Molecular underpinning of intracellular pH regulation on TMEM16F

Pengfei Liang¹, Huanghe Yang¹,²*

Affiliations:
¹Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, USA
²Department of Neurobiology, Duke University Medical Center, Durham, North Carolina, USA

*Correspondence to:
Huanghe Yang
Email: huanghe.yang@duke.edu
Telephone: 919-684-1406
Abstract

TMEM16F, a dual functional phospholipid scramblase and ion channel, is important in blood coagulation, skeleton development, HIV infection and cell fusion. Despite the advances in understanding its structure and activation mechanism, how TMEM16F is regulated by intracellular factors remains largely elusive. Here we report that TMEM16F lipid scrambling and ion channel activities are strongly influenced by intracellular pH (pH_i). We find that low pH_i attenuates whereas high pH_i potentiates TMEM16F activation. Our biophysical characterizations pinpoint that the pH_i regulatory effects on TMEM16F stem from protonation and deprotonation of the Ca^{2+} binding sites, which in turn reduces and enhances Ca^{2+} binding affinity, respectively. We further demonstrate that intracellular alkalization of 0.5 pH_i can significantly promote TMEM16F activities in a choriocarcinoma cell line. Our findings thus uncover a regulatory mechanism of TMEM16F by pH_i and shine light on understanding the pathophysiological roles of TMEM16F in diseases with dysregulated pH_i including cancer.

Key Words:
TMEM16F, ANO6, scramblase, CaPLSase, pH regulation, intracellular pH, phosphatidylserine
Introduction

The mammalian TMEM16 family consists of ten members. TMEM16A and TMEM16B are Ca$^{2+}$-activated Cl$^-$ channels (CaCCs), which participate in fluid secretion, smooth muscle contraction, gut motility, nociception, motor learning, anxiety and cancer (Hartzell, Putzier and Arreola, 2005; Caputo et al., 2008; Yang et al., 2008; Schroeder et al., 2008; Berg, Yang and Jan, 2012; Cho et al., 2012; Huang et al., 2012; Pedemonte and Galietta, 2014; Oh and Jung, 2016; Whitlock and Hartzell, 2017; Zhang et al., 2017; Crottés and Jan, 2019; Li et al., 2019). Majority of the other TMEM16 members are likely not CaCCs (Suzuki et al., 2010; Yang et al., 2012; Huang et al., 2013; Suzuki, Imanishi and Nagata, 2014; Whitlock et al., 2018; Bushell, Ashley C.W. Pike, et al., 2019). As one of the most studied TMEM16 proteins, TMEM16F is a dual functional Ca$^{2+}$-activated non-selective ion channel and Ca$^{2+}$-activated phospholipid scramblase (CaPLSase), which mediates phospholipids flip-flop across membrane lipid bilayer and rapidly destroy the asymmetric distribution of phospholipids on cell membranes (Suzuki et al., 2010; Yang et al., 2012). TMEM16F-mediated cell surface exposure of phosphatidylserine (PS), an aminophospholipid concentrated in the inner leaflet of the plasma membrane, is essential for a number of cellular and physiological processes including blood coagulation (Suzuki et al., 2010; Yang et al., 2012), skeleton development (Ehlen et al., 2013; Ousingsawat et al., 2015), viral infection (Zaitseva et al., 2017), membrane microparticle release (Fuji et al., 2015), cell-cell fusion and placental development (Zhang et al., 2020). The loss-of-function mutations of human TMEM16F cause Scott Syndrome, a mild bleeding disorder characterized by a deficiency in CaPLSase-mediated PS exposure and subsequent defects on prothrombinase assembly, thrombin generation and blood coagulation (Suzuki et al., 2010; Castoldi et al., 2011). On the other hand, the TMEM16F deficient mice resist thrombotic challenges, suggesting that TMEM16F CaPLSase has the potential to serve as a promising therapeutic target for thrombotic disorders such as stroke, deep vein thrombosis and heart attack (Yang et al., 2012). Given its importance in health and disease, it is thus urgent to understand the molecular mechanisms and cellular functions of TMEM16F.

Recent structural and functional studies have advanced our understanding of the molecular architecture and the activation mechanism of TMEM16 CaPLSases (Pedemonte and Galietta, 2014; Brunner, Schenck and Dutzler, 2016; Whitlock and Hartzell, 2017; Falzone et al., 2018). The pore-gate domain of TMEM16 proteins consists of not only the permeation pathway for phospholipids and ions, but also two highly conserved Ca$^{2+}$ binding sites (Yu et al., 2012; Brunner et al., 2014; Tien et al., 2014). Binding of intracellular Ca$^{2+}$ (Ca$^{2+}$i) triggers conformational changes, which lead to the opening of the activation gates and subsequent lipid and ion permeation (Dang et al., 2017; Paulino et al., 2017; Alvadia et al., 2019; Bushell, Ashley C. W. Pike, et al., 2019; Feng et al., 2019; T. Le et al., 2019). In addition to Ca$^{2+}$i, membrane depolarization also facilitates the activation of TMEM16 CaCCs and TMEM16F ion channels (Yang et al., 2008, 2012; Dang et al., 2017; Paulino et al., 2017). In contrast to the comprehensive understanding of their activation mechanisms, the regulatory mechanisms of TMEM16 proteins just began to emerge.

Phosphatidylinositol (4,5)-bisphosphate (PIP$_2$) was recently shown to plays a critical role in regulating TMEM16A and TMEM16F ion channel rundown or desensitization (Ta et al., 2017; De Jesús-Pérez et al., 2018; Ye et al., 2018; S. C. Le et al., 2019; Tembo et al., 2019; Yu et al., 2019). In addition, both intracellular and extracellular pH have also been reported to regulate endogenous CaCCs (Arreola, Melvin and Begenisich, 1995; Park and Brown, 1995; Qu and
Hartzell, 2000) and heterologous expressed TMEM16A CaCCs (Chun et al., 2015; Cruz-Rangel et al., 2017; Segura-Covarrubias et al., 2020). The Oh laboratory recently reported that intracellular proton can inhibit TMEM16A CaCC by competing with Ca\(^{2+}\) on binding to the Ca\(^{2+}\) binding sites instead of affecting intracellular histidine residues (Chun et al., 2015). Nevertheless, it is not known if TMEM16F’s phospholipid scrambling and ion channel activities can also be regulated by pH\(_{i}\). Interestingly, TMEM16F is highly expressed in various tumor cells including the pancreatic ductal adenocarcinoma cells (Wang et al., 2018), glioma cells (Xuan, Wang and Xie, 2019), and choriocarcinoma BeWo cells (Zhang et al., 2020). Consistent with its high expression level, TMEM16F has been implicated in tumor cell proliferation, migration and metastasis (Jacobsen et al., 2013; Wang et al., 2018; Xuan, Wang and Xie, 2019). Given the fact that pH dysregulation (intracellular alkalization to pH 7.3-7.6 and extracellular acidification to pH 6.8-7.0) is one of the hallmarks of cancer (Webb et al., 2011; White, Grillo-Hill and Barber, 2017), it is thus critical to understand whether pH\(_{i}\) can regulate TMEM16F ion channel and CaPLSase activities.

In this study, we utilized patch clamp and fluorescence imaging methods to systematically characterize the impacts of pH\(_{i}\) on TMEM16F activation. Our results show that intracellular acidification attenuates whereas intracellular alkalization potentiates both TMEM16F ion channel and CaPLSase activities. pH\(_{i}\) mainly affects TMEM16F Ca\(^{2+}\)-dependent activation with negligible effect on its voltage-dependent activation. Our biophysical analysis and mutagenesis studies further demonstrates that the pH\(_{i}\) regulatory effects on TMEM16F stem from the protonation and deprotonation of the Ca\(^{2+}\) binding residues, which in turn reduces and enhances Ca\(^{2+}\) binding affinity, respectively. We also show that intracellular alkalization by 0.5 pH\(_{i}\) from physiological pH\(_{i}\) significantly promotes TMEM16F ion channel and CaPLSase activities in BeWo choriocarcinoma cells. Our findings thus uncover a new regulatory mechanism of TMEM16F, which will facilitate our understanding of the physiological and pathological functions of TMEM16F and other TMEM16 family members in health and disease.

**Results**

**pH\(_{i}\) regulates both TMEM16A and TMEM16F ion channel activities**

In order to evaluate pH\(_{i}\) effect on TMEM16F ion channel activity, we first used TMEM16A CaCC to establish an optimized electrophysiology protocol for quantification. Instead of using the gap-free protocol at fixed membrane voltages in a previous study (Chun et al., 2015), we used inside-out patches to measure TMEM16A current activation across a wide range of voltages under different pH\(_{i}\) so that the conductance-voltage (G-V) relationships can be constructed and the pH\(_{i}\) effects are compared at different pH\(_{i}\), voltages and Ca\(^{2+}\). Consistent with the previous report (Chun et al., 2015), our analysis also shows that low pH\(_{i}\) (=6.1) greatly suppresses whereas high pH\(_{i}\) (=8.9) significantly potentiates TMEM16A activation in the presence of 0.5 µM Ca\(^{2+}\); as evidenced by the dramatic leftward and rightward shifts of the G-V curves, respectively (Fig.1, A, B). By plotting
the mean conductance under different pHs and voltages, which are normalized to the maximum conductance under +100 mV and at pH = 8.9, we constructed the conductance-pH (G-pH) relation (Fig. 1C). By fitting the G-pH relation with linear regression, we obtained the slopes of ~0.3 for activation voltages from +20 to +100 mV. The slopes of the G-pH plots represent the apparent pH sensitivity under 0.5 µM Ca\(^{2+}\), which indicates that each pH unit change or every 10-fold change of intracellular H\(^+\) concentration from physiological pH can lead to ~30% change of TMEM16A channel activation across different activation voltages. The almost identical slopes of the G-pH plots under different membrane potentials suggest that pH has negligible effect on TMEM16A voltage-dependent activation (Fig. 1C).

