Vergarajauregui et al., 2008).

The *MCOLN2* and *MCOLN3* genes encode mucolipin-2 (TRPML2) and mucolipin-3 (TRPML3), respectively, which together with TRPML1 make up the TRPML subfamily of TRP channels. Recently, TRPML2 has been shown to traffic via the Arf6-associated pathway and to co-localize with major histocompatibility protein class I and glycosylphosphatidylinositol-anchored proteins (Karacsonyi et al., 2007). Spontaneous mutations in *MCOLN3* lead to the varitint-waddler (Va) mouse phenotype, characterized by early deafness, vestibular and pigmentation defects, and perinatal lethality (Di Palma et al., 2002). Given the observed mouse phenotype, TRPML3 was shown to localize to the cytoplasmic compartment of hair cells and the plasma membrane of stereocilia and to be critical for early cochlear hair cell development. Recently, three independent groups determined that the Va A419P mutation of TRPML3 generates a constitutively active channel in the plasma membrane, with strong inward rectification (Grimm et al., 2007; Kim et al., 2007; Xu et al., 2007; Nagata et al., 2008).

In this report, we show that human wild-type TRPML2 and TRPML3 co-localize with TRPML1 when over-expressed in CHO cells. We further demonstrate the homo- and hetero-multimerization of TRPMLs by co-immunoprecipitation (co-IP). The TRPML2 and TRPML3 proteins display channel properties with smaller single channel conductances than that of TRPML1 (Raychowdhury et al., 2004). More importantly, our studies show for the first time that hetero-multimers of the TRPMLs form functional channel complexes with distinct properties.

Materials and Methods

Plasmids

TRPML1-GFP and TRPML1-Flag were previously described (LaPlante et al., 2002). TRPML1, TRPML2, and TRPML3 fused to the V5 epitope were constructed as follows. Plasmids containing TRPML1 and TRPML3 cDNAs and TRPML2 cDNA made from human placenta RNA (Clontech, Mountain View, CA) were used as templates for TRPML1, TRPML3, and TRPML2, respectively.

Cyntia Curcio-Morelli and Peng Zhang contributed equally to this work.

Horacio F. Cantiello and Susan A. Slaugenhaupt are co-senior authors of this work.

Contract grant sponsor: National Institute of Neurological Disorders and Stroke.
Contract grant sponsor: MGH Fund for Medical Discovery;
Contract grant number: NS39995.

*Correspondence to: Susan A. Slaugenhaupt, Center for Human Genetic Research, Massachusetts General Hospital and Harvard Medical School, 185 Cambridge Street, Boston, MA 02114.
E-mail: slaugenhaupt@chgr.mgh.harvard.edu

Received 16 January 2009; Accepted 8 September 2009

Published online in Wiley InterScience (www.interscience.wiley.com.), 2 November 2009.
DOI: 10.1002/jcp.21956

TRPML1, TRPML2, or TRPML3 were amplified with the BD Advantage PCR amplification kit (Clontech) and cloned using the Gateway PCR system following the manufacturer's instructions (Invitrogen, Carlsbad, CA). The resulting mammalian expression vectors contain an NH_2 terminus V5 epitope fused either to TRPML1, TRPML2, or TRPML3, and are called V5-TRPML1, V5-TRPML2, and V5-TRPML3, respectively. GFP not fused to any protein was used for co-transfections in some experiments or transfected alone as a control for transfection efficiency.

Cell culture and transfections

CHO cells were split 1 day before transfection and grown in 6-well plates or 4-chamber culture slides (BD Falcon). Transfections were done in Ham's F12 medium supplemented with 10% fetal bovine serum using FuGENE6 reagent following the manufacturer's instructions (Roche, Indianapolis, IN). Cells were harvested 48 h after transfection. To check for transfection efficiency, a positive control GFP-tagged vector was also transfected and green fluorescence intensity was observed in every experiment.

In vivo co-immunoprecipitation (co-IP) and Western blot analysis

Transfected CHO cells were re-suspended in lysis buffer containing 0.1 M Tris–HCl pH 8.0, 0.15 M NaCl, 0.1% IGEPAL (Sigma, St. Louis, MO), type I protease inhibitor cocktail set (Calbiochem, San Diego, CA), 1 mM phenymethylsulfonyl fluoride (PMSF), 1 mM iodoacetamide (IodAc), and 1 mM *N*-ethylmaleimide (NEM) (Calbiochem). Lysis of the cells was done for 30 min at 4°C. Cell lysates were centrifuged at 9,000*g* for 10 min and the cleared supernatant was incubated overnight with the specific antibody (see dilutions below) plus 20 μl of rProtein G beads (Invitrogen). Immunoprecipitation pellets (IP pellets) were washed twice with buffer A containing 25 mM Tris–HCl pH 8.0, 300 mM NaCl, 1 mM $CaCl_2$, 0.5% Triton X-100 and twice in buffer B containing 25 mM Tris–HCl (pH 8), 140 mM NaCl, and 1 mM $CaCl_2$. Pellets were re-suspended in sample loading buffer, boiled for 4 min, resolved in 10% SDS–PAGE, and transferred to a polyvinyl membrane (BioRad, Hercules, CA). Blots were probed with the specific antibody (see below). Anti-mouse IgG secondary antibody HRP-conjugated and peroxidase substrate were used following manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Antibodies

Anti-GFP antibody (Roche) was used on IP (0.01 μg/μl) and Western blot analysis (1:1,000 dilution). Anti-V5 antibody (Invitrogen) was used for IP (1:250 dilution) and Western blot analysis (1:5,000 dilution). Anti-FLAG antibody (Sigma) was used for IP (1:250 dilution).

Immunofluorescence and microscopy

CHO cells transiently expressing TRPML1-GFP, TRPML2-V5, or TRPML3-V5 alone or co-expressing TRPML1-GFP plus TRPML2-V5 or TRPML3-V5 were fixed for 15 min in 4% paraformaldehyde, permeabilized with 0.05% Triton for 1 h and blocked with Image-iT Fx signal enhancer (Molecular Probes, Invitrogen, Carlsbad, CA) for 30 min followed by 2% BSA for 5 min. Cells were incubated with anti-V5 antibody at 1:200 dilution for 1 h, washed with PBS (1×), and incubated with Cy3 labeled goat anti-mouse (H + L) antibody at a dilution of 1:500 for 30 min. Specimens were analyzed using a Leica SP5 confocal microscope.

