Figures and figure supplements

Identification of TMEM206 proteins as pore of PAORAC/ASOR acid-sensitive chloride channels

Florian Ullrich et al
Figure 1. Identification of TMEM206 as ASOR component. (A) Screening assay. Engineered HeLa cells inducibly expressing iodide-sensitive E2GFP and FMRFamide-gated Na⁺ channel FaNaC were acutely exposed to an acidic solution (pH 5) containing 20 μM FMRFamide and 100 mM I⁻. Iodide influx through ASOR is stimulated both by acidic pH and the depolarization caused by FaNaC-mediated Na⁺-influx and induces quenching of E2GFP fluorescence. Fluorescence quenching is reduced by ASOR knock-down. (B) Assay verification under the conditions used for screening. Addition of I⁻ and FMRFamide at pH 5 (arrow) induces rapid quenching of fluorescence. Less rapid quenching upon omission of either I⁻, FMRFamide or acidic pH suggests that it is caused by I⁻ influx through ASOR, as further supported by inhibition by PS (pregnenolone sulfate) and DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid) (Figure 1—figure supplement 1). (C) Fluorescence curves from a 384-well plate treated with siRNA against 280 genes, including TMEM206. Fluorescence quenching is specifically slowed by siRNA against TMEM206. (D) Distribution of median Z-scores (mean of 3 replicates) from filtered hits (see Materials and methods). TMEM206 was the top hit. (E) Tissue expression of TMEM206 extracted from the GTEx database (https://gtexportal.org/home/; TPM, transcripts per million). (F) Dendrogram depicting similarity between TMEM206 orthologs from human (Homo sapiens), African naked mole-rat (Heterocephalus glaber), chicken (Gallus gallus), green anole lizard (Anolis carolinensis), zebrafish (Danio rerio) and from the hemichordate acorn worm (Saccoglossus kowalewskii). Amino-acid sequence identity to human TMEM206 given in brackets. Dendrogram based on a Clustal Omega protein alignment fed into the Simple Phylogeny tool at EMBL-EBI (https://www.ebi.ac.uk/services).

DOI: https://doi.org/10.7554/eLife.49187.002
Figure 1—figure supplement 1. Verification of the assay for the genome-wide siRNA screen. (A–D) Controls in which E2GFP-2A-FaNaC-expressing cells were subjected to solutions containing I- (A, B) or gluconate (C, D) at pH 5 (A, C) or pH 7.4 (B, D). Only depolarization induced by activation of FaNaC by FMRFamide in the presence of I- and acidic pH (A) produced rapid quenching of E2GFP fluorescence. (E–F) ASOR inhibitors pregnenolone sulfate (PS, (E) and DIDS (F)) inhibited fluorescence quenching. PS was added acutely together with the acidic solution. Cells were preincubated with DIDS, which induced a large increase in the absolute signal due to its intrinsic fluorescence.

DOI: https://doi.org/10.7554/eLife.49187.003
Figure 2. Subcellular localization and transmembrane topology of TMEM206. (A) Subcellular localization of TMEM206 (fused to GFP at the C-terminus) in transfected HeLa cells. A similar localization was observed when the tag was attached to the N-terminus. Scale bar: 10 μm. (B) Hydropathy analysis of TMEM206 using the TMPred server (https://embnet.vital-it.ch/software/TMPRED_form.html) suggests the presence of two transmembrane domains. (C, D) Detection of GFP by its fluorescence (green) or immunocytochemistry (red) in cells transfected with GFP-TMEM206 (C) or TMEM206-GFP (D), without (top panels) or with (lower panels) plasma membrane permeabilization. Scale bars: 10 μm. (E) A HA-epitope inserted after residue 271 between TM1 and TM2 (G) in the TMEM206-exHA mutant was detected in non-permeabilized cells. (F) Western blot of membranes from HEK cells transfected with TMEM206-GFP or TMEM206(Dglyc)-GFP in which all four predicted N-linked glycosylation sites between TM1 and TM2 were disrupted by mutagenesis. Note the lower molecular weight of TMEM206(Dglyc)-GFP. Deglycosylation of membrane proteins by PNGaseF reduced the molecular weight of the TMEM206-GFP.
WT, but not the TMEM206(Δglyc)-GFP protein. (G) Schematic topology of TMEM206. Predicted glycosylation sites (N), and phosphorylated sites (P) identified in mass spectrometry (https://www.phosphosite.org/) are indicated, as well as the location of added epitopes.