Using the same patch clamp protocol, we next examined whether pH can regulate TMEM16F ion channel activity. As TMEM16F is less sensitive to Ca\(^{2+}\) than TMEM16A (Yang et al., 2012), we activated TMEM16F channels stably expressed in HEK293 cells using 100 µM Ca\(^{2+}\) and membrane depolarization. Our inside-out patch clamp recordings show that pH also regulates TMEM16F channel activation as it does to TMEM16A (Fig. 1D, E). Alkalized pH (8.0 and 8.9) greatly potentiates TMEM16F current compared to a physiological pH of 7.3. In contrast, acidic pH (5.1, 6.1 and 6.7) suppresses TMEM16F channel activation in a pH-dependent fashion. The apparent pH sensitivity of TMEM16F channel was assessed by plotting the G-pH relationship (Fig. 1F). Our linear regression fittings result in a slope range from 0.19 to 0.21, indicating that for each pH unit reduction, TMEM16F channel activation will be reduced by ~20%. Similar to TMEM16A, TMEM16F pH sensitivity is also voltage independent as evidenced by the parallel G-pH relationships at different voltages. By extrapolating the fittings of the G-pH relationships, we predict that TMEM16F channel activation would be completely abolished when pH drops below 4. As TMEM16F channel subjects to PIP\(_2\)-dependent rundown or desensitization under inside-out configuration (Ye et al., 2018), we thus estimated the influence of rundown on our measurements of the pH effects. The peak currents elicited by a voltage-step protocol only reduced ~20% (Fig. S1A-B) within the time window to complete all the recordings shown in Fig. 1D (about 1 minute). This result suggests that TMEM16F channel rundown only has small effects on our quantification of the pH effects. It is worth mentioning that we sequentially perfused different pH solutions from low pH to high pH and the normalized TMEM16F current to the maximum current in pH 8.9 (Fig. 1E). Thus, if taken channel rundown into account, the pH sensitivity of TMEM16F channel in our measurements would have been underestimated. Taking together, our patch clamp recordings reveal that pH is an important intracellular factor to regulate both TMEM16A and TMEM16F ion channel activities.

**pH regulates TMEM16F scrambling activity**

Having shown that pH can regulate TMEM16F ion channel activity, we next sought to examine whether pH can also influence TMEM16F lipid scrambling activity. In order to quantify TMEM16F lipid scrambling in different pHs, we established a modified fluorescence imaging assay (Le, Le and Yang, 2019; T. Le et al., 2019) to overcome the difficulties in precisely controlling pH and Ca\(^{2+}\) (Fig. 2A). In this lipid scrambling fluorometry assay, pH and Ca\(^{2+}\) are
accurately controlled by solution exchange between the glass pipettes and the cytosol of the whole-cell patch clamped cells. Once we break the patch membrane into whole-cell configuration, the pipette solution with fixed pH_{i} and Ca^{2+} starts to diffuse into the cytosol and activate TMEM16F CaPLSases. Fluorescently conjugated Annexin V (AnV-CF 594) is simultaneously perfused to the patched cell. After a delay during which Ca^{2+} reaches the threshold to activate TMEM16F CaPLSase, AnV starts to be attracted to cell surface by the externalized PS (Fig. 2B). The fluorescence signal accumulated on cell surface increases over time and exhibit a sigmoid relationship with time (Fig. 2C). By fitting the curve with a generalized logistic function, we can obtain t_{1/2}, the time needed to reach half-maximum fluorescence when the macroscopic CaPLSase activity reaches maximum speed. At physiological pH_{i} of 7.3, 100 µM Ca^{2+} activates TMEM16F lipid scrambles with an averaged t_{1/2} of 20.3±2.4 minutes (Fig. 2B-D and Movie S1). We found that intracellular alkalization (pH_{i}=8.9) markedly shortened t_{1/2} to about 11.6±3.5 minutes. In stark contrast, intracellular acidification (pH_{i}=6.1) significantly prolonged t_{1/2} to 29.1±3.0 minutes. The changes of t_{1/2} under different pH_{i} indicate that low pH_{i} attenuates and high pH_{i} enhances TMEM16F CaPLSase activities. We also quantified the maximum lipid scrambling rate at t_{1/2} or the slope (k) of the ‘linear phase’ on the sigmoid curves under different pH_{i}. We found that low pH_{i} (pH_{i}=6.1) significantly reduced slope k from 0.26±0.04 at physiological pH_{i} to 0.19±0.04, whereas high pH_{i} of 8.9 increased slope k to 0.4±0.02 (Fig. 2E), suggesting that pH_{i} affects the maximum lipid scrambling rate of TMEM16F. To ensure the enhanced PS externalization at high pH_{i} is mediated by TMEM16F CaPLSases instead of through other mechanisms such as alkalization induced apoptosis (Lagadic-Gossmann, Huc and Lecureur, 2004), we used the same assay to test our TMEM16F knockout (KO) HEK293 cells (Le, Le and Yang, 2019) in pH_{i} 8.9. In stark contrast to fast lipid scrambling in TMEM16F stable HEK293 cells (Fig. 2C-D), high pH_{i} did not induce AnV binding to the TMEM16F KO cells over time course of 12.5 minutes (Fig. S1C-D and Movie S2). This experiment suggests that apoptosis-induced PS exposure is unlikely to contribute to the pH_{i} effects on TMEM16F CaPLSases under our experimental conditions.

Taken together, our results show that pH_{i} regulates both TMEM16F ion channel and scrambling activities. In comparison to physiological pH_{i}, intracellular acidification suppresses TMEM16F activation, whereas intracellular alkalization enhances TMEM16F activation. This implies that the pH_{i} effects on TMEM16F ion channel and lipid scrambling might share the same molecular mechanism.

**pH_{i} has no effect on voltage-dependent activation of TMEM16A and TMEM16F channels**

We next set out to dissect the molecular mechanism underlying pH_{i} regulation on TMEM16 ion channels and scrambles. TMEM16A and TMEM16F channel activation requires both intracellular Ca^{2+} and membrane depolarization (Pedemonte and Galietta, 2014). We first tested whether pH_{i} has any effect on their voltage-dependent activation of the two channels. We have shown that the G-pH_{i} plots are parallel over a wide range of activation voltages under given Ca^{2+} (Fig. 1C and 1F), suggesting that pH_{i} is likely to have minimal effect on the voltage-dependent activation of TMEM16A and TMEM16F channels. To further support this observation,
we utilized the gain-of-function mutations in the pore-gate domain, namely TMEM16A-Q645A, TMEM16A-L543Q (Fig. 3A), TMEM16F-F518K and TMEM16F-Y563K (Fig. 3D) (Peters et al., 2018; T. Le et al., 2019). As shown in Fig. 3B and 3E, all these GOF mutations can be activated by membrane depolarization in the absence of Ca\(^{2+}\), which allows us to explicitly dissect out the effects of pH\(_i\) on voltage dependent activation in zero Ca\(^{2+}\) (Fig. 3B). When voltage is the only stimulus, pH\(_i\) effects on the TMEM16A (Fig. 3B-C) and TMEM16F mutant channels (Fig. 3E-F) are almost entirely abolished, as evidenced by the nearly flat \(I-pH\) curves for all the GOF mutations (Fig. 3C and 3F). Based on these results, we conclude that pH\(_i\) exerts no obvious effect on voltage-dependent activation of TMEM16A and TMEM16F ion channels.

**pH\(_i\) regulation of TMEM16F ion channel activity is Ca\(^{2+}\) dependent**

As lowering pH\(_i\) has been reported to inhibit Ca\(^{2+}\)-dependent activation of TMEM16A (Chun et al., 2015), we next examined whether pH\(_i\) effect on TMEM16F channel activation is also through influencing its Ca\(^{2+}\) dependence by comparing the pH\(_i\) effects under 5, 100 and 1000 µM Ca\(^{2+}\). Under 5 µM Ca\(^{2+}\), TMEM16F channel activation is strongly pH\(_i\) dependent (Fig. 4A and 4C). However, the pH\(_i\) dependence under 5 µM Ca\(^{2+}\) is apparently different from that under 100 µM Ca\(^{2+}\) (Fig. 1D-E): no TMEM16F channel activity can be observed at low pH\(_i\) (=6.1), and the threshold voltage to activate TMEM16F current is at higher voltage (~80mV) than the threshold activation voltage under 100 µM Ca\(^{2+}\) (Fig. 3A and C). The apparent pH\(_i\) sensitivity at 100 mV under 5 µM Ca\(^{2+}\) increases to 0.32 compared to the pH\(_i\) sensitivity of 0.20 under 100 µM Ca\(^{2+}\), suggesting that pH\(_i\) sensitivity is enhanced when Ca\(^{2+}\) is low (Fig. 4E). On the contrary, the pH\(_i\) effect is almost completely diminished when the Ca\(^{2+}\) is increased to 1000 µM, as seen by the robust and almost identical activation of TMEM16F current from pH\(_i\) 6.1 to 8.9 and nearly flat G-V relationships (Fig. 4B and 4D). Consistently, when saturating Ca\(^{2+}\) (100 µM) was applied to TMEM16A-CaCC at different pH\(_i\), the pH\(_i\) effect on TMEM16A also diminished (Fig. S2). Our results thus demonstrate that the pH\(_i\) regulation of TMEM16F and TMEM16A channel is highly Ca\(^{2+}\) dependent in which saturating Ca\(^{2+}\) (1000 µM for TMEM16F and 100 µM for TMEM16A) eliminates their pH\(_i\) sensitivity, whereas lower Ca\(^{2+}\) boosts pH\(_i\) sensitivity.

**pH\(_i\) regulation of TMEM16F lipid scrambling activity is Ca\(^{2+}\) dependent**

We next addressed whether the pH\(_i\) regulation on TMEM16F lipid scrambling is also Ca\(^{2+}\) dependent using our lipid scrambling fluorometry assay (Fig. 2A). Under 5 µM Ca\(^{2+}\), TMEM16F lipid scrambling is strongly pH\(_i\) dependent (Fig. 5A-B and Movie S3). However, comparing with 100 µM Ca\(^{2+}\), \(t_{1/2}\) under 5 µM Ca\(^{2+}\) is significantly delayed at pH\(_i\) of 7.3 and 8.9 (Fig. 5C and Fig. S3A). In addition, the maximum lipid scrambling rates as quantified by slope k are significantly reduced under 5 µM Ca\(^{2+}\) compared to 100 µM Ca\(^{2+}\) (Fig. 5D and Fig. S3B). TMEM16F scrambling activity under 5 µM Ca\(^{2+}\) is completely abolished when pH\(_i\) was 6.1. This is distinct from TMEM16F lipid scrambling in 100 µM Ca\(^{2+}\) and pH\(_i\) 6.1, under which condition TMEM16F-mediated lipid scrambling can be clearly observed (Fig. 2B-D). On the contrary, when saturated intracellular Ca\(^{2+}\) (1000 µM) was applied, fast and robust PS exposure were detected at all
intracellular pH (Fig. 5E and Movie S4). In addition, the t1/2 and the maximum lipid scrambling rates (slope k) of TMEM16F lipid scrambling are comparable regardless of pH (Fig. 5G-H and Fig. S3). Our imaging experiments thus demonstrate that similar to TMEM16F channel activity (Fig. 4), pH regulation of TMEM16 CaPLSase activity is also highly Ca\textsuperscript{2+} dependent (Fig. S3).