In vitro translation

TRPML1, TRPML2, and TRPML3 were in vitro translated with TNT T7 Coupled Reticulocyte Lysate Systems following the manufacturer's instructions (Promega, Madison, WI). Briefly, 1 μg of each plasmid was mixed with rabbit reticulocyte lysate, T7 RNA polymerase and amino acid mixture in a tube according to manufacturer's instructions, followed by a 90 min incubation period at 30°C. The translated proteins were aliquoted and stored at −70°C.

Electrophysiology

Electrophysiological data were acquired using either an AxoPatch 200B amplifier (Axon Instruments, Union City, CA) or a PC501A amplifier (Warner Instruments Corp., Hamden, CT), low-pass filtered at 10 kHz, and digitized with 1400A Digidata (Axon Instruments). The pClamp 10.0 software (Axon Instruments) was used to acquire and analyze the data. In some cases, tracings were further filtered with a low-pass 8-pole Bessel filter at 100 Hz for display purpose only.

Both in vitro translated TRPML proteins and eluent of co-IP material were re-constituted into a lipid bilayer system to assess for channel properties. A lipid mixture was prepared with a content of 70% 1-palmitoyl-2-oleoyl-phosphatidylcholine and 30% 1-palmitoyl-2-oleoyl phosphatidyl-ethanolamine in *n*-decane (~20–25 mg/ml). In vitro translated material or IP pellets from in vivo transiently expressed mucolipins were mixed with the above lipid solution at 1:1 ratio followed by brief sonication to form liposomes. The liposomes were painted with a glass rod to the aperture (150 μm diameter) of a polystyrene cuvette (CP13-150) that fits in a lipid bilayer chamber (model BCH-13, Warner Instruments Corp.). The *cis* side of the lipid bilayer was bathed with 150 mM KCl and 10 mM HEPES (pH 7.4), while the *trans* side of the bilayer was bathed with 15 mM KCl and 10 mM HEPES (pH 7.4).

Calculations and statistical analysis. The single channel data from current-to-voltage relationships were fitted to the Goldman–Hodgkin–Katz (GHK) equation for either single cation (Eq. 1, solid lines in Fig. 2B), or both cation and anion permeability (Eq. 2, dashed lines in Fig. 2B):

$$I = \left(\frac{zF}{RT}\right)\frac{V\left(C_i - C_o\exp\left(\frac{zF}{RT} * V\right)\right)}{\left(1 - \exp\left(\frac{zF}{RT} * V\right)\right)} \quad (1)$$

$$I = \frac{P_i z_i FaV(C_{ii} - C_{io}\exp(aV))}{1 - \exp(aV)} + \frac{P_i z_i FbV(C_{ij} - C_{jo}\exp(bV))}{1 - \exp(bV)} \quad (2)$$

where I is the current at a certain voltage V, C_i, and C_o is the concentration of ions in *cis* and *trans* side, respectively, z, z_i, and z_j are charges of ions, P_i and P_j are permeabilities for a specific ion, $a = z_iF/RT$, $b = z_jF/RT$, and F, R, and T have their usual meaning.

Data comparisons were made by paired *t*-test analysis, while other statistical analysis was conducted as unpaired *t*-test for groups of similar size (Snedecor and Cochran, 1973).

Results

TRPML1 co-localizes and hetero-multimerizes with TRPML2 and TRPML3

To study the cellular co-localization of TRPML proteins, CHO cells were used to over-express TRPML1-GFP and TRPML2-V5 or TRPML3-V5 tagged proteins (Fig. 1A–C). Fluorescent imaging of transfected cells revealed a high degree of co-localization between TRPML1 and TRPML2 (Fig. 1B) or TRPML3 (Fig. 1C). Expression of each TRPML individually expressed is shown in Figure 1A. Co-localization of TRPML proteins suggests that these proteins may interact with each other to form channel complexes. To test this, we conducted co-IP experiments using TRPML1 tagged with three different epitopes (GFP, Flag, and V5), which confirmed that TRPML1 can form homo-multimers (Fig. 1D,E). High molecular weight bands (>150 KDa) in the SDS–PAGE for TRPML1 corroborates the formation of TRPML1 multimers and reveals the presence of homo-multimeric complexes that are hard to dissociate to monomers even under denaturing conditions. Co-IP experiments performed using TRPML1 plus TRPML2 or