DOI: https://doi.org/10.7554/eLife.49187.005
Figure 3. TMEM206 mediates $I_{\text{Cl,H}}$ ASOR currents. (A) Voltage-clamp traces of non-transfected (n.t.) and TMEM206-GFP transfected HEK cells at $pH_o = 4.8$, using the protocol shown at the top. (B) Current densities ($I/C$, at indicated $pH_o$) at +80 and -80 mV for non-transfected (n.t.) HEK cells and cells transfected with human TMEM206 (T6) fused N- or C-terminally to GFP, or co-expressing untagged TMEM206 with GFP from a separate pEGFP-N1 vector. Transfection increased current levels by 5- to 15-fold (bars, mean). (C) CRISPR-Cas9 mediated genomic disruption of TMEM206 in HEK cells (by guide RNA g1) abolished native acid-activated ASOR currents ($I_{\text{Cl,H}}$) as determined at $pH_o 4.8$. Clamp protocol as in (A). (D) Outward and (smaller) inward $I_{\text{Cl,H}}$ currents rapidly activate in WT, but not TMEM206$^{-/-}$ HEK cells when $pH_o$ is lowered from 7.4 to 4.8. (E) Native $I_{\text{Cl,H}}$ of HEK cells is abolished in three independent TMEM206$^{-/-}$ cell lines generated with different guide RNAs (g1 – g3). (bars, mean; ***, p<0.001, one-way ANOVA with Bonferroni correction) (F) Voltage-clamp traces of TMEM206$^{-/-}$ HEK cells transfected with human TMEM206 reveal large $I_{\text{Cl,H}}$. Clamp protocol as in (A). (G) Rapid activation and deactivation of $I_{\text{Cl,H}}$ in TMEM206-transfected TMEM206$^{-/-}$ HEK cells when $pH_o$ is changed between 7.4 and 4.8. Currents monitored using a ramp protocol. (H) $I_{\text{Cl,H}}$ densities at indicated voltages and $pH_o$ of non-transfected (n.t.) or TMEM206-transfected TMEM206$^{-/-}$ HEK cells (bars, mean; ***, p<0.001, one-way ANOVA with Bonferroni correction). (I) $pH_o$-dependence of $I_{\text{Cl,H}}$ (at +80 mV) from native HEK cells and TMEM206-transfected TMEM206$^{-/-}$ HEK cells. $pH_{50}$, $pH_o$ at which current is half maximal.

DOI: https://doi.org/10.7554/eLife.49187.006
Figure 3—figure supplement 1. TMEM206 mediates $I_{Cl,H}$ ASOR currents in HeLa cells. (A–C) Voltage-clamp traces of wild type (WT, (A)) and TMEM206$^{-/-}$ HeLa cells (B) at pH$_o$ = 4.8, using the protocol shown in (C). (D) Outward and (smaller) inward $I_{Cl,H}$ currents rapidly activate in WT, but not
Figure 3—figure supplement 1 continued

TMEM206<sup>−/−</sup> HeLa cells when pH<sub>o</sub> is lowered from 7.4 to 4.8. (E) Current densities (at pH<sub>i</sub> 7.4 and pH<sub>o</sub> 4.8) at +80 and −80 mV for non-transfected WT HeLa cell, three independent TMEM206<sup>−/−</sup> clones, and TMEM206-transfected TMEM206<sup>−/−</sup> HeLa cells (rescue). Rescue current densities exceeded those in WT cells by ~10 fold (g1–g3: guide RNAs used for CRISPR-Cas9-mediated gene disruption, see Table 1). Bars, mean, **, p<0.05; ***, p<0.001; one-way ANOVA with Bonferroni correction.

