Supporting Information

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SI Materials and Methods

cDNA Cloning. Mouse TMEM16A(abc), TMEM16A(ac), and TMEM16B–GFP (pEGFP–N1) were generous gifts from Criss Hartzell (Emory University). The TMEM16B cDNA used has 89 residues truncated from the N terminus. mCherry was amplified using PCR. The mCherry-tagged TMEM16A isoforms were made by switching EGFP with mCherry using BamHI and NotI sites. TMEM16A(a)–GFP, TMEM16A(c)–GFP, and TMEM16A(0)–GFP were made by selectively deleting *a* or *c* segment from TMEM16A(ac)–GFP, using the In-Fusion Cloning kit (Clontech). To create mCherry–CaM–$C1_{PKC\gamma}$, we used overlap extension PCR to fuse $C1_{PKC\gamma}$ to the C terminus of calmodulin. The fusion product was cloned into pcDNA4.1 (Invitrogen) using BamHI and XhoI sites, after which mCherry was PCR amplified and cloned upstream using KpnI and BamHI sites. Point mutation in mCherry–CaM_{1234}–$C1_{PKC\gamma}$ was introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene). To create Xpress–YFP–CaM, YFP and calmodulin were amplified by PCR and sequentially cloned into pcDNA4.1 (Invitrogen) downstream of the Xpress tag sequence using KpnI/BamHI and BamHI/NotI sites, respectively. All PCR products were verified by sequencing. The four alternatively spliced segments are *a* (residues 1–116), *b* (268–289), *c* (470–473), and *d* (498–523).

Cell Culture and Transfection. Low passage number HEK293 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS and 100 $\mu g \cdot ml^{-1}$ penicillin–streptomycin. For electrophysiology experiments, HEK293 cells cultured in 6-cm tissue culture dishes were transiently transfected with the indicated TMEM16A isoform (6 μg), T antigen (2 μg), and the appropriate CaM construct (4 μg), using the calcium phosphate precipitation method. Cells were washed with PBS 4–8 h after transfection, maintained in supplemented DMEM, and replated onto fibronectin-coated glass coverslips 24 h after transfection.

Electrophysiology. Whole-cell recordings of HEK cells were conducted 48–72 h after transfection using an EPC8 or EPC10 patch clamp amplifier (HEKA Electronics) controlled by PULSE software (HEKA). Micropipettes were fashioned from 1.5-mm thin-walled glass with filament (WPI Instruments) and filled with internal solution containing (in millimoles): 130 CsCl, 1 $MgCl_2$, 10 EGTA, 2 MgATP (added fresh), 10 Hepes (pH 7.4), and $CaCl_2$ to obtain the desired free Ca^{2+} concentration (maxchelator.stanford.edu/CaMgATPEGTA-TS.htm). Series resistance was typically 1.5–2 MΩ. There was no electronic series resistance compensation. External solution contained (in millimoles): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 15 glucose and 10 Hepes (pH 7.4). Whole-cell *I–V* curves were generated from a family of step potentials (−100 to +100 mV from a holding potential of 0 mV). Currents were sampled at 25 kHz and filtered at 5 or 10 kHz. Traces were acquired at a repetition interval of 4 s. Leak and capacitive currents were subtracted using a P/8 protocol.

Confocal Microscopy. Static images of TMEM16A iosforms and calmodulin were observed using a Leica TCS SPL AOBS MP confocal microscope system and a 40× oil objective (HCX PL APO 1.25–0.75 NA). HEK293 cells expressing GFP and mCherry fusion proteins were imaged using 488- and 543-nm argon laser line, respectively, for excitation.

Immunoprecipitation and Immunoblotting. Confluent cultures of HEK293 cells plated in 6-cm tissue culture dishes were harvested 48 h after transfection. Cells were washed in PBS and resuspended in 0.5 mL cold lysis buffer (50 mmol/L Tris·HCl, 150 mmol/L NaCl, 1% IGEPAL CA-630) containing 1× protease inhibitor mixture for 30 min. For high calcium experiments, transfected cells were incubated for 30 min with A23187 (5 μM) in the presence of 2 mM external $CaCl_2$ before solubilization in lysis buffer (50 mmol/L Tris·HCl, 150 mmol/L NaCl, 1% IGEPAL CA-630, 2mM $CaCl_2$). Cell lysates were centrifuged at 10,000 × *g* for 15 min at 4 °C and 50 μL of the supernatant removed to be used as input. The remaining 450 μL supernatant was precleared by incubation with 50 μL protein G bead slurry for 1 h on a rotator. The mixture was centrifuged and the resulting supernatant incubated with 4 μg primary antibody (anti-Xpress, Life Technologies) and 50 μL protein G slurry overnight on a rotator. The mixture was again centrifuged, and the pellet washed four times with lysis buffer. A total of 50 μL Laemmli sample buffer was added to the bead pellet and the mixture was vortexed and heated (90–100 °C for 10 min). The sample was centrifuged and the supernatant loaded onto a gel for subsequent SDS/PAGE and Western blot analyses. For input lanes, 5× sample buffer was added to the previously saved supernatant, the mixture was vortexed and heated, and 50 μL was loaded on a gel for SDS/PAGE and Western blot analyses. For immunoblots, primary antibodies (anti-GFP, Invitrogen; 1:10,000 dilution) were detected by horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, Thermo Scientific; 1:5,000 dilution) and enhanced chemiluminescence.