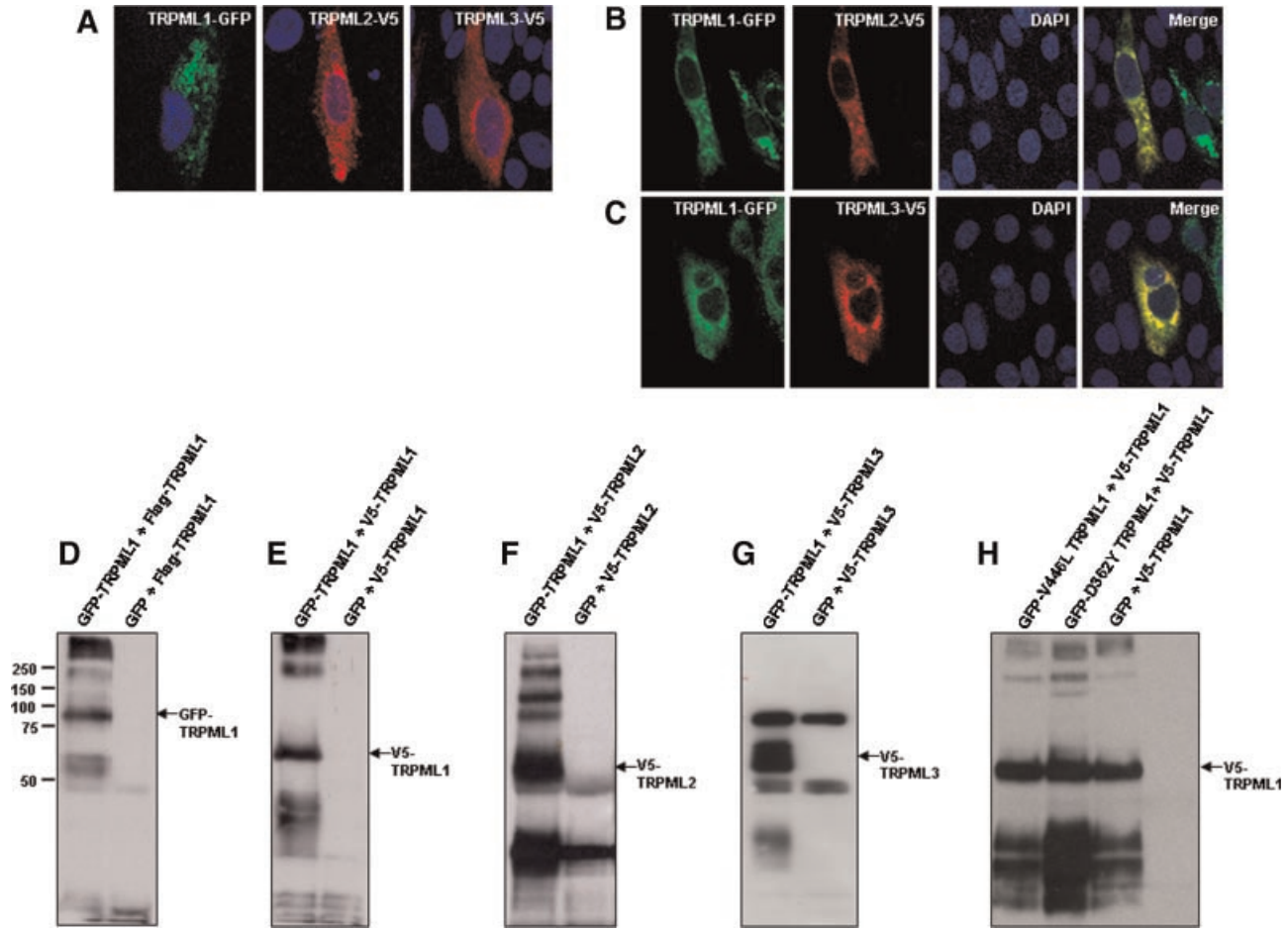

Fig. 1. TRPML1 co-localizes and forms homo-multimers and hetero-multimers with TRPML2 and TRPML3 in CHO cells. CHO cells transiently expressing (A) each TRPML1-GFP, TRPML2-V5, or TRPML3-V5; or (B) co-expressing TRPML1-GFP plus TRPML2-V5; or (C) TRPML1-GFP plus TRPML3-V5 were probed with anti-V5 antibody. Merged images show a high degree of co-localization between TRPML1-GFP and TRPML2-V5 or TRPML3-V5. D: CHO cells transiently expressing GFP-TRPML1 or GFP and TRPML1-Flag were processed for IP with anti-Flag antibody and probed with anti-GFP antibody for Western Analysis. E: Cells transiently expressing GFP-TRPML1 or GFP and TRPML1-V5 were processed for IP with anti-GFP antibody. The pellets and aliquots of the supernatants were probed with anti-V5 antibody for Western analysis. Cells transiently expressing GFP-TRPML1 or GFP and (F) TRPML2-V5 or (G) TRPML3-V5 were processed for IP with anti-GFP antibody. The pellets and aliquots of the supernatants were probed with anti-V5 antibody for Western blot analysis. H: Wild-type TRPML1 interacts with TRPML1 mutants V446L, F408del, and D362Y.

TRPML1 plus TRPML3, show that TRPML1 is capable of forming hetero-complexes with TRPML2 (Fig. 1F) and TRPML3 (Fig. 1G). These results show that the TRPML family is present in vivo as both homo- and hetero-multimeric complexes.

Wild-type TRPML1 co-immunoprecipitates with TRPML1 mutants

The F408del, V446L, and D362Y MLIV mutations were characterized in MLIV patients and their mRNA expression level is comparable with wild-type TRPML1 expression found in cells from normal donors (Sun et al., 2000). Using CHO cells transiently expressing GFP tagged F408del TRPML1, V446L TRPML1, D362Y TRPML1, and V5-tagged wild-type TRPML1, we observed that V5-TRPML1 co-immunoprecipitated with all three TRPML1 mutants, but no interaction was seen in the negative controls expressing only GFP (Fig. 1H). Both deletion and point mutations in TRPML1 had no effect on the homo-multimerization pattern, suggesting that these mutations do not compromise the assembly of the TRPML1 channel. This indicates that some mutations in MLIV may preserve TRPML1 protein structure, permitting normal interactions with other proteins.

Single channel properties of TRPML2 and TRPML3

To determine whether TRPML2 and TRPML3 display the functional properties of ion channels, we turned to an in vitro testing system, which eliminates the contribution of other channels and regulatory proteins in the cell-based expression system. Both TRPML2 and TRPML3 were individually in vitro translated and tested in a lipid bilayer re-constitution system in the presence of a KCl chemical gradient (150:15 mM, *cis–trans* compartment, respectively) (Fig. 2). Both proteins had single channel properties, with a small single channel conductance, cation permeability, and partial permeability to anions, as indicated by fitting experimental data to the GHK equation (Fig. 2B,D, solid lines are fitted with GHK equation for a single cation, while dashed lines are fitted with GHK equation for both cation and anion permeabilities). The fitted data also indicate