DOI: https://doi.org/10.7554/eLife.49187.007
Figure 4. Ion selectivity and inhibitor sensitivity of TMEM206/ASOR channels. (A) Example traces of $I_{Cl,H}$ (elicited by voltage ramps) from TMEM206-transfected TMEM206$^{−/−}$ HEK cells upon equimolar replacement of extracellular NaCl (150 mM) by the sodium salts of the indicated anions. The intracellular solution contained 150 mM CsCl. The voltage at which $I = 0$ defines the reversal potential. (B) Reversal potentials $E_{rev}$. (C) Upon replacement of extracellular Cl$^−$ by gluconate, or (D) by HSO$_4^−$ /SO$_4^{2−}$, no currents discernible from background were detected (at pH 4.8). (E) Mean inhibition (%) of $I_{Cl,H}$ from TMEM206-transfected TMEM206$^{−/−}$ HEK cells (measured at +80 mV, pH 4.8) by various inhibitors of ASOR. PS, pregnenolone sulfate; DIDS, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid; NFA, niflumic acid. (F) Fast and reversible block of $I_{Cl,H}$ by PS. (G) Example I/V curves of $I_{Cl,H}$ from TMEM206-transfected TMEM206$^{−/−}$ HEK cells exposed to different PS concentrations. (H) Same for block by DIDS. DOI: https://doi.org/10.7554/eLife.49187.010

DOI: https://doi.org/10.7554/eLife.49187.010

Ullrich et al. eLife 2019;8:e49187. DOI: https://doi.org/10.7554/eLife.49187
Figure 5. Properties of TMEM206 orthologs. (A) Typical voltage clamp-traces of indicated TMEM206 orthologs transiently expressed in TMEM206−/− HEK cells at pH 7.4 (top traces) or pH 5.0 (bottom traces). Clamp protocol as in Figure 3A. (B) Mean current amplitudes of tested orthologs at pH 7.4.

Figure 5 continued on next page
and (C) pH$_o$ 5.0. The ortholog from acorn worm failed to give currents because of its retention in the endoplasmic reticulum (Figure 5—figure supplement 1F). (D) pH$_o$-dependence of currents from various orthologs at +80 mV (top) and −80 mV (bottom) as determined from voltage ramps. (E–G) Reversal potentials $E_{\text{rev}}$ of indicated orthologs with external NaCl (E), Nal (F) and Na$_2$SO$_4$ (G). Significant currents with Na$_2$SO$_4$ could be measured only for green anole and zebrafish. $\Delta E_{\text{rev}}$, difference to $E_{\text{rev}}$ for NaCl. All currents measured at pH$_o$ 5.25. (H) Current densities (at +80 mV, pH$_o$ 5.25) with external Na$_2$SO$_4$ (I) Example traces showing determination of $E_{\text{rev}}$ (indicated by arrows) for human, chicken and zebrafish orthologs. bg, background current at pH$_o$ 7.4 (n = 8–13 cells; *, $p<0.033$; ***, $p<0.001$; Kruskal-Wallis test, Dunn’s multiple comparison correction, error bars, SD (B,C) or SEM (D–H)).

DOI: https://doi.org/10.7554/eLife.49187.012
Figure 5—figure supplement 1. Subcellular localization of different GFP-tagged TMEM206 orthologs after transfection into HeLa TMEM206−/− cells. All proteins were fused with GFP at the N-terminus to allow their detection by fluorescence. Scale bars: 10 µm.
DOI: https://doi.org/10.7554/eLife.49187.013
Figure 6. Substituted cysteine accessibility scan of TMEM206 transmembrane domains. (A) Effect of MTSES on currents from WT and mutant TMEM206-transfected TMEM206−/− cells. MTSES had no effect on WT TMEM206, but acutely and irreversibly decreased or increased $I_{Cl,H}$ of I307C and L315C mutants, respectively. Note that acidic pH does not interfere with MTSES reactivity. The protocol was designed to be able to detect potential effects of MTSES at pH 7.4. (B) Ratio of current before ($I_{\text{ctrl}}$) and after ($I_{\text{MTSES}}$) exposure to MTSES, at +80 and −80 mV. Mutants are grouped by location within the two TMDs of TMEM206. For current amplitudes of non-modified cysteine mutants, see Figure 6—figure supplement 1. (C) Current traces of selected mutants elicited by voltage ramps (top) before and after MTSES-application. (D–F) Cysteine mutants showing currents at pH 7.4. (D) Voltage-clamp traces of WT TMEM206 and indicated mutants at pH 7.4. Clamp protocol as in Figure 3A. (E) Mean current densities at pH 7.4. (F) All mutants still responded to low pH.

DOI: https://doi.org/10.7554/eLife.49187.015
Figure 6—figure supplement 1. Acid-induced current densities of cysteine mutants. (A, B) Maximal current densities (I/C) at +80 mV and pH 5.25 for all cysteine mutants in transmembrane domains 1 (TM1) and 2 (TM2) are shown. Bars, mean.