**pH alters Ca\textsuperscript{2+} sensitivity of TMEM16F ion channel**

Having shown that pH\textsubscript{i} regulation of TMEM16F is Ca\textsuperscript{2+} dependent but not voltage dependent, we next tested the hypothesis that pH\textsubscript{i} may directly influence the Ca\textsuperscript{2+} binding sites of TMEM16F following the same pH\textsubscript{i} regulation mechanism of TMEM16A. If this is the case, protonation and deprotonation of the Ca\textsuperscript{2+} binding residues would reduce or increase the Ca\textsuperscript{2+} sensitivity of TMEM16F, respectively. In order to test this hypothesis, we measured the apparent Ca\textsuperscript{2+} sensitivity of TMEM16F under different pH\textsubscript{i} using inside-out patches (Fig. 6A). Our results showed that the EC\textsubscript{50} of TMEM16F Ca\textsuperscript{2+}-dose response under physiological pH\textsubscript{i} is 6.2±2.2 µM (Fig. 6B-C), comparable with previous studies (Yang et al., 2012). When pH\textsubscript{i} drops to 6.1, TMEM16F Ca\textsuperscript{2+} sensitivity decreases more than 20 folds (EC\textsubscript{50} = 144.1±6.8 µM). In stark contrast, when pH\textsubscript{i} is switched to 8.9, TMEM16F Ca\textsuperscript{2+} sensitivity is dramatically enhanced (EC\textsubscript{50} = 1.2±1.2 µM). These results thus further support that pH\textsubscript{i} works on the Ca\textsuperscript{2+} binding sites to exert its regulatory effects.

Next, we extracted the relative conductance (G/G\textsubscript{Max}) from Fig. 6B and plotted the G-pH\textsubscript{i} relationship for each Ca\textsuperscript{2+} (Fig. 6D). The G-pH\textsubscript{i} relationships under different Ca\textsuperscript{2+} are not parallel, which is in stark contrast to the parallel G-pH\textsubscript{i} relationships under different membrane voltages (Fig. 1F). As the slope of the G-pH\textsubscript{i} relationship represents the apparent pH\textsubscript{i} sensitivity, we constructed the pH\textsubscript{i} sensitivity-Ca\textsuperscript{2+} relationship curves in Fig. 6E. Interestingly, the pH\textsubscript{i} sensitivity-Ca\textsuperscript{2+} curve displays a bell-shaped distribution, which peaks around 15 µM Ca\textsuperscript{2+}. Recapitulating the bell-shaped Ca\textsuperscript{2+} response curves of Ins(1,4,5)P\textsubscript{3} (IP\textsubscript{3})- and Ca\textsuperscript{2+}-gated IP\textsubscript{3}R and ryanodine receptor (RyR) channels (Bezprozvanny, Watras and Ehrlich, 1991), the bell-shaped pH\textsubscript{i} sensitivity-Ca\textsuperscript{2+} curve of TMEM16F demonstrates that TMEM16F is strictly pH\textsubscript{i} sensitive under physiological range of Ca\textsuperscript{2+}. TMEM16F channel activation becomes more and more sensitive to pH\textsubscript{i} when Ca\textsuperscript{2+} elevates from resting level of 0.1 µM until its pH\textsubscript{i} sensitivity peaks around 15 µM Ca\textsuperscript{2+}. When Ca\textsuperscript{2+} increases beyond 15 µM Ca\textsuperscript{2+}, TMEM16F pH\textsubscript{i} sensitivity sharply decreases. Our analysis thus defines the physiological range of TMEM16F pH\textsubscript{i} regulation.

**Partially disrupting the Ca\textsuperscript{2+} binding sites enhances pH\textsubscript{i} sensitivity**

To further prove that pH\textsubscript{i} directly affects Ca\textsuperscript{2+} binding residues, we examine the pH\textsubscript{i} sensitivity of E667Q, the Ca\textsuperscript{2+} binding site mutation that markedly reduces TMEM16F Ca\textsuperscript{2+} sensitivity with EC\textsubscript{50} of 2.8 mM (Yang et al., 2012) (Fig. 7 A). In stark contrast to the lack of pH\textsubscript{i} sensitivity of wildtype (WT) TMEM16F under 1000 µM Ca\textsuperscript{2+} (Fig. 4B, 4D and 4E), the activation of the loss-of-function (LOF) E667Q becomes strongly pH\textsubscript{i} dependent in 1000 µM Ca\textsuperscript{2+} (Fig. 7B-D). Similarly, another Ca\textsuperscript{2+} binding site LOF mutation E670Q also exhibits markedly enhanced pH\textsubscript{i} sensitivity under 1000 µM Ca\textsuperscript{2+} (Fig. S4). As a control, we examined the pH\textsubscript{i} sensitivity of
Q559K, a mutation of the pore lining residue Q559 that affects TMEM16F ion selectivity without obvious effect on Ca\(^{2+}\) binding (Fig. 7A) (Yang et al., 2012; Ye et al., 2019). As shown in Fig. 7E-G, the pH\(_i\) sensitivity of Q559K under 100 µM Ca\(^{2+}\) is almost identical with the pH\(_i\) sensitivity of WT TMEM16F channel. Consistent with what we observed in TMEM16F, the LOF mutation of a TMEM16A Ca\(^{2+}\) binding residue E730Q also renders the CaCC with strong pH\(_i\) sensitivity under 100 µM Ca\(^{2+}\) compared with the lack of pH\(_i\) sensitivity of WT TMEM16A under this saturating Ca\(^{2+}\) (Fig. S2 and Fig. S5). Taken together, our systematic biophysical characterizations and mutagenesis experiments explicitly illustrate a pH\(_i\) regulatory mechanism for both TMEM16F and TMEM16A. According to this mechanism, pH\(_i\) regulates the activation of these TMEM16 proteins through protonation and deprotonation of their Ca\(^{2+}\) binding sites, which in turn reduces and enhances their Ca\(^{2+}\) binding affinity, respectively (Fig. 7G). As the Ca\(^{2+}\) binding residues are highly conserved, this pH\(_i\) regulatory mechanism may also apply to other TMEM16 family members.

**Intracellular alkalization promotes TMEM16F activities in a cancer cell line**

Given that intracellular alkalization is one of the hallmarks of cancer cells (Cardone, Casavola and Reshkin, 2005; White, Grillo-Hill and Barber, 2017) and TMEM16F is highly expressed in a wide spectrum of tumors (Jacobsen et al., 2013; Wang et al., 2018; Xuan, Wang and Xie, 2019), we next sought to examine whether endogenous TMEM16F in tumor cells can also be strongly promoted by intracellular alkalization as we observed in the HEK293 cells exogenously expressed with TMEM16F. To address this, we utilized a human choriocarcinoma cell line BeWo that highly expresses TMEM16F CaPLSases to enable cell-cell fusion of the placental trophoblast tumor cells (Zhang et al., 2020). As shown in Fig. 8, mild intracellular alkalization from pH\(_i\) 7.2 to 7.7, the pH\(_i\) range observed in many cancer cells (Webb et al., 2011) can significantly enhance both TMEM16F ion channel (Fig. 8A-C) and CaPLSase activities (Fig. 8D-G and Movie S5). In contrast, intracellular alkalization to pH\(_i\) 7.7 is incapable of inducing any TMEM16F-like current or CaPLSase activity in our TMEM16F deficient (KO) BeWo cells (Zhang et al., 2020) (Fig. S6 and Movie S6). Our characterizations of the endogenous TMEM16F in BeWo cancer cell line thus demonstrate that intracellular alkalization indeed enhances endogenous TMEM16F activities in tumor cells.

**Discussion**

In this study, we report that pH\(_i\) can effectively regulate TMEM16F CaPLSase and ion channel activities. Through systematic investigation of TMEM16F and comparison with TMEM16A-CaCC that is inhibited by intracellular protonation, we found that pH\(_i\) regulates TMEM16F and TMEM16A activation through the same molecular mechanism. Our biophysical characterizations and mutagenesis studies explicitly show that the Ca\(^{2+}\) binding residues within the pore-gate domains of the TMEM16 proteins serve as the pH\(_i\) sensors (Fig. 7H). Protonation of the carboxylate groups of the Ca\(^{2+}\) binding residues prevents Ca\(^{2+}\) binding, thereby hindering...
TMEM16 activation (Fig. 6C). On the contrary, deprotonation of the carboxylate groups of the Ca\(^{2+}\) binding residues facilitates Ca\(^{2+}\) binding, thereby promoting TMEM16 activation.

Histidine, the most titratable amino acid under physiological pH\(_i\) range, is unlikely to be the pH\(_i\) sensor for TMEM16F activation. The previous mutagenesis study of TMEM16A demonstrates that all the intracellular histidine to alanine mutations do not alter the inhibitory effect of proton on TMEM16A activation (Chun et al., 2015). As some of these histidine residues are conserved between TMEM16F and TMEM16A, it is thus plausible to assert that the equivalent histidine residues also do not contribute to TMEM16F pH\(_i\) sensing. In addition, our characterizations of the GOF mutations and LOF mutations in this study further support that the intracellular histidine residues are unlikely to involve in pH\(_i\) regulation. For the GOF mutations shown in Fig. 3, pH\(_i\) losses its effects on TMEM16F and TMEM16A activation in the absence of Ca\(^{2+}\). If some of the histidine residues contribute to pH\(_i\) sensing, we would still be able to see some residual pH\(_i\) sensitivity. In addition, the dramatically enhanced pH\(_i\) sensitivity for the LOF mutations of the Ca\(^{2+}\)-binding sites in TMEM16F (Figs. 7B-C and S4) and TMEM16A (Fig. S5) further support that the Ca\(^{2+}\) binding residues but not the intracellular histidine residues are responsible for pH\(_i\) sensing.