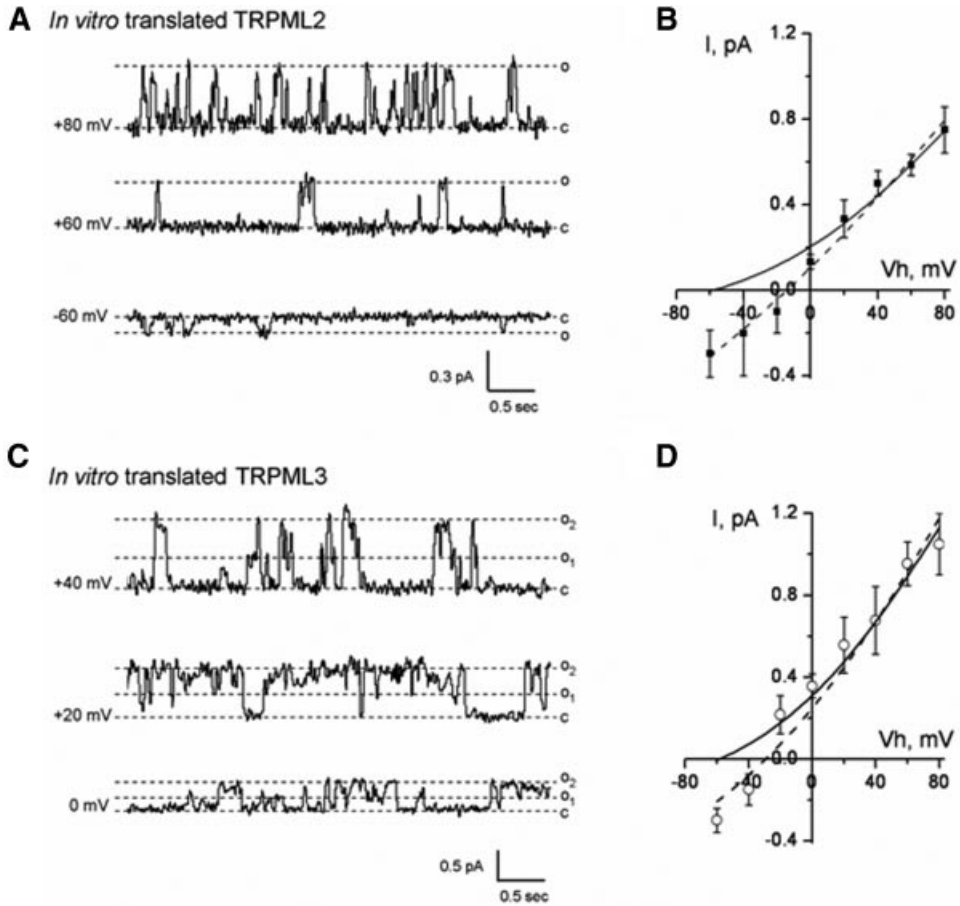

Fig. 2. Single channel properties of in vitro translated TRPML2 and TRPML3. In vitro translated TRPML2 (A,B) and TRPML3 (C,D) were re-constituted in a lipid bilayer system and single channel activity was examined in a K^+ gradient (150:15 mM, *cis–trans* compartment, respectively). The conductance was calculated from the linear region of cation conductance (large positive potentials). Both TRPML2 and TRPML3 showed small single channel conductance, cation permeability, and partial permeability to anions, as indicated by fitting experimental data to the Goldman–Hodgkin–Katz (GHK) equation (B,D). Solid lines were fitted with GHK equation for a single cation, while dashed lines were fitted with GHK equation for both cation and anion permeabilities. The single channel conductance calculated from the linear region of cation conductance, was 7.44 ± 1.29 pS for TRPML2 (n = 11) and 10.2 ± 1.52 pS for TRPML3 (n = 3).

that both TRPML2 and TRPML3 had a much bigger permeability for K^+ than Cl^-. The single channel conductance calculated from the linear region of cation conductance, was 7.44 ± 1.29 pS for TRPML2 (Fig. 2A,B, n = 11) and 10.2 ± 1.52 pS for TRPML3 (Fig. 2C,D, n = 3). The single channel conductances of both TRPML2 and TRPML3 were lower than that of previously observed by TRPML1, which has a larger single channel conductance (35 pS) and more evident cation selectivity (Raychowdhury et al., 2004).

Electrophysiological properties of TRPML homo- and hetero-multimers

Given the channel properties of TRPML2 and TRPML3 and the multimerization capabilities of the various TRPML isotypes in cells, we further investigated whether or not TRPML homo- or hetero-multimers also function as ion channels. Channel activity of in vitro translated wild-type TRPML1, TRPML2, and TRPML3 were assessed with a lipid bilayer re-constitution system (Fig. 3). For the individual mucolipins and the combinations of TRPML1 with TRPML2 or TRPML3, channel activity was consistently found in 10–30% of the experiments. In contrast, no channel activity was found in any of the control experiments performed (n = 20), where samples were re-constituted from in vitro translation reactions where the plasmids had been omitted. Mean currents of in vitro translated TRPML alone and complexes, were all significantly higher than the control mean currents (n = 20, Student's *t*-test, Fig. 3C). Interestingly, mean currents of in vitro translated TRPML1 were 412.5% larger than those from TRPML2 ($P < 0.01$) or 356.4% larger than those from TRPML3 ($P < 0.01$). In addition, TRPML1 mean currents appeared larger than the combination of TRPML1 with either TRPML2 or TRPML3, which is consistent with a prevalent finding that most of these heteromeric combinations, while functional, were in average, more similar to the TRPML2 or TRPML3 isotype phenotype, with rare instances of TRPML1 single channel currents (Fig. 3A,B).

To further assess the in vivo channel properties of TRPML hetero-multimers, we re-constituted TRPML2 and TRPML3 expressed from CHO cells (Fig. 4), and co-IP pellets of TRPML1 + 1, TRPML1 + 2, and TRPML1 + 3 (Fig. 5, as shown on Fig. 1E–G) in the lipid bilayer system. The material from cells expressing either TRPML2 (Fig. 4A) or TRPML3 (Fig. 4B) alone, showed ion channel activity with a very small conductance, similar to the in vitro translated proteins (Fig. 2). These cell-expressed proteins were further used for co-IP assays (Fig. 1) and the co-IP pellets were re-constituted to lipid bilayers