Data and Statistical Analyses. Data were analyzed off-line using PulseFit (HEKA), Microsoft Excel, and Origin software. Statistical analyses were performed in Origin using built-in functions. Statistically significant differences between means ($P < 0.05$) were determined using Student *t* test for comparisons between two groups, or one-way ANOVA followed by pairwise means comparisons using Bonferroni test for multiple groups. Data are presented as means ± SEM.

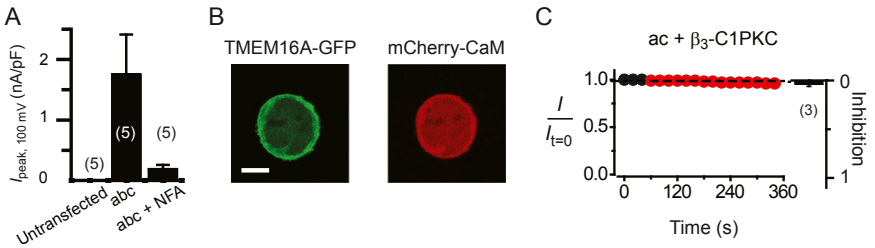

Fig. S1. Control experiments. (*A*) Bar graph showing peak amplitude of Cl^- currents recorded from HEK293 cells either untransfected or expressing TMEM16A (abc) ± 100 μM niflumic acid (NFA). (*B*) Representative confocal images showing the colocalization of TMEM16A(abc)–GFP and mCherry–CaM. (Scale bar, 5 μm.) (*C*) Lack of effect of 1 μM PdBu on calcium-activated chloride currents from cells coexpressing TMEM16A(ac) and $Ca_V\beta_3$–C1PKC.

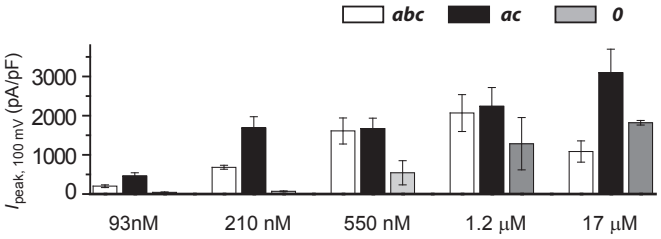

Fig. S2. Distinctive Ca^{2+}-dependent regulation of TMEM16A splice variants. Comparison of $[Ca^{2+}]_i$ dependence of TMEM16A(abc), TMEM16A(ac), and TMEM16A(0) activation. Steady-state current density was recorded at +100 mV plotted vs. free $[Ca^{2+}]_i$.

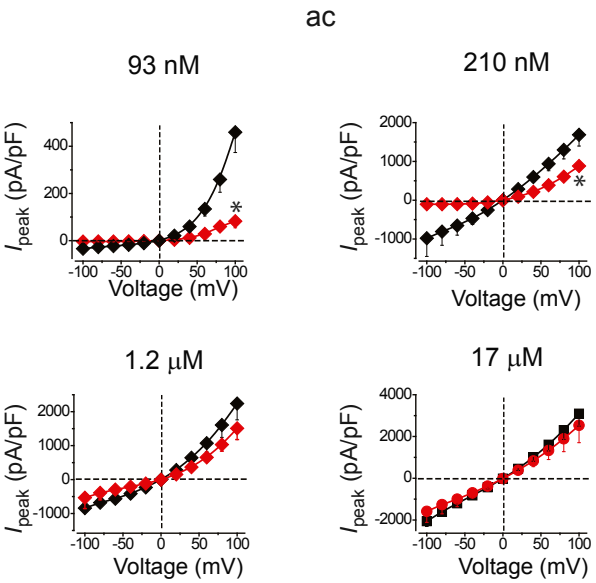

Fig. S3. Impact of CaM and CaM_{1234} on TMEM16A(ac) gating. Population I_{peak}–*V* relationships for TMEM16A(ac) coexpressed with either WT CaM (black diamond) or CaM_{1234} (red diamond). Data are means ± SEM; $n = 5$ for each point. *$P < 0.05$ compared with TMEM16A(ac) + CaM using two-tailed unpaired Student *t* test.