Interestingly, pH\(_i\) sensitivity of TMEM16F is highly Ca\(^{2+}\) dependent and exhibits a bell-shaped relationship with Ca\(^{2+}\)\(_i\) (Fig. 6E). According to this relationship, saturating Ca\(^{2+}\)\(_i\) can override the pH\(_i\) effects on the protonation states of the Ca\(^{2+}\) binding residues, thereby eliminates pH\(_i\) sensitivity for WT TMEM16F and TMEM16A (Figs. 4E, 5F and Fig. S2). Mutating the Ca\(^{2+}\) binding residues dramatically reduces TMEM16F and TMEM16A apparent Ca\(^{2+}\) sensitivities (Yang et al., 2012; Yu et al., 2012; Tien et al., 2014; Alvdia et al., 2019). This disrupts the balance between pH\(_i\) and Ca\(^{2+}\)\(_i\) so that the protonation states of Ca\(^{2+}\) binding site in these LOF mutant proteins gain more weight on influencing Ca\(^{2+}\) binding affinity. Therefore, the Ca\(^{2+}\) binding site mutations become pH\(_i\) sensitivity under high Ca\(^{2+}\)\(_i\), which are saturating for WT TMEM16 proteins (Fig. 7D, Figs. S4D and S5D). On the other hand, when Ca\(^{2+}\)\(_i\) is very low and voltage plays a more prominent role in activating the TMEM16 proteins, the pH\(_i\) sensitivities of TMEM16F and TMEM16A reduces. This is likely because pH\(_i\) has negligible impact on voltage-dependent activation of these proteins as evidenced by the near parallel G-pH\(_i\) relationships across wide range of activation voltages (Fig. 1C and 1F), as well as the lack of pH\(_i\) effects on the voltage dependent activation of the GOF mutations of TMEM16F and TMEM16F in the absence of Ca\(^{2+}\)\(_i\) (Fig. 3).

For TMEM16F, the pH\(_i\) sensitivity gradually increases from resting 0.1 µM Ca\(^{2+}\)\(_i\) to its maximum sensitivity at ~15 µM Ca\(^{2+}\)\(_i\). It is worth noting that all our experiments were conducted at room temperature. According to a recent publication (Lin et al., 2019), TMEM16F is more sensitive to Ca\(^{2+}\)\(_i\) at 37°C. It is therefore plausible that pH\(_i\) may be more sensitive to Ca\(^{2+}\)\(_i\) under physiological temperature.

Intracellular alkalization is one of the hallmarks of cancer cells (Webb et al., 2011). A number of distinct ion transporters and pumps, including the Na\(^+\)-H\(^+\) exchangers (Lauritzen et al., 2012), the H\(^+\)/K\(^+\)-ATPase proton pump (Goh, Sleptsova-Freidrich and Petrovic, 2014) and the
Na\textsuperscript{+}-driven bicarbonate transporters (McIntyre et al., 2016), have been known to contribute to intracellular alkalization. Different from the extensive understanding of pH\textsubscript{i} dysregulation in cancer cells, how intracellular alkalization affects cancer cell function is still elusive. Here we show that a mere 0.5 pH\textsubscript{i} unit increase from the physiological pH\textsubscript{i} of 7.2 can significantly enhance both TMEM16F CaPLSase and ion channel activation at room temperature. Thus, the dual functional TMEM16F CaPLSase and ion channel can be a new pH\textsubscript{i} sensing protein that responds to intracellular alkalization.

TMEM16F has been identified in a wide variety of cancer cells; and according to the Human Protein Atlas (http://www.proteinatlas.org), the high expression level of TMEM16F is associated with the overall prognosis of a number cancers including breast and cervical cancers. Although it is unclear how TMEM16F contributes to tumor growth and cancer progression, it has been reported that genetic manipulations of TMEM16F can change cancer cell proliferation and migration (Jacobsen et al., 2013; Wang et al., 2018; Xuan, Wang and Xie, 2019). Interestingly, loss of membrane phospholipid asymmetry is a salient feature of many cancerous cells, whose cell surfaces display an elevated amount of PS (Riedl et al., 2011; Zhao et al., 2011). It is still unclear which phospholipid transporters are responsible for the enhanced PS exposure on the surfaces of the cancer cells. Nevertheless, no sign of apoptosis (Fidler et al., 1991) and the beneficial effects of PS exposure to cancer cell survival (Schröder-Borm, Bakalova and Andrä, 2005; He et al., 2009; Kenis and Reutelingsperger, 2009; Gerber et al., 2011; Blanco et al., 2014; Zhang et al., 2014) suggest that CaPLSases instead of caspase-dependent lipid scramblases may play important roles in facilitating PS exposure in cancer cells. We show in this study that the CaPLSase and ion channel activities of the endogenous TMEM16F in a human choriocarcinoma cell line indeed can be pronouncedly promoted by intracellular alkalization (Fig. 8). Our current investigation of the mechanism of TMEM16F pH\textsubscript{i} regulation thus lays a foundation to further understand the role of TMEM16F in cancer and other physiological or pathological conditions, in which pH\textsubscript{i} fluctuates or is dysregulated. The shared molecular mechanism of pH\textsubscript{i} regulation between TMEM16F and TMEM16A identified in this study will also facilitate our understanding the regulatory mechanism and physiological functions of other TMEM16 family members.

Methods and Materials

Cell lines and culture

HEK293T and BeWo cells are authenticated by Duke Cell Culture Facility. HEK293T cell line with stable expression of C-terminally eGFP-tagged mTMEM16F was a gift from Dr. Min Li. The TMEM16F and BeWo deficient (KO) HEK293T cell line were generated using CRISPR-Cas9 and have been validated in our recent studies (Le, Le and Yang, 2019; T. Le et al., 2019; Zhang et al., 2020). All our mutagenesis studies were conducted in the TMEM16F-KO HEK293T cells. HEK293T cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM; Gibco, #11995-065) supplemented with 10% fetal bovine serum (Sigma, cat. F2442) and 1% penicillin–
streptomycin (Gibco, #15-140-122). BeWo cells were cultured in Dulbecco's Modified Eagle Medium-Hams F12 (DMEM/F12) medium (Gibco, #11320-033), supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were cultured in a humidified atmosphere with 5% CO2 at 37°C.

Mutagenesis and transfection
The murine TMEM16F (Open Biosystems cDNA # 6409332) and murine TMEM16A (Open Biosystems cDNA # 30547439) coding sequences are in pEGFP-N1 vector, resulting in eGFP tags on their C-termini. Single point mutations were generated using QuikChange site-directed mutagenesis kit (Agilent) and majorities of them have been reported in previous publications. The plasmids were transiently transfected to the TMEM16F-KO HEK 293T cells by using X-tremeGENE9 transfection reagent (Millipore-Sigma). Cells grown on poly-L-lysine (PLL, Sigma) coated coverslips placed in a 24-well plate. Medium was changed 6 hours post transfection with either regular (Gibco, #11995-065) or Ca2+-free DMEM (Gibco, #21068-028). Experiments were proceeded 24–48 hours after transfection.

Electrophysiology
All currents were recorded in either inside-out or whole-cell configurations 24-48 hours after transfection using an Axopatch 200B amplifier (Molecular Devices) and the pClamp software package (Molecular Devices). The glass pipettes were pulled from borosilicate capillaries (Sutter Instruments) and fire-polished using a microforge (Narishge) to reach resistance of 2–3 MΩ.

For inside-out patch, the pipette solution (external) contained (in mM): 140 NaCl, 10 HEPES, 1 MgCl2, adjusted to pH 7.3 (NaOH), and the bath solution contained 140 mM NaCl, 10 mM HEPES, 5 EGTA, adjusted to pH 7.3 (NaOH). Zero Ca2+ internal solution contains (in mM): 140 NaCl and 10 HEPES, 5 EGTA. Due to the difficulties to accurately measure and control free Ca2+ under different pHi, we directly added CaCl2 into a solution containing (in mM): 140 NaCl and 10 HEPES in the absence of Ca2+ chelator. We may have underestimated the free Ca2+ concentrations. But this seems to be the only way to make sure the free Ca2+ concentrations keep the same under different pHi. Different pH levels were adjusted either by NaOH or HCl as required.

For whole cell recordings, the pipette solution (internal) contained (in mM): 140 CsCl, 1 MgCl2, 10 HEPES, plus CaCl2 to obtain the desired free Ca2+ concentration. pH was adjusted either by CsOH or HCl as desired. The bath solution contained 140 mM NaCl, 10 mM HEPES, 1 MgCl2 and adjusted to pH 7.3 (NaOH). Procedures for solution application were as employed previously (S. C. Le et al., 2019). Briefly, a perfusion manifold with 100–200 µm tip was packed with eight PE10 tubes. Each tube was under separate valve control (ALA-VM8, ALA Scientific Instruments), and solution was applied from only one PE10 tube at a time onto the excised patches or whole-cell clamped cells. All experiments were at room temperature (22–25°C). All the chemicals for solution preparation were obtained from Sigma-Aldrich.

Phospholipid scrambling fluorometry
The phospholipid scrambling fluorometry was adapted from the method developed by the Hartzell laboratory (Yu et al., 2015). Instead of delivering and detecting current, the glass pipettes under whole-cell configuration were used to achieve precise control of intracellular pH and Ca\(^{2+}\). Briefly, the cells were seeded and transfected on poly-L-lysine (Sigma)-coated coverslips prior to the experiments. Glass pipettes were prepared and filled with internal solution as mentioned previously in the electrophysiology section. After focusing on the cell surface eGFP signal from the expressed TMEM16F-eGFP, the light filter set was switched to detect Annexin V-CF594 signal (594 nm). Annexin V-CF594 (Biotium #29011) was diluted at 0.5 µg ml\(^{-1}\) with Annexin V-binding solution (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl\(_2\), pH 7.4) and then added into the ALA perfusion system as mentioned above. The perfusion manifold was lowered next to the cell to be patched. Once the whole-cell configuration was established, the perfusion valve was simultaneously opened and Anv solution was flushed to the patch-clamped cell. At the same time, image acquisition started with intervals of 5-10 seconds.

**Data Analysis**

\(G-V\) curves were constructed from tail currents measured 200-400 µs after repolarization. In the cases when the tail currents were tiny, the steady-state peak currents were used to build the \(I-V\) relation. For the \(G-V\) curves obtained from the same patch, the conductance was normalized to the maximal conductance obtained at pH 8.9 and the highly depolarization voltage. Individual \(G-V\) curves were fitted with a Boltzmann function,

\[
G(V) = \frac{G_{\text{max}}}{1 + e^{-\frac{ZF(V-V_{0.5})}{RT}}}
\]

(1)

where \(G_{\text{max}}\) denotes the fitted value for maximal conductance at a given pH, \(V_{0.5}\) denotes the voltage of half maximal activation of conductance, \(z\) denotes the net charge moved across the membrane during the transition from the closed to the open state and \(F\) denotes the faraday constant.