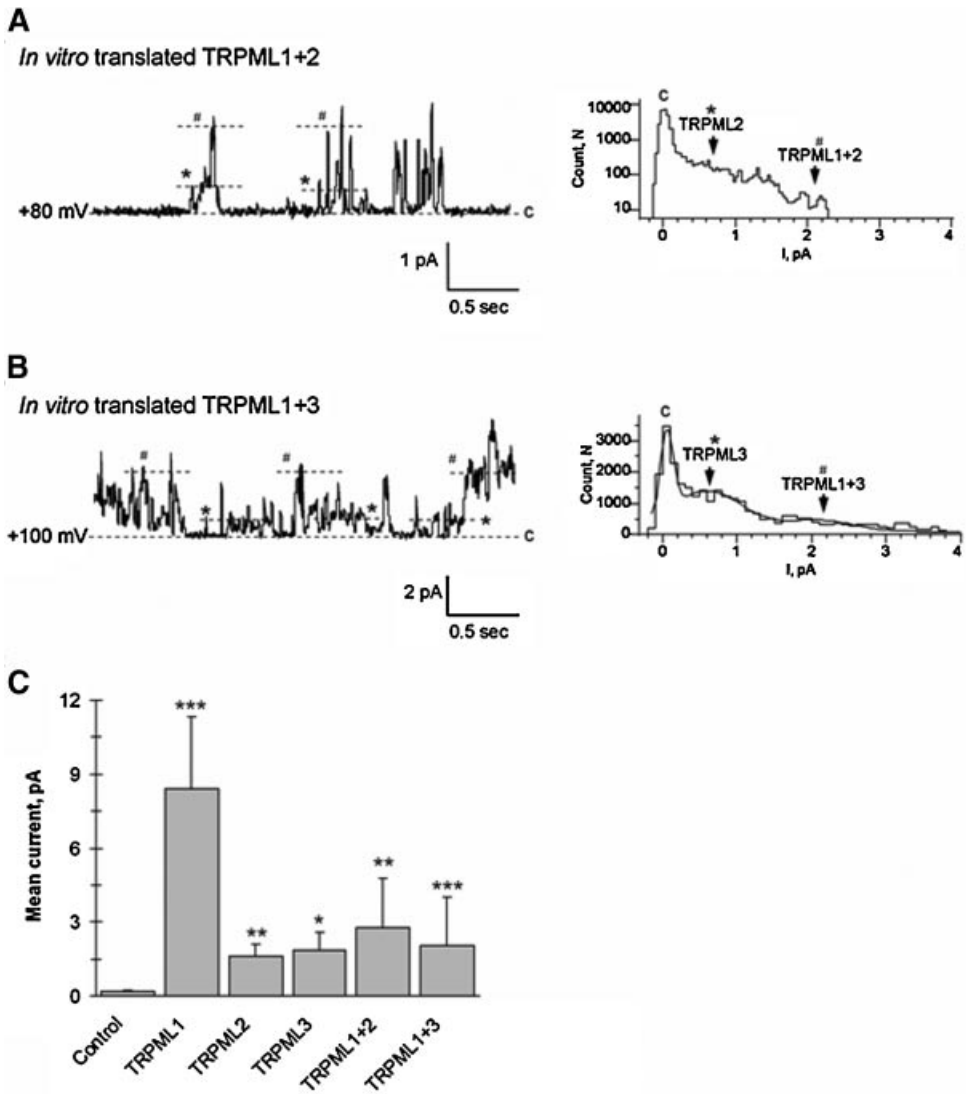

Fig. 3. Single channel properties of in vitro translated TRPML hetero-complexes. In vitro translated TRPML1 + TRPML2 complexes (A) and TRPML1 + TRPML3 complexes (B) both contained multiple subconductances of various sizes, as shown with representative tracings (Left) and amplitude histograms (Right). The smaller conductances (*) are likely contributed by TRPML2 or TRPML3, while the larger conductances (#) are likely contributed by TRPML1. C: Bar graph shows the mean currents at +60 mV of TRPML alone and complexes. The control is a sample from in vitro translation reactions where the plasmids had been omitted, and as a result no channel was found in any of the control experiments performed (n = 20). TRPML1-3 each showed various levels of channel activities, while the combination of TRPML1 and 2 or 1 and 3 altered the channel properties. The mean currents of TRPML alone and complexes were all significantly larger than the mean current of control (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$ with Student's *t*-test). The data are expressed as mean ± SEM. The number of cases for each sample was as follows: Control (n = 20), TRPML1 (n = 12), TRPML2 (n = 24), TRPML3 (n = 22), TRPML1 + 2 (n = 5), and TRPML1 + 3 (n = 2).

for accessing channel properties of TRPML multimers (Fig. 5). As a control, we also re-constituted co-IP pellets using empty GFP vector versus TRPML1-3, which should contain no TRPML proteins (as shown in Fig. 1E–G, right lanes). As expected, we detected no channel activity in most of the experiments (Fig. 5E, GFP + V5-TRPML1-3). We did, however, observed an endogenous Cl^- conductance that equally contaminated all samples ($N_{total} = 47$, including 17 experiments using empty GFP vector + TRPML1, 14 experiments using empty GFP vector + TRPML2, and 16 experiments using empty GFP vector + TRPML3). In contrast, co-IP pellets using TRPML1 versus TRPML1-3 (Fig. 1E–G, left lanes) yielded higher cation-selective channel activity (Fig. 5E, $P < 0.05$), also as expected. Next, we conducted an electrophysiological analysis of co-immunoprecipitates obtained from TRPMLs over-expressing CHO cells. We observed clear electrophysiological differences, including single channel conductance and kinetic behavior for the single channel currents for TRPML1 + TRPML1 (V5-GFP), TRPML1 + TRPML2, or TRPML1 + TRPML3. GFP-TRPML1 + V5-TRPML1 complexes had a most frequent conductance of approximately 35 pS and a much smaller subconductance (~10 pS) (Fig. 5A), consistent with our previous report (Raychowdhury et al., 2004). GFP-TRPML 1 + V5-TRPML2 complexes displayed two subconductances that are presumably contributed by TRPML2 (smaller conductance) and TRPML1 (larger conductance), respectively (Fig. 5B). Similarly, GFP-TRPML1 + V5-TRPML3 complexes also contained both a small conductance (presumably contributed by TRPML3) and a large conductance (presumably

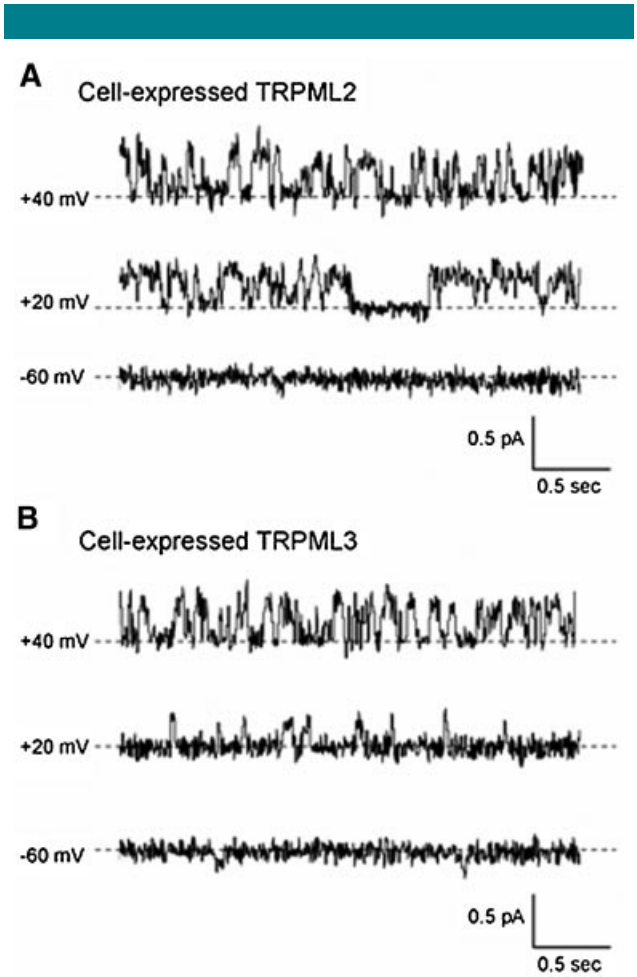

Fig. 4. Single channel activity of TRPML2 (A) and TRPML3 (B) transiently expressed in CHO cells. Cell-expressed TRPML2 and 3 show similar small conductance as in vitro expressed materials (Fig. 3).