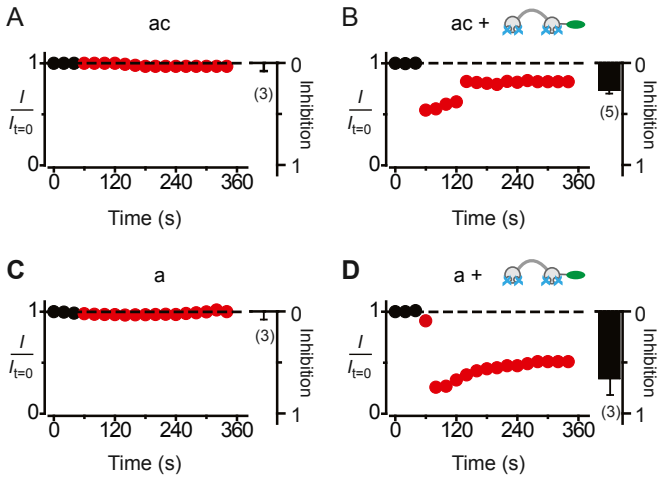

Fig. S4. ChIMP assay for TMEM16A(ac) and TMEM16A(a). (*A*) Diary plots and population bar chart showing effect of 1 μM PdBu on TMEM16A(ac) channels. (*B–D*) Data for TMEM16A(ac) + mCherry–CaM_{1234}–$C1_{PKC}$, TMEM16A(a), and TMEM16A(a) + mCherry–CaM_{1234}–$C1_{PKC}$; same format as *A*.

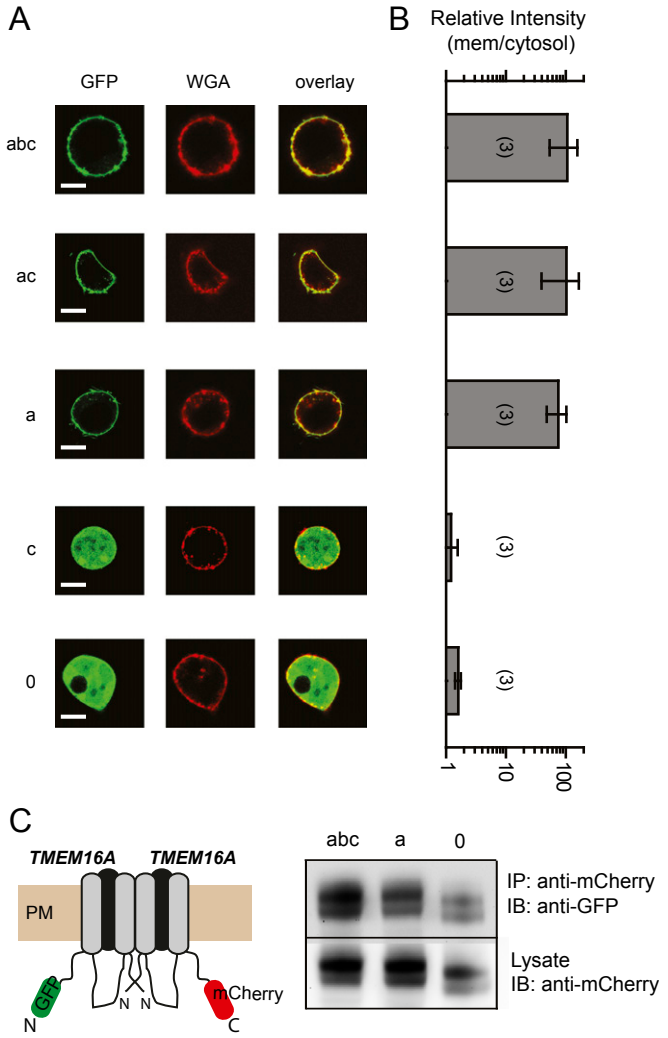

Fig. S5. Splice segment *a* is required for efficient TMEM16A plasma membrane trafficking but not channel dimerization. (*A*) Representative confocal images showing subcellular localization of distinct TMEM16A–GFP splice variants expressed in HEK293 cells. (Scale bar, 5 μm.) The plasma membrane was stained with Alexa 543-tagged wheat germ agglutinin (WGA; red images). (*B*) Relative membrane to cytosol fluorescence intensity ratios for various TMEM16A–GFP splice variants in HEK293 cells. (*C*, *Left*) Schematic showing principle of dimerization assay. (*Right*) Coimmunoprecipitation of GFP-tagged TMEM16A splice variants with mCherry-tagged counterparts.