Dose response curves were fitted with Hill equation,

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + \frac{[EC_{50}]}{[Ca^{2+}]^{H}}}\]

(2)

where \(G/G_{\text{max}}\) denotes current normalized to the max current elicited by 1000 µM Ca\(^{2+}\) at given pH\(_i\), [Ca\(^{2+}\)] denotes free Ca\(^{2+}\) concentration, \(H\) denotes Hill coefficient, and \(EC_{50}\) denotes the half-maximal activation concentration of Ca\(^{2+}\).

Bell-shape fitting was performed with bell-shaped dose-response in Prism software (GraphPad). Briefly, the pH\(_i\) sensitivity (Y) and Ca\(^{2+}\) concentration (X) were fitted with the following equations,

\[Y = \text{Peak} + \text{Section1} + \text{Section2}\]

(3)
Section1 = \frac{\text{Span1}}{1 + 10^{(\text{LogEC50}_1 - X)} \cdot nH_1} \quad (4)

Section2 = \frac{\text{Span2}}{1 + 10^{(\text{LogEC50}_2 - X)} \cdot nH_2} \quad (5)

\text{Span1} = \text{Plateau1} - \text{Peak} \quad (6)

\text{Span2} = \text{Plateau2} - \text{Peak} \quad (7)

where, \text{Plateau1} and \text{Plateau 2} denote the plateaus at the left and right ends of the curve, which are set to be 0 in this study; \text{Peak} is the maximum value of the curve. \text{X} is the Ca^{2+} concentration. \text{LogEC50}_1 and \text{LogEC50}_2 are the concentrations that give half-maximal stimulatory and inhibitory effects in the same units as \text{X}; \text{nH}_1 and \text{nH}_2 are the unitless slope factors or Hill slopes, which are set to be 1 in this study.

PDB coordinate files were downloaded from the Protein Data Bank website https://www.rcsb.org/. All figures were generated in Pymol software (Schrödinger, Inc.).

Quantifying phospholipid scrambling activity

To analyze the accumulation of AnV fluorescent signal on cell surfaces over time, a previously reported MATLAB (Mathworks) code was used (T. Le et al., 2019). Briefly, a region of interest (ROI) was manually chosen around the scrambling cells, and the AnV fluorescent intensity was calculated using the following equation for each frame,

\[ F = \sum_{n=1}^{N} f \quad (8) \]

where \( f \) equals the fluorescent intensity of each pixel and \( N \) is the number of the pixels in the ROI.

The time-dependent increase of AnV fluorescence was fitted with generalized logistic equation (Yin et al., 2003),

\[ F = \frac{F_{\text{max}}}{1 + e^{-k(t-t_{1/2})}} \quad (9) \]

where \( F_{\text{max}} \) is the maximum normalized AnV fluorescent intensity, which is set to 100% in this study; \( k \) is the maximum growth rate or the ‘slope’ of the ‘linear phase’ in the sigmoidal curve; \( t_{1/2} \) is the time point at which the growth rate reaches its maximum and the fluorescent intensity reaches 1/2 of its maximum value.

Statistical analysis

All statistical analyses were performed with Clampfit 10.7, Excel and Prism software (GraphPad). Two-tailed Student’s t-test was used for single comparisons between two groups (paired or
unpaired), and one-way ANOVA following by Tukey’s test was used for multiple comparisons. Data were represented as mean ± standard error of the mean (SEM). Symbols *, **, ***, **** and ns denote statistical significance corresponding to p-value <0.05, <0.01, <0.001, <0.0001 and no significance, respectively.

Data Availability Statement.
All data discussed in the paper will be made available to readers upon request.

Acknowledgement
We are grateful to Son C. Le for his help on electrophysiology and plotting the structural models. We thank Drs. Jianmin Cui and Jorg Grandl their constructive suggestions on the projects. We appreciate Trieu Le, Yang Zhang and Ping Dong for their generous help and comments on this project. This work was supported by NIH-DP2-GM126898 (to H.Y.).

Author Contributions
H.Y. conceived and supervised the project. P.L. performed phospholipid scrambling and electrophysiology experiments, as well as data analysis. P.L. and H.Y. wrote the manuscript.

Competing Interests
The authors declare no competing financial interests.

References
Alvadia, C. et al. (2019) ‘Cryo-EM structures and functional characterization of the murine lipid scramblase TMEM16F’, eLife. eLife Sciences Publications Ltd, 8. doi: 10.7554/eLife.44365.
Arreola, J., Melvin, J. E. and Begenisich, T. (1995) ‘Inhibition of Ca2+-dependent Cl- channels from secretory epithelial cells by low internal pH’, The Journal of Membrane Biology. Springer-Verlag, 147(1), pp. 95–104. doi: 10.1007/BF00235400.
Berg, J., Yang, H. and Jan, L. Y. (2012) ‘Ca2+-activated Cl-channels at a glance’, Journal of Cell Science. Company of Biologists, 125(6), pp. 1367–1371. doi: 10.1242/jcs.093260.
Bezprozvanny, I., Watras, J. and Ehrlich, B. E. (1991) ‘Bell-shaped calcium-response curves of lnsl(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum’, Nature Publishing Group, 351(6329), pp. 751–754. doi: 10.1038/351751a0.
Blanco, V. M. et al. (2014) ‘Phosphatidylserine-selective targeting and anticancer effects of SapC-DOPS nanovesicles on brain tumors’, Oncotarget. Impact Journals LLC, 5(16), pp. 7105–7118. doi: 10.18632/oncotarget.2214.
Brunner, J. D. et al. (2014) ‘X-ray structure of a calcium-activated TMEM16 lipid scramblase’, Nature, 516(7530), pp. 207–212. doi: 10.1038/nature13984.
Brunner, J. D., Schenck, S. and Dutzler, R. (2016) ‘Structural basis for phospholipid scrambling in the TMEM16 family’, Current Opinion in Structural Biology. Elsevier Ltd, pp. 61–70. doi:
1610.1016/j.sbi.2016.05.020.

Bushell, S. R., Pike, Ashley C. W., et al. (2019) ‘The structural basis of lipid scrambling and inactivation in the endoplasmic reticulum scramblase TMEM16K’, Nature Communications. Nature Publishing Group, 10(1), pp. 1–16. doi: 10.1038/s41467-019-11753-1.

Bushell, S. R., Pike, Ashley C. W., et al. (2019) ‘The structural basis of lipid scrambling and inactivation in the endoplasmic reticulum scramblase TMEM16K’, Nature Communications. Springer Science and Business Media LLC, 10(1). doi: 10.1038/s41467-019-11753-1.

Caputo, A. et al. (2008) ‘TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity’, Science, 322(5901), pp. 590–594. doi: 10.1126/science.1163518.

Cardone, R. A., Casavola, V. and Reshkin, S. J. (2005) ‘The role of disturbed pH dynamics and the NA+/H+ exchanger in metastasis’, Nature Reviews Cancer, pp. 786–795. doi: 10.1038/nrc1713.

Castoldi, E. et al. (2011) ‘Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome’, Blood. American Society of Hematology, pp. 4399–4400. doi: 10.1182/blood-2011-03-32502.

Cho, H. et al. (2012) ‘The calcium-activated chloride channel anoctamin 1 acts as a heat sensor in nociceptive neurons’, Nature Neuroscience. Nature Publishing Group, 15(7), pp. 1015–1021. doi: 10.1038/nn.3111.

Chun, H. et al. (2015) ‘Protons inhibit anoctamin 1 by competing with calcium’, Cell Calcium. Churchill Livingstone, 58(5), pp. 431–441. doi: 10.1016/j.ceca.2015.06.011.

Crottès, D. and Jan, L. Y. (2019) ‘The multifaceted role of TMEM16A in cancer’, Cell Calcium. Elsevier Ltd. doi: 10.1016/j.ceca.2019.06.004.

Cruz-Rangel, S. et al. (2017) ‘Extracellular protons enable activation of the calcium-dependent chloride channel TMEM16A’, Journal of Physiology, 595(5), pp. 1515–1531. doi: 10.1113/JP273111.

Dang, S. et al. (2017) ‘Cryo-EM structures of the TMEM16A calcium-activated chloride channel’, Nature. Nature Publishing Group, 552(7685), pp. 426–429. doi: 10.1038/nature25024.

Ehlen, H. W. A. et al. (2013) ‘Inactivation of anoctamin-6/Tmem16f, a regulator of phosphatidylserine scrambling in osteoblasts, leads to decreased mineral deposition in skeletal tissues’, Journal of Bone and Mineral Research, 28(2), pp. 246–259. doi: 10.1002/jbmr.1751.

Falzone, M. E. et al. (2018) ‘Known structures and unknown mechanisms of TMEM16 scramblases and channels’, Journal of General Physiology. Rockefeller University Press, 150(7), pp. 933–947. doi: 10.1085/jgp.201711957.

Feng, S. et al. (2019) ‘Cryo-EM Studies of TMEM16F Calcium-Activated Ion Channel Suggest Features Important for Lipid Scrambling’, Cell Reports. Elsevier B.V., 28(2), pp. 567-579.e4. doi: 10.1016/j.celrep.2019.06.023.

Fidler, I. J. et al. (1991) ‘Elevated Expression of Phosphatidylserine in the Outer Membrane Leaflet of Human Tumor Cells and Recognition by Activated Human Blood Monocytes’, Cancer
Research. American Association for Cancer Research, 51(11), pp. 3062–3066.

Fujii, T. et al. (2015) ‘TMEM16F is required for phosphatidylserine exposure and microparticle release in activated mouse platelets’, Proceedings of the National Academy of Sciences of the United States of America. National Academy of Sciences, 112(41), pp. 12800–12805. doi: 10.1073/pnas.1516594112.

Gerber, D. E. et al. (2011) ‘Phase I safety and pharmacokinetic study of bavituximab, a chimeric phosphatidylserine-targeting monoclonal antibody, in patients with advanced solid tumors’, Clinical Cancer Research. American Association for Cancer Research, 17(21), pp. 6888–6896. doi: 10.1158/1078-0432.CCR-11-1074.

Goh, W., Sleptsova-Freidrich, I. and Petrovic, N. (2014) ‘Use of proton pump inhibitors as adjunct treatment for triple-negative breast cancers. An introductory study’, Journal of Pharmacy and Pharmaceutical Sciences. Canadian Society for Pharmaceutical Sciences, 17(3), pp. 439–446. doi: 10.18433/J34608.