contributed by TRPML1) (Fig. 5C). However, the large conductance was larger than that of TRPML1 + TRPML2, suggesting there could be different stoichiometries in the various channel combinations. These data clearly show that the various hetero-complexes display distinct channel properties, which are reflected in the different mean currents (Fig. 5E). To further prove the functional nature of the complexes, either the V5- or the GFP-antibody was used to assess changes in electrical activity of the hetero-complexes.
TRPML1 + TRPML3 hetero-complex currents were selectively blocked by addition of the V5 antibody (Fig. 5D), or the GFP antibody (data not shown), which discloses the contribution of either TRPML subunit in the hetero-complex. Taken together, the data indicate that TRPML1 forms functional hetero-complexes with both TRPML2 and TRPML3, having distinct properties from both the TRPML1 homo-complexes, and the individual proteins.

Discussion

Many TRP channels form either homo- or hetero-multimers, an essential feature for their proper and distinct channel activity (Venkatachalam and Montell, 2007). The first evidence that TRPML1 forms complexes was based on the observation of multi-conductance states of the single TRPML1 channel currents (Raychowdhury et al., 2004). High molecular weight complexes containing TRPML1 are also frequently seen on Western blots (top bands on Fig. 1 D,E) (Miedel et al., 2006; Vergarajauregui and Puertollano, 2006). Hetero-multimeric complexes between the TRPML family members were recently detected by FRET analysis in cells over-expressing mucolipins (Venkatachalam et al., 2006). In the present study, we confirmed that TRPML1 could form both homo- and hetero-multimers with other TRPML members and showed that at least three of the MLIV-causing mutations on TRPML1 did not affect such interactions.

Although the vast majority of the MLIV cases are caused by mutations that render a complete loss of protein (Wang et al., 2002; Goldin et al., 2004; Vergarajauregui and Puertollano, 2006) there are several independent mutations described in the *MCOLN1* gene that result in defective proteins. These defective proteins result in phenotypic variations observed in MLIV disease (Slaugenhaupt, 2002; Goldin et al., 2004; Bach et al., 2005; Dobrovolny et al., 2007). Herein, we show that wild-type TRPML1 oligomerizes with both other TRPML isotypes, and MLIV-causing mutated TRPML1. The F408del, V446L, and D362Y mutants co-immunoprecipitate with normal V5-TRPML1, indicating that these mutations do not affect their capacity to multimerize (Fig. 1H). In that sense, it is possible that inactive monomers of TRPML1, with an intact capacity to multimerize, could associate with normal TRPML2 or TRPML3 and alter their function. It could also mean that some mutants could multimerize and preserve some of their function. F408del is the only MLIV mutation known to preserve function in patients that have normal neurological development despite having high gastrin and ophthalmologic defects (Altarescu et al., 2002). Interestingly, it has been shown that channel function is indeed preserved in F408del and V446L mutations but not the sharp inhibition by lowering pH (Raychowdhury et al., 2004). TRPML1 D362Y mutation has been shown to remain in the endoplasmic reticulum suggesting that this mutation causes MLIV because the channel is not targeted to the lysosomes (Kiselyov et al., 2005). This indicates that these mutations do not affect the capacity of the TRPML subunits to multimerize and suggests the possibility that, in vivo, some TRPML1 mutants could multimerize with either TRPML2 or TRPML3, and potentially modulate phenotypic expression, although this has yet to be demonstrated.

The individual channel properties of each one of the TRPML proteins were assessed in a system devoid of other channels and/or regulatory proteins. Using the lipid bilayer re-constitution system, we demonstrated that wild-type TRPML2 and TRPML3 have intrinsic channel capabilities, and that their electrophysiological properties are different from wild-type TRPML1 in both single channel conductance and cation perm-selectivity. While the TRPML1 channel is a large conductance channel (130–150 pS), which shows smaller single levels of conductance in the order of approximately 35 pS (Raychowdhury et al., 2004), the TRPML2 and TRPML3 channels display only small single channel conductances (~10 pS); further, neither TRPML2 nor TRPML3 channels are strictly cation-selective. Further, mixtures of TRPML1 with either one of the other TRPML proteins, showed distinct channel properties, confirming functional oligomerization.

The fact that the hetero-multimers retain channel activity supports the idea that multimerization itself may play an important role in TRPML channel function. On average, different mean currents were found for TRPML1 + TRPML1, TRPML1 + TRPML2, and TRPML1 + TRPML3 (Fig. 5E). The simplest explanation is that both TRPML2 and TRPML3 affect TRPML1 function, assuming similar levels of the proteins were expressed in both in vitro and in vivo methods. Moreover, at the single channel level, these homo- and hetero-multimeric