DOI: https://doi.org/10.7554/eLife.49187.016
Figure 6—figure supplement 2. Localization of studied TMEM206 residues. (A) Visualization of the human TMEM206 protein (using software provided by http://wlab.ethz.ch/protter) and helical wheel diagrams of TM1 and TM2. Selected residues are color-coded as explained. In helical wheels, (N) and (C) label the N- and C-terminal end of the displayed polypeptide segments. (B) Alignment of protein sequences encoding TM1 and TM2 from the TMEM206 orthologs studied in this work. Residues color-coded as in (A). With the exception of R87, all residues found to have interesting functional effects on TMEM206/ASOR currents are conserved.

DOI: https://doi.org/10.7554/eLife.49187.017
Figure 7. Ion selectivity changes of mutants in transmembrane domains. (A–D) Current traces of WT (A), L84C (B), I307W (C) and L315D (D) TMEM206 expressed in TMEM206−/− HEK cells measured with indicated extracellular solutions at pH 5.25. Reversal potentials $E_{\text{rev}}$ indicated by arrows. With WT and L315D TMEM206 currents in Na$_2$SO$_4$ were too small for $E_{\text{rev}}$ determination. Currents were elicited by voltage ramps as in Figure 6C. (E) Reversal potential $E_{\text{rev}}$ with extracellular NaCl (***, p<0.001 vs. WT; one-way ANOVA, Bonferroni correction). (F, G) Shift of reversal potentials ($\Delta E_{\text{rev}}$) with extracellular NaI (F) or Na$_2$SO$_4$ (G) relative to that measured with NaCl (NA, not applicable (currents not significantly above background or $E_{\text{rev}}$ not stable over time (I307C)). (H) Current densities at +80 mV with extracellular Na$_2$SO$_4$ at pH 5.25. *, p<0.033 and ***, p<0.001 vs. WT; one-way ANOVA, Bonferroni correction. No statistical analysis was performed on the data in (G). DOI: https://doi.org/10.7554/eLife.49187.019
Figure 7—figure supplement 1. Acid-induced current densities of TMEM206 mutants. Maximal current densities (I/C) at +80 mV and −80 mV and pH 5.25 for the indicated mutants. Note segmented y axis. Bars, mean.
DOI: https://doi.org/10.7554/eLife.49187.020
Figure 8. Role of TMEM206/ASOR channels in acid-induced cell death and volume regulation. (A) Representative pictures of WT and TMEM206/−/− HEK293 cells (clone 2E5) double stained with propidium iodide (PI, red) and Hoechst 33342 (blue) after incubation for 2 hr with control (pH 7.4) or acidic (pH 4.5) solution, also containing the potent inhibitor of TMEM206 pregnenolone sulfate (PS) at 100 µM or not. In this assay, dead cells are stained with PI, membrane impermeant, whereas Hoechst 33342, which is permeant, stains nuclei of all cells. Scale bars: 100 µm. (B) Quantification of PI positive cells over the total number of cells, determined by Hoechst staining, in WT and two independent TMEM206+/− HEK clones generated with different guide RNAs (g2, g3). For quantification, 10x objective microscopic fields were randomly chosen. Bars represent mean ± SEM. Data were acquired in three independent experiments and analyzed by using one-way ANOVA with post hoc multiple comparisons using the Tukey’s test: ***p<0.001. (C) Influence of TMEM206 ablation (C) or block by 100 µM pregnenolone sulfate (PS) (D) on acid-induced cell volume changes of HEK293 cells as monitored by calcein fluorescence. Mean of eight measurements; error range, SEM. Similar results were obtained in three experiments, and similar effects were observed with HeLa cells (Figure 8—figure supplement 1). DOI: https://doi.org/10.7554/eLife.49187.022
Figure 8—figure supplement 1. Role of TMEM206/ASOR ablation or block on acid-induced cell volume changes in HeLa cells. Influence of TMEM206 ablation (A) or block by 100 μM pregnenolone sulfate (PS) (B) on acid-induced cell volume changes of HeLa cells as monitored by calcein fluorescence. Mean of eight measurements; error range, SEM.

DOI: https://doi.org/10.7554/eLife.49187.023