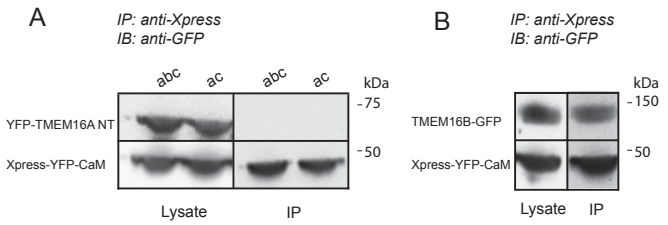

Fig. S6. CaM interaction with TMEM16B. (*A*) Coimmunoprecipitation of YFP-tagged N terminus of TMEM16A(abc) and TMEM16A(ac) with Xpress–YFP–CaM. (*B*) Coimmunoprecipitation of GFP-tagged TMEM16B with Xpress–YFP–CaM.

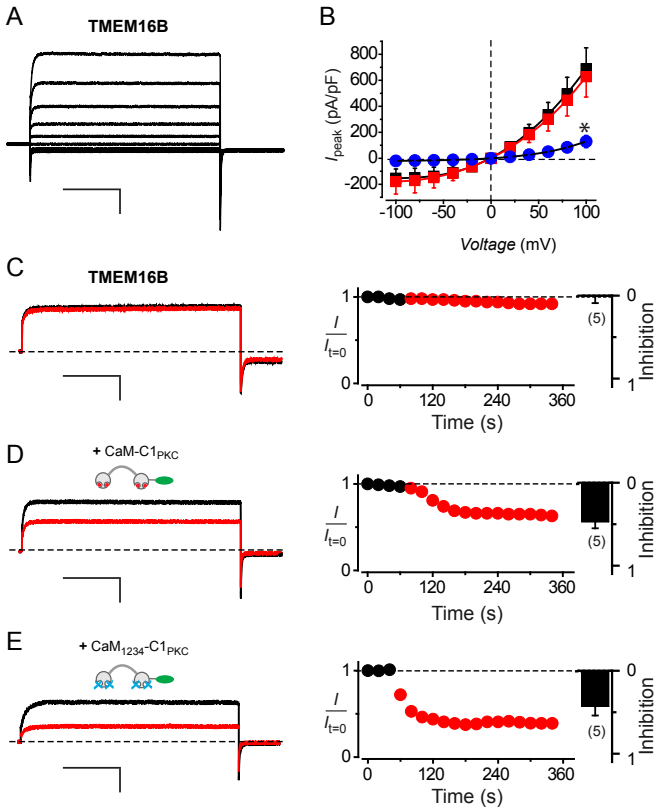

Fig. S7. Preassociated apoCaM regulates TMEM16B. (*A*) Family of TMEM16B channel Cl^- currents elicited by −100- to +100-mV test pulses at a free intracellular Ca^{2+} concentration of 1.2 μM. (Scale bar, 1.5 nA, 200 ms.) (*B*) Population steady-state current–voltage (I_{peak}–*V*) relationships for TMEM16B expressed alone (■) or together with either WT CaM (red square) or CaM1234 (blue dot). Data are means ± SEM; n = 5–7 for each point. *$P < 0.05$ compared with TMEM16B using two-tailed unpaired Student t test. (*C*) ChIMP assay. (*Left*) Exemplar currents before (black trace) and after (red trace) exposure to PdBu in a HEK293 cell transfected with only TMEM16B cDNA. (Scale bar, 0.5 nA, 200 ms.) (*Right*) Diary plots and population bar charts showing PdBu does not affect TMEM16B channels. (*D*) Data for cells coexpressing TMEM16B + CaM–C1$_{PKC\gamma}$; same format as *C*. (Scale bar, 0.5 nA, 200 ms.) (*E*) Data for cells coexpressing TMEM16B + CaM$_{1234}$–C1$_{PKC\gamma}$; same format as *C*. (Scale bar, 0.2 nA, 200 ms.)