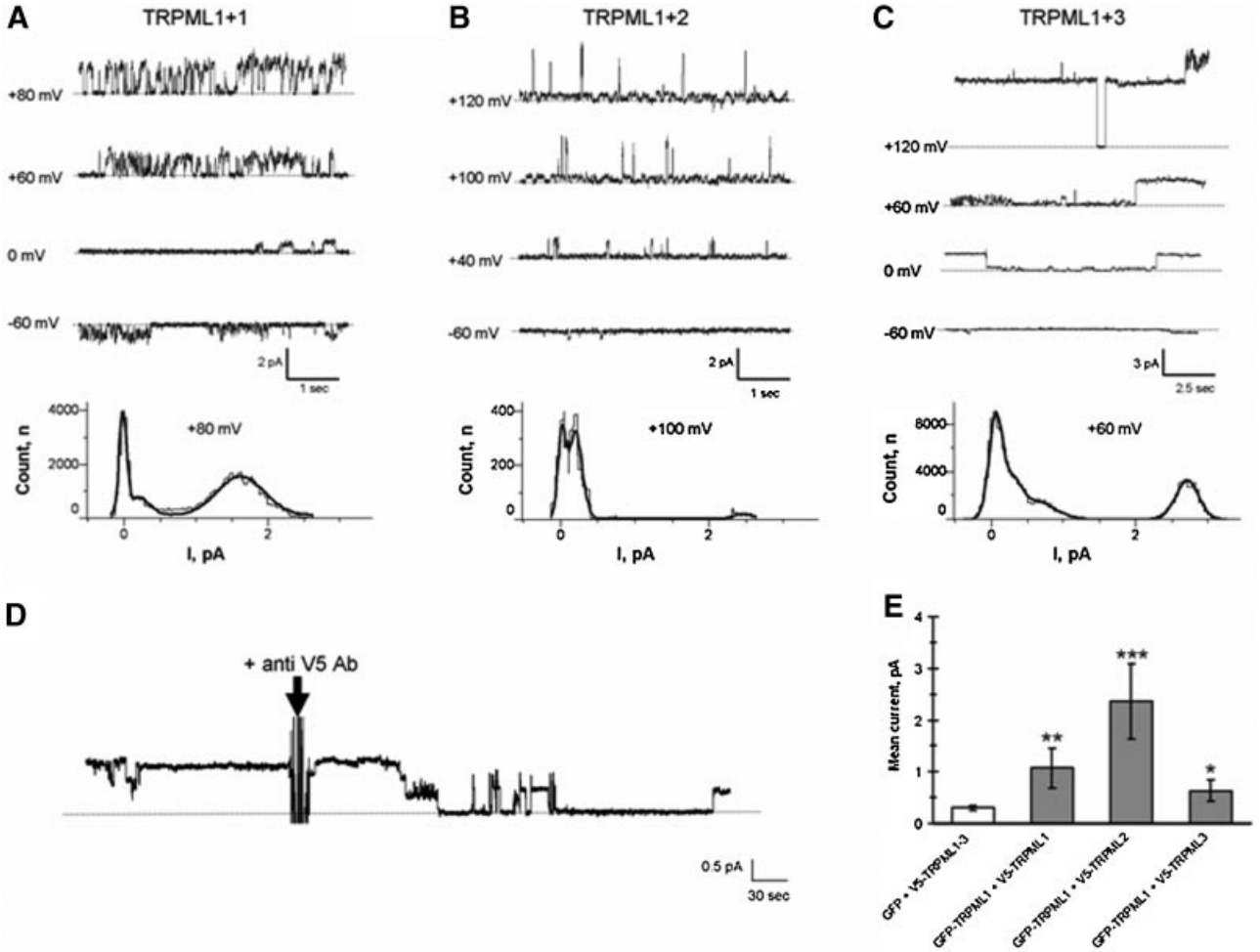

Fig. 5. Channel activity of co-IP samples obtained from TRPLMs transiently expressed in CHO cells. The eluent from co-IP pellets was re-constituted in a lipid bilayer system and channel activity was recorded in the presence of a K^+ gradient (150:15 mM, *cis–trans* compartments, respectively). The channel activity of the co-IP samples was determined with conditions similar to those used for the in vitro translated samples. Single channel currents for all (A) TRPML1 + TRPML1, (B) TRPML1 + TRPML2, or (C) TRPML1 + TRPML3 showed clear differences in single channel conductance and kinetic properties. D: TRPML1 + TRPML3 currents were blocked by addition of 1:100 V5 antibody (at +60 mV). E: The mean currents of controls (co-IP pellets of empty GFP vector + either one of V5-TRPML1-3) and the various hetero-complexes of TRPML channel proteins from co-IP pellets (TRPML1 + 1, TRPML1 + 2, or TRPML1 + 3), were calculated at +60 mV by averaging currents of at least 15 sec for each experiment. The differences in the mean current suggest that the hetero-complexes display distinct channel properties. The data are expressed as mean ± SEM. The number of the cases for each sample was as follows: GFP + V5-TRPML1-3 (N_{total} = 47, including 17 experiments using empty GFP vector + TRPML1, 14 experiments using empty GFP vector + TRPML2, and 16 experiments using empty GFP vector + TRPML3); GFP-TRPML1 + V5-TRPML1 (n = 27); GFP-TRPML1 + V5-TRPML2 (n = 26); and GFP-TRPML1 + V5-TRPML3 (n = 21). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$ with Student's *t*-test comparing with the control (GFP + V5-TRPML1-3).

complexes of TRPML show clear differences in channel activity, supporting that the interaction between TRPML proteins modifies channel function. Another piece of evidence in favor of functional hetero-multimerization is based on the fact that the antibody against V5 partially blocked single channel currents of the complexes, therefore demonstrating interaction among the channel's monomers in the complex. Most preparations, including in vitro translated materials and co-IP samples, provided solid evidence that complexes are functional. Further experiments will be required to fully characterize the biophysical properties of the hetero-complexes, particularly with regards to the effect(s) of Cl^- and pH, voltage dependence, permeability to calcium and other divalent cations, even anions, as well as the stoichiometry of the channel complexes.

Hetero-multimerization is considered an effective way of modulating channel function, subcellular localization, and biophysical properties of interacting TRP channels. It is possible that variable assembly of TRPML proteins, which are differentially expressed in target tissues, results in tissue-specific channel characteristics. *MCOLN2* is highly expressed in heart, liver, and pancreas, with moderate levels of expression in skeletal muscle, kidney, and placenta (data not shown). *MCOLN3* has low levels of expression in most tissues, but can be detected in pancreas, kidney, skeletal muscle, and the inner ear of mice. Since *MCOLN1* expression is ubiquitous, it is possible that there are tissue-specific combinations of TRPML homo- and hetero-multimers, and that the loss of these multimers plays a role in the pathogenesis of MLIV. The recently developed MLIV mouse model, in combination with mice lacking *Mcoln2* and *Mcoln3*, will provide an extremely valuable tool in order to further characterize the physiological relevance of mucolipin family multimerization (Venugopal et al., 2007).

Literature Cited

Altarescu G, Sun M, Moore DF, Smith JA, Wiggs EA, Solomon BI, Patronas NJ, Frei KP, Gupta S, Kaneski CR, Quarrell OW, Slaugenhaupt SA, Goldin E, Schiffmann R. 2002. The neurogenetics of mucolipidosis type IV. Neurology 59:306–313.

Bach G, Webb MB, Bargal R, Zeigler M, Ekstein J. 2005. The frequency of mucolipidosis type IV in the Ashkenazi Jewish population and the identification of 3 novel MCOLN1 mutations. Hum Mutat 26:591.