Hartzell, C., Putzier, I. and Arreola, J. (2005) ‘CALCIUM-ACTIVATED CHLORIDE CHANNELS’, Annual Review of Physiology. Annual Reviews, 67(1), pp. 719–758. doi: 10.1146/annurev.physiol.67.032003.154341.

He, J. et al. (2009) ‘Antiphosphatidylserine antibody combined with irradiation damages tumor blood vessels and induces tumor immunity in a rat model of glioblastoma’, Clinical Cancer Research. American Association for Cancer Research, 15(22), pp. 6871–6880. doi: 10.1158/1078-0432.CCR-09-1499.

Huang, F. et al. (2013) ‘TMEM16C facilitates Na + -activated K + currents in rat sensory neurons and regulates pain processing’, Nature Neuroscience. Nature Publishing Group, 16(9), pp. 1284–1290. doi: 10.1038/nn.3468.

Huang, W. C. et al. (2012) ‘Calcium-Activated Chloride Channels (CaCCs) Regulate Action Potential and Synaptic Response in Hippocampal Neurons’, Neuron, 74(1), pp. 179–192. doi: 10.1016/j.neuron.2012.01.033.

Jacobsen, K. S. et al. (2013) ‘The role of TMEM16A (ANO1) and TMEM16F (ANO6) in cell migration’, Pflugers Archiv European Journal of Physiology, 465(12), pp. 1753–1762. doi: 10.1007/s00424-013-1315-z.

De Jesús-Pérez, J. J. et al. (2018) ‘Phosphatidylinositol 4,5-bisphosphate, cholesterol, and fatty acids modulate the calcium-activated chloride channel TMEM16A (ANO1)’, Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. Elsevier B.V., 1863(3), pp. 299–312. doi: 10.1016/j.bbalip.2017.12.009.

Kenis, H. and Reutelingsperger, C. (2009) ‘Targeting Phosphatidylserine in Anti-Cancer Therapy’, Current Pharmaceutical Design. Bentham Science Publishers Ltd., 15(23), pp. 2719–2723. doi: 10.2174/138161209788923903.

Lagadic-Gossmann, D., Huc, L. and Lecureur, V. (2004) ‘Alterations of intracellular pH homeostasis in apoptosis: Origins and roles’, Cell Death and Differentiation. pp. 953–961. doi: 10.1038/sj.cdd.4401466.

Lauritzen, G. et al. (2012) ‘The Na +/H + exchanger NHE1, but not the Na +, HCO3-
cotransporter NBCn1, regulates motility of MCF7 breast cancer cells expressing constitutively active ErbB2’, *Cancer Letters*, 317(2), pp. 172–183. doi: 10.1016/j.canlet.2011.11.023.

Le, S. C. *et al.* (2019) ‘Molecular basis of PIP2-dependent regulation of the Ca2+-activated chloride channel TMEM16A’, *Nature Communications*. Nature Publishing Group, 10(1), pp. 1–12. doi: 10.1038/s41467-019-11784-8.

Le, T. *et al.* (2019) ‘An inner activation gate controls TMEM16F phospholipid scrambling’, *Nature Communications*. Nature Publishing Group, 10(1), p. 1846. doi: 10.1038/s41467-019-09778-7.

Le, T., Le, S. C. and Yang, H. (2019) ‘Drosophila Subdued is a moonlighting transmembrane protein 16 (TMEM16) that transports ions and phospholipids’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 294(12), pp. 4529–4537. doi: 10.1074/jbc.AC118.006530.

Li, K. X. *et al.* (2019) ‘TMEM16B regulates anxiety-related behavior and GABAergic neuronal signaling in the central lateral Amygdala’, *eLife*. eLife Sciences Publications Ltd, 8. doi: 10.7554/eLife.47106.

Lin, H. *et al.* (2019) ‘Temperature-dependent increase in the calcium sensitivity and acceleration of activation of ANO6 chloride channel variants’, *Scientific Reports*. Nature Publishing Group, 9(1), p. 6706. doi: 10.1038/s41598-019-43162-1.

McIntyre, A. *et al.* (2016) ‘Disrupting hypoxia-induced bicarbonate transport acidifies tumor cells and suppresses tumor growth’, *Cancer Research*. American Association for Cancer Research Inc., 76(13), pp. 3744–3755. doi: 10.1158/0008-5472.CAN-15-1862.

Oh, U. and Jung, J. (2016) ‘Cellular functions of TMEM16/anoctamin’, *Pflugers Archiv European Journal of Physiology*. Springer Verlag, pp. 443–453. doi: 10.1007/s40424-016-1790-0.

Ousingsawat, J. *et al.* (2015) ‘Anoctamin-6 Controls Bone Mineralization by Activating the Calcium Transporter NCX1 * □ S’. JBC Papers in Press. doi: 10.1074/jbc.M114.602979.

Park, K. and Brown, P. D. (1995) ‘Intracellular pH modulates the activity of chloride channels in isolated lacrimal gland acinar cells’, *American Journal of Physiology - Cell Physiology*, 268(3 37-3). doi: 10.1152/ajpcell.1995.268.3.c647.

Paulino, C. *et al.* (2017) ‘Activation mechanism of the calcium-activated chloride channel TMEM16A revealed by cryo-EM’, *Nature*, 552(7685), pp. 421–425. doi: 10.1038/nature24652.

Pedemonte, N. and Galietta, L. J. V. (2014) ‘Structure and Function of TMEM16 Proteins (Anoctamins)’, *Physiological Reviews*. American Physiological Society Bethesda, MD, 94(2), pp. 419–459. doi: 10.1152/physrev.00039.2011.

Peters, C. J. *et al.* (2018) ‘The Sixth Transmembrane Segment Is a Major Gating Component of the TMEM16A Calcium-Activated Chloride Channel.’, *Neuron*. NIH Public Access, 97(5), pp. 1063-1077.e4. doi: 10.1016/j.neuron.2018.01.048.

Qu, Z. and Hartzell, H. C. (2000) ‘Anion Permeation in Ca 2+ -Activated Cl− Channels’, *The Journal of General Physiology*, 116(6), pp. 825–844. doi: 10.1085/jgp.116.6.825.
Richards, F. J. (1959) ‘A flexible growth function for empirical use’, *Journal of Experimental Botany*, 10(2), pp. 290–301. doi: 10.1093/jxb/10.2.290.

Riedl, S. *et al.* (2011) ‘In search of a novel target - Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy’, *Biochimica et Biophysica Acta - Biomembranes*. Elsevier, 1808(11), pp. 2638–2645. doi: 10.1016/j.bbamem.2011.07.026.

Schröder-Born, H., Bakalova, R. and Andrä, J. (2005) ‘The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine’, *FEBS Letters*. John Wiley & Sons, 579(27), pp. 6128–6134. doi: 10.1016/j.febslet.2005.09.084.

Schroeder, B. C. *et al.* (2008) ‘Expression Cloning of TMEM16A as a Calcium-Activated Chloride Channel Subunit’, *Cell*, 134(6), pp. 1019–1029. doi: 10.1016/j.cell.2008.09.003.

Segura-Covarrubias, G. *et al.* (2020) ‘Voltage-Dependent Protonation of the Calcium Pocket Enable Activation of the Calcium-Activated Chloride Channel Anoctamin-1 (TMEM16A)’, *Scientific Reports*. Nature Publishing Group, 10(1), p. 6644. doi: 10.1038/s41598-020-62860-9.

Suzuki, J. *et al.* (2010) ‘Calcium-dependent phospholipid scrambling by TMEM16F’, *Nature*, 468(7325), pp. 834–840. doi: 10.1038/nature09583.

Suzuki, J., Imanishi, E. and Nagata, S. (2014) ‘Exposure of Phosphatidylserine by Xk-related Protein Family Members during Apoptosis’, *Journal of Biological Chemistry*, 289(44), pp. 30257–30267. doi: 10.1074/jbc.M114.583419.

Ta, C. M. *et al.* (2017) ‘Contrasting effects of phosphatidylinositol 4,5-bisphosphate on cloned TMEM16A and TMEM16B channels’, *British Journal of Pharmacology*. John Wiley and Sons Inc., 174(18), pp. 2984–2999. doi: 10.1111/bph.13913.

Tembo, M. *et al.* (2019) ‘Phosphatidylinositol 4,5-bisphosphate (PIP2) and Ca2 are both required to open the Cl channel TMEM16A’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 294(33), pp. 12556–12564. doi: 10.1074/jbc.RA118.007128.

Tien, J. *et al.* (2014) ‘A comprehensive search for calcium binding sites critical for TMEM16A calcium-activated chloride channel activity’, 3, p. 2772. doi: 10.7554/eLife.02772.

Wang, L. *et al.* (2018) ‘CCR7 regulates ANO6 to promote migration of pancreatic ductal adenocarcinoma cells via the ERK signaling pathway’, *Oncology Letters*. Spandidos Publications, 16(2), pp. 2599–2605. doi: 10.3892/ol.2018.8962.

Webb, B. A. *et al.* (2011) ‘Dysregulated pH: A perfect storm for cancer progression’, *Nature Reviews Cancer*. Nature Publishing Group, pp. 671–677. doi: 10.1038/nrc3110.

White, K. A., Grillo-Hill, B. K. and Barber, D. L. (2017) ‘Cancer cell behaviors mediated by dysregulated pH dynamics at a glance’, *Journal of Cell Science*. Company of Biologists Ltd, 130(4), pp. 663–669. doi: 10.1242/jcs.195297.

Whitlock, J. M. *et al.* (2018) ‘Anoctamin 5/TMEM16E facilitates muscle precursor cell fusion’, *Journal of General Physiology*. Rockefeller University Press, 150(11), pp. 1498–1509. doi:
Whitlock, J. M. and Hartzell, H. C. (2017) ‘Anoctamins/TMEM16 Proteins: Chloride Channels Flirting with Lipids and Extracellular Vesicles’, Annual Review of Physiology. Annual Reviews, 79(1), pp. 119–143. doi: 10.1146/annurev-physiol-022516-034031.

Xuan, Z. B., Wang, Y. J. and Xie, J. (2019) ‘ANO6 promotes cell proliferation and invasion in glioma through regulating the ERK signaling pathway’, OncoTargets and Therapy. Dove Medical Press Ltd., 12, pp. 6721–6731. doi: 10.2147/OTT.S211725.

Yang, H. et al. (2012) ‘TMEM16F forms a Ca2+-activated cation channel required for lipid scrambling in platelets during blood coagulation.’, Cell, 151(1), pp. 111–122. doi: 10.1016/j.cell.2012.07.036.

Yang, Y. D. et al. (2008) ‘TMEM16A confers receptor-activated calcium-dependent chloride conductance’, Nature, 455(7217), pp. 1210–1215. doi: 10.1038/nature07313.

Ye, W. et al. (2018) ‘Phosphatidylinositol-(4, 5)-bisphosphate regulates calcium gating of small-conductance cation channel TMEM16F’, Proceedings of the National Academy of Sciences of the United States of America. National Academy of Sciences, 115(7), pp. E1667–E1674. doi: 10.1073/pnas.1718728115.

Ye, W. et al. (2019) ‘Dynamic change of electrostatic field in TMEM16F permeation pathway shifts its ion selectivity’, eLife. eLife Sciences Publications Ltd, 8. doi: 10.7554/eLife.45187.

Yin, X. et al. (2003) ‘A flexible sigmoid function of determinate growth’, Annals of Botany, 91(3), pp. 361–371. doi: 10.1093/aob/mcg029.

Yu, K. et al. (2012) ‘Explaining calcium-dependent gating of anoctamin-1 chloride channels requires a revised topology’, Circulation Research. Lippincott Williams & WilkinsHagerstown, MD, 110(7), pp. 990–999. doi: 10.1161/CIRCRESAHA.112.264440.

Yu, K. et al. (2015) ‘Identification of a lipid scrambling domain in ANO6/TMEM16F’, eLife, 4. doi: 10.7554/eLife.06901.

Yu, K. et al. (2019) ‘A network of phosphatidylinositol 4,5-bisphosphate binding sites regulates gating of the Ca2+-activated Cl− channel ANO1 (TMEM16A)’, Proceedings of the National Academy of Sciences of the United States of America. National Academy of Sciences, 116(40), pp. 19952–19962. doi: 10.1073/pnas.1904012116.

Zaitseva, E. et al. (2017) ‘Fusion Stage of HIV-1 Entry Depends on Virus-Induced Cell Surface Exposure of Phosphatidylserine’, Cell Host and Microbe. Cell Press, 22(1), pp. 99-110.e7. doi: 10.1016/j.chom.2017.06.012.

Zhang, L. et al. (2014) ‘Phosphatidylserine-targeted bimodal liposomal nanoparticles for in vivo imaging of breast cancer in mice’, Journal of Controlled Release. Elsevier, 183(1), pp. 114–123. doi: 10.1016/j.jconrel.2014.03.043.

Zhang, Y. et al. (2017) ‘Inferior Olivary TMEM16B Mediates Cerebellar Motor Learning’, Neuron. Elsevier, 95(5), pp. 1103-1111.e4. doi: 10.1016/J.NEURON.2017.08.010.

Zhang, Y. et al. (2020) ‘TMEM16F phospholipid scramblase mediates trophoblast fusion and
placental development’, *Science Advance*, 6(eaba0310).

Zhao, D. *et al.* (2011) ‘Near-infrared Optical Imaging of Exposed Phosphatidylserine in a Mouse Glioma Model’, *Translational Oncology*. Neoplasia Press Inc, 4(6), pp. 355–364. doi: 10.1593/tlo.11178.
Figure 1. pH\textsubscript{i} regulates both TMEM16A and TMEM16F ion channel activities. (A) Representative TMEM16A currents recorded from inside-out patches perfused with intracellular solutions containing 0.5 µM Ca\textsuperscript{2+} at different pH\textsubscript{i}. Currents were elicited by voltage steps from −100 to +100 mV with 20 mV increments. The holding potential was −60 mV. All the traces shown were from the same patch. (B) Mean G-V relations of the TMEM16A channels under different pH\textsubscript{i} at 0.5 µM Ca\textsuperscript{2+}. Relative conductance was determined by measuring the amplitude of tail currents 400 μs after repolarization to a fixed membrane potential (−60 mV). The smooth curves represent Boltzmann fits \( \frac{G}{G\text{max}} = \frac{1}{1 + \exp(-ze(V-V_{1/2}))/kT} \). \( G\text{max} \) is tail current amplitude in response to depolarization to +100 mV in 0.5 µM Ca\textsuperscript{2+} at pH\textsubscript{i}=8.9. Error bar represents SEM (n=7). (C) Mean conductance of TMEM16A at different pH\textsubscript{i} was normalized to the maximum conductance at pH\textsubscript{i}=8.9 at different voltages and then plotted as a function of pH\textsubscript{i} (G-pH\textsubscript{i} relationship). Data were fitted with linear regression and the mean slopes from fittings were 0.27, 0.28, 0.29, 0.3 and 0.29 for 20, 40, 60, 80 and 100 mV, respectively (n=7). (D) Representative TMEM16F currents recorded from inside-out patches perfused with intracellular solutions containing 100 µM Ca\textsuperscript{2+} at different pH\textsubscript{i}. Currents were evoked by voltage steps from −100 to +100 mV with 20 mV increments and the holding potential was −60 mV. All traces shown were from the same patch. (E) Mean G-V relations of TMEM16F current under different pH\textsubscript{i}. The smooth curves represent Boltzmann fits similar as in (B). Error bar represents SEM (n=7). (F) G-pH\textsubscript{i} relationship for TMEM16F channels. Data were fitted with linear regression and the mean slopes from fittings were 0.19, 0.20 and 0.21 for 60, 80 and 100 mV, respectively (n=7).
Figure 2. pH$_i$ regulates TMEM16F lipid scrambling activity. (A) Schematic design of the lipid scrambling fluorometry assay. CaPLSase activity is monitored by cell surface accumulation of fluorescently tagged Annexin-V (AnV), a phosphatidylserine (PS) binding protein that is continuously perfused through a perfusion manifold. AnV fluorescence remains dim in bulk solution and will strongly fluoresces after being recruited by cell surface PS, which is externalized by CaPLSases. We utilize whole-cell patch pipettes to deliver intracellular solutions into the cytosol to achieve precise control of intracellular pH and Ca$^{2+}$. Once breaking into whole-cell configuration, the pipette solution will rapidly diffuse into the cell and activate CaPLSases. The AnV fluorescence signal on cell surface will be continuously recorded with 5-second interval. (B) Representative lipid scrambling fluorometry images of the HEK293T cells stably expressed with TMEM16F-eGFP (left, green signal) at different pH$_i$. For the AnV-CF 594 signal on the right, the first column is fluorescence signal immediately after forming whole-cell configuration; the second column is the time when fluorescence intensity reached half maximum ($t_{1/2}$) and the last column is the time when fluorescence signal roughly reach plateau. The time point (minutes followed by seconds) of each image after breaking into whole-cell configuration are shown on the top right corner. The pipette solution contained 100 µM Ca$^{2+}$ and holding potential was -60 mV. (C) The time course of AnV fluorescence signal at different pH$_i$ as shown in (B). The AnV signal was normalized to the maximum AnV fluorescence intensity at the end of each recording. The smooth curves represent fits to generalized logistic equation, $F= F_{\text{max}}/[1+ \exp(-k(t-t_{1/2}))]$. (D) Under 100 µM Ca$^{2+}$, $t_{1/2}$ for pH$_i$ = 6.1,7.3 and 8.9 are 29.1±3.0 minutes; 20.3±2.4 minutes and 11.6±3.5 minutes (n=5), respectively. (E) Under 100 µM Ca$^{2+}$, slopes for pH$_i$ = 6.1,7.3 and 8.9 are 0.19±0.04, 0.26±0.04 and 0.40±0.02 (n=5), respectively. Values represent as mean ± SEM. * =p<0.05, ** =p<0.01 and **** = p < 0.0001, using one-way ANOVA followed by Tukey’s test.
Figure 3. pH\text{\textsubscript{i}} has no effect on TMEM16A or TMEM16F when Ca\textsuperscript{2+} is absent. (A) Locations of L543 and Q645 on the TMEM16A structure (PDB 5OYB). (B) Representative TMEM16A-L543Q and TMEM16A-Q645A currents recorded from inside-out patches perfused with intracellular solutions containing 0 Ca\textsuperscript{2+} at different pH\textsubscript{i}. (C) I-pH\textsubscript{i} curve of TMEM16A mutations L543Q and Q645A at 100 mV. Slopes from linear fit for L543Q and Q645A are -0.02 and -0.04, respectively. The G-pH\textsubscript{i} curve of WT under 0.5 µM Ca\textsuperscript{2+} was replotted as the black dash line. Error bars represent standard deviation (n=7). (D) Locations of Y563 and F518 on the TMEM16F structure (PDB 6QP6). (E) Representative TMEM16F-Y563K and TMEM16F-F518K currents recorded from inside-out patches perfused with intracellular solutions containing 0 Ca\textsuperscript{2+} at different pH\textsubscript{i}. (F) The I-pH\textsubscript{i} relationship of TMEM16F mutations Y563K and F518K at 100 mV. Slopes from linear fit for Y563K and F518K are 0.03 and 0.01, respectively. G-pH\textsubscript{i} curve of WT under 100 µM Ca\textsuperscript{2+} was replotted as black dash line. Error bars represent standard deviation (n=5). Note that the gain-of-function mutations do not have obvious tail current under 0 Ca\textsuperscript{2+}, thereby I-pH\textsubscript{i} relations not G-pH\textsubscript{i} were plotted to evaluate their pH\textsubscript{i} sensitivities.
Figure 4. pH<sub>i</sub> regulation of TMEM16F channel activity is Ca<sup>2+</sup> dependent. (A), (B) Representative TMEM16F currents recorded from inside-out patches perfused with intracellular solutions containing 5 µM and 1000 µM Ca<sup>2+</sup>, respectively. All traces shown in each panel were from the same patch. Currents were elicited by voltage steps from −100 to +100 mV with 20 mV increments. The holding potential was -60 mV. (C), (D) Mean G-V relations of the TMEM16F currents from (A) and (B), respectively. Relative conductance was determined by measuring the amplitude of tail currents 400 µs after repolarization to a fixed membrane potential (−60 mV). The smooth curves represent Boltzmann fits. Error bars represent SEM (n = 5). (E) pH<sub>i</sub> sensitivity of TMEM16F current at +100 mV was measured by the slope of the the G-pH<sub>i</sub> relationship. Mean conductance at different Ca<sup>2+</sup> concentration was normalized to the maximum conductance at pH<sub>i</sub>=8.9 and +100 mV. Averaged slopes from linear fit for 5 and 1000 µM Ca<sup>2+</sup> are 0.32 and 0.04, respectively. The G-pH<sub>i</sub> curve at 100 µM Ca<sup>2+</sup> was replotted as black line for reference. Error bars represent standard deviation (n=5).
Figure 5. pH\textsubscript{i} regulation of TMEM16F scrambling activity is Ca\textsuperscript{2+} dependent. (A) Representative images of TMEM16F-eGFP scrambling activity under 5 μM intracellular Ca\textsuperscript{2+} with different pH\textsubscript{i}. The white dotted rectangles in the top row demarcate the patch clamped TMEM16F-eGFP expressing cells. For the AnV-CF 594 signals on the right, the first column is fluorescence signal immediately after forming whole-cell configuration; the second column is the time point (minutes followed by seconds, top right corner) when fluorescence intensity reaches half maximum (t\textsubscript{1/2}) and the last column is the time point when fluorescence reaches plateau. No obvious AnV-CF 594 signal can be detected in pH\textsubscript{i} 6.1 over 40 minutes. (B) The time courses of the AnV fluorescent intensity for TMEM16F activated by 5 μM Ca\textsuperscript{2+} under different pH\textsubscript{i} in (A). The smooth curves represent fits to the logistic equation similar to Fig.2B. (C) The t\textsubscript{1/2} at different pH\textsubscript{i} under 5 μM Ca\textsuperscript{2+}. N/S at pH\textsubscript{i}=6.1 represents no scrambling. Error bars represent SEM (n = 5). (D) The slopes at different pH\textsubscript{i} under 5 μM Ca\textsuperscript{2+}. (E) Representative images of TMEM16F-eGFP scrambling activity under 1000 μM intracellular Ca\textsuperscript{2+} with different pH\textsubscript{i}. For the AnV-CF 594 signal on the right, the first column is fluorescence signal immediately after forming whole-cell configuration; the second column is the time point when fluorescence intensity reaches half maximum (t\textsubscript{1/2}) and the last column is the time point when fluorescence reaches plateau. (F) The time courses of the AnV fluorescent intensity for TMEM16F activated by 1000 μM Ca\textsuperscript{2+} under different pH\textsubscript{i} in (E). The smooth curves represent fits to the logistic equation. (G) The t\textsubscript{1/2} of lipid scrambling at different pH\textsubscript{i} under 1000 μM Ca\textsuperscript{2+}. (H) The slopes k at different pH\textsubscript{i} under 1000 μM Ca\textsuperscript{2+}. Error bars represent SEM (n = 5). Statistics were done using one-way ANOVA followed by Tukey’s test. **=p<0.01, ***=p<0.001 and ns (not significant): p>0.05.
**Figure 6.** pH alters Ca\(^{2+}\) binding affinity of TMEM16F. (A) Representative TMEM16F currents recorded from inside-out patches perfused with intracellular solutions containing 0.1, 1, 5, 100, 1000 and 5000 (pH=6.1 only) µM Ca\(^{2+}\) at different pH. Currents were elicited by voltage steps from −100 to +100 mV with 20 mV increments. The holding potential was -60 mV. (B) Ca\(^{2+}\) dose-response of mTMEM16F channel at +100 mV with different pH. The smooth curves represent the fits to the Hill equation: \(G/G_{\text{max}} = G_{1000}/(1+(K_D/[Ca^{2+}])^H)\), wherein \(K_D\) is the apparent dissociation constant, \(H\) is the Hill coefficient, and \(G_{1000}\) is the conductance with 1000 µM Ca\(^{2+}\) at given pH. The error bars represent SEM (n=5). (C) Half maximal effective concentrations of Ca\(^{2+}\) (EC\(_{50}\)) at pH 6.1, 7.3 and 8.9 were 144.1±6.8, 6.2±2.2 and 1.2±1.2 µM, respectively. p-values were determined with Tukey test comparisons following one-way ANOVA. **** p < 0.0001. (D) The G-pH\(_i\) relationship of TMEM16F current at +100 mV under different Ca\(^{2+}\) concentrations. Solid lines represent linear fits. (E) The relationship of pH\(_i\) sensitivity and intracellular Ca\(^{2+}\) concentration. The pH\(_i\) sensitivity values were slopes from linear fit shown in (D) under different Ca\(^{2+}\) concentrations. The smooth line was fitted with bell-shaped dose response curve using Graphpad Prism with peak pH sensitivity of 0.33 at ~15 µM Ca\(^{2+}\).
Figure 7. pH$_i$ specifically works on the Ca$^{2+}$ binding sites of TMEM16F to regulate its activities. (A) Locations of the pore-lining residue Q559 and the Ca$^{2+}$ binding residue E667 on the TMEM16F structure (PDB ID: 6QP6). (B) Representative TMEM16F-E667Q currents recorded from inside-out patches perfused with intracellular solutions containing 1000 µM Ca$^{2+}$ at different pH$_i$. (C) The I-V curve of E667Q currents at different pH$_i$. (D) The pH$_i$ sensitivity of E667Q (EQ) evaluated by the I-pH$_i$ relationship. The slope of the I-pH$_i$ relationship for E667Q at 1000 µM Ca$^{2+}$ is 0.30±0.02 shown in red solid line. The I-pH$_i$ curves of WT at different Ca$^{2+}$ concentrations were plotted as dashed lines as references. (E) Representative TMEM16F-Q559K currents recorded from inside-out patches perfused with intracellular solutions containing 100 µM Ca$^{2+}$ at different pH$_i$. (F) The G-V curves of Q559K currents at different pH$_i$. (G) The pH$_i$ sensitivity of Q559K (QK) evaluated by the G-pH$_i$ relationship. The slope of the G-pH$_i$ relationship for Q559K at 100 µM Ca$^{2+}$ is 0.20±0.02 shown in red solid line. The G-pH$_i$ curves of WT at different Ca$^{2+}$ concentrations were also plotted as dashed lines as references. (H) A cartoon illustration of pH$_i$ regulation of TMEM16 channels and scramblases. Under low pH$_i$ condition, protonation of Ca$^{2+}$ binding residues can significantly reduce Ca$^{2+}$ binding affinity of TMEM16 proteins and suppress their activation. In contrast, high pH$_i$ deprotonates the Ca$^{2+}$ binding residues, enhances Ca$^{2+}$ binding and TMEM16 activation. The size of arrows represents the open probability of TMEM16 proteins. The size of Ca$^{2+}$ ions represents the strength of apparent Ca$^{2+}$ binding affinity.
Figure 8. Intracellular alkalization potentiates the activation of endogenous TMEM16F CaPLSase in choriocarcinoma BeWo cells. (A) Representative whole-cell currents recorded from wildtype (WT) BeWo cells perfused with intracellular solutions containing 5 µM Ca\(^{2+}\) at different pH\(_i\) (7.2 and 7.7). (B) Mean I-V relation using tail currents at different pH\(_i\). Error bars represent SEM (n=5). (C) Comparison of the tail currents at +200 mV under different pH\(_i\). Error bar represents SEM (n=5). (D) Representative CaPLSase activity of WT BeWo cells at different pH\(_i\). BF, bright field. For AnV-CF 594 signals, the first column is recorded immediately after forming whole-cell patch configuration; the second column is the time point when fluorescence intensity reaches half maximum (\(t_{1/2}\)) and the last column is the time point when fluorescence reaches plateau. The time on the top right corner shows minutes followed by seconds. (E) The time course of endogenous CaPLSases under pH\(_i\) 7.2 and 7.7. The fluorescence signal was normalized to its maximum intensity for each pH\(_i\). The smooth curves represent fits to the logistic equation. (F) \(t_{1/2}\) at different pH\(_i\). Averaged \(t_{1/2}\) for pH\(_i\)=7.2 and 7.7 are 27.7±3.3 minutes (n=5) and 20.1±2.5 minutes (n=5), respectively. (G) The slopes at different pH\(_i\). Averaged slopes for pH\(_i\)=7.2 and 7.7 are 0.26±0.03 (n=5) and 0.30±0.02 (n=5), respectively. Values represent mean±SEM and statistics were done using one-way ANOVA followed by Tukey’s test. * =p<0.05 and ** =p<0.01.
Supplementary information for

**Molecular underpinning of intracellular pH regulation on TMEM16F**

Pengfei Liang¹, Huanghe Yang１,²*

**Affiliations:**

¹Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, USA

²Department of Neurobiology, Duke University Medical Center, Durham, North Carolina, USA

*Correspondence to:
Huanghe Yang
Email: huanghe.yang@duke.edu
Telephone: 919-684-1406

**This PDF file includes:**

- Figures. S1-S6
- Captions for Movies S1-S6

**Other supplementary materials for this manuscript include the following:**

- Movies S1-S6
Figure S1. TMEM16F shows less rundown using voltage-step protocol and the knockout (KO) HEK293T cells show no scrambling activity even at high pH_i. (A) Representative TMEM16F currents recorded from inside-out patches perfused with intracellular solutions containing 100 μM Ca^{2+} at pH_i, 7.3. The interval between each trace was 20 seconds and all the recordings were from the same patch. (B) The G-V relationship of currents shown in (A). Error bars represent mean ± SEM (n=3). (C) Representative images of lipid scrambling in TMEM16F-KO HEK293T cells. The patch pipette contained 100 μM free Ca^{2+} at pH_i=8.9. No CaPLSase activity was observed in TMEM16F-KO HEK293T cells. (D) Representative fluorescent intensity of AnV binding for TMEM16F-eGFP stable HEK293T and TMEM16F-KO HEK293T cells at pH_i=8.9 (n=4).
Figure S2. TMEM16A-CaCC losses pHi regulation under saturating pHi. (A) Representative TMEM16A currents recorded from inside-out patches perfused with intracellular solutions containing 100 µM Ca²⁺ at different pHi. (B) G-V relationships at different pHi under 100 µM Ca²⁺. All conductance were normalized to the maximum conductance at pHi=8.9 and +100 mV. Error bar represents standard deviation (n=5). (C) G-pHi curve of TMEM16A at 100 µM Ca²⁺ (red, solid line) and 0.5 µM Ca²⁺ (black, dotted line). The slopes from linear fits are 0.02 and 0.3, respectively. Error bar represents standard deviation (n=5).
Figure S3. pHᵢ regulation on TMEM16F scrambling activity is Ca²⁺ dependent. (A) The t₁/₂ of lipid scrambling at different pHᵢ