Bargal R, Avidan N, Ben-Asher E, Olender Z, Zeigler M, Frumkin A, Raas-Rothschild A, Glusman G, Lancet D, Bach G. 2000. Identification of the gene causing mucolipidosis type IV. Nat Genet 26:118–123.

Cantiello HF, Montalbetti N, Goldmann WH, Raychowdhury MK, Gonzalez-Perrett S, Timpanaro GA, Chasan B. 2005. Cation channel activity of mucolipin-1: The effect of calcium. Pflugers Arch 451:304–312.

Di Palma F, Belyantseva IA, Kim HJ, Vogt TF, Kachar B, Noben-Trauth K. 2002. Mutations in Mcoln3 associated with deafness and pigmentation defects in varitint-waddler (Va) mice. Proc Natl Acad Sci USA 99:14994–14999.

Dobrovolny R, Liskova P, Ledvinova J, Poupetova H, Asfaw B, Filipec M, Jirsova K, Kraus J, Elleder M. 2007. Mucolipidosis IV: Report of a case with ocular restricted phenotype caused by leaky splice mutation. Am J Ophthalmol 143:663–671.

Goldin E, Stahl S, Cooney AM, Kaneski CR, Gupta S, Brady RO, Ellis JR, Schiffmann R. 2004. Transfer of a mitochondrial DNA fragment to MCOLN1 causes an inherited case of mucolipidosis IV. Hum Mutat 24:460–465.

Grimm C, Cuajungco MP, van Aken AF, Schnee M, Jors S, Kros CJ, Ricci AJ, Heller S. 2007. A helix-breaking mutation in TRPML3 leads to constitutive activity underlying deafness in the varitint-waddler mouse. Proc Natl Acad Sci USA 104:19583–19588.

Karacsonyi C, Miguel AS, Puertollano R. 2007. Mucolipin-2 localizes to the Arf6-associated pathway and regulates recycling of GPI-APs. Traffic 8:1404–1414.

Kim HJ, Li Q, Tjon-Kon-Sang S, So I, Kiselyov K, Muallem S. 2007. Gain-of-function mutation in TRPML3 causes the mouse varitint-waddler phenotype. J Biol Chem 282:36138–36142.

Kiselyov K, Chen J, Rbaibi Y, Oberdick D, Tjon-Kon-Sang S, Shcheynikov N, Muallem S, Soyombo A. 2005. TRP-ML1 is a lysosomal monovalent cation channel that undergoes proteolytic cleavage. J Biol Chem 280:43218–43223.

LaPlante JM, Falardeau J, Sun M, Kanazirska M, Brown EM, Slaugenhaupt SA, Vassilev PM. 2002. Identification and characterization of the single channel function of human mucolipin-1 implicated in mucolipidosis type IV, a disorder affecting the lysosomal pathway. FEBS Lett 532:183–187.

Miedel MT, Weixel KM, Bruns JR, Traub LM, Weisz OA. 2006. Posttranslational cleavage and adaptor protein complex-dependent trafficking of mucolipin-1. J Biol Chem 281:12751–12759.

Nagata K, Zheng L, Madathany T, Castiglioni AJ, Bartles JR, Garcia-Anoveros J. 2008. The varitint-waddler (Va) deafness mutation in TRPML3 generates constitutive, inward rectifying currents and causes cell degeneration. Proc Natl Acad Sci USA 105:353–358.

Raychowdhury MK, Gonzalez-Perrett S, Montalbetti N, Timpanaro GA, Chasan B, Goldmann WH, Stahl S, Cooney A, Goldin E, Cantiello HF. 2004. Molecular pathophysiology of mucolipidosis type IV: pH dysregulation of the mucolipin-1 cation channel. Hum Mol Genet 13:617–627.

Slaugenhaupt SA. 2002. The molecular basis of mucolipidosis type IV. Curr Mol Med 2:445–450.

Slaugenhaupt SA, Acierno JS, Jr., Helbling LA, Bove C, Goldin E, Bach G, Schiffmann R, Gusella JF. 1999. Mapping of the mucolipidosis type IV gene to chromosome 19p and definition of founder haplotypes. Am J Hum Genet 65:773–778.

Snedecor GW, Cochran WG. 1973. Statistical methods. Ames, IA: Iowa State University Press.

Soyombo AA, Tjon-Kon-Sang S, Rbaibi Y, Bashllari E, Bisceglia J, Muallem S, Kiselyov K. 2006. TRP-ML1 regulates lysosomal pH and acidic lysosomal lipid hydrolytic activity. J Biol Chem 281:7294–7301.

Sun M, Goldin E, Stahl S, Falardeau JL, Kennedy JC, Acierno JS, Jr., Bove C, Kaneski CR, Nagle J, Bromley MC, Colman M, Schiffmann R, Slaugenhaupt SA. 2000. Mucolipidosis type IV is caused by mutations in a gene encoding a novel transient receptor potential channel. Hum Mol Genet 9:2471–2478.

Venkatachalam K, Montell C. 2007. TRP channels. Annu Rev Biochem 76:387–417.

Venkatachalam K, Hofmann T, Montell C. 2006. Lysosomal localization of TRPML3 depends on TRPML2 and the mucolipidosis-associated protein TRPML1. J Biol Chem 281:17517–17527.

Venugopal B, Browning MF, Curcio-Morelli C, Varro A, Michaud N, Nanthakumar N, Walkley SU, Pickel J, Slaugenhaupt SA. 2007. Neurologic, gastric, and opthalmologic pathologies in a Murine Model of mucolipidosis type IV. Am J Hum Genet 81:1070–1083.

Vergarajauregui S, Puertollano R. 2006. Two di-leucine motifs regulate trafficking of mucolipin-1 to lysosomes. Traffic 7:337–353.

Vergarajauregui S, Oberdick R, Kiselyov K, Puertollano R. 2008. Mucolipin 1 channel activity is regulated by protein kinase A-mediated phosphorylation. Biochem J 410: 417–425.

Wang ZH, Zeng B, Pastores GM, Raksadawan N, Ong E, Kolodny EH. 2002. Rapid detection of the two common mutations in Ashkenazi Jewish patients with mucolipidosis type IV. Genet Test 5:87–92.

Xu H, Delling M, Li L, Dong X, Clapham DE. 2007. Activating mutation in a mucolipin transient receptor potential channel leads to melanocyte loss in varitint-waddler mice. Proc Natl Acad Sci USA 104:18321–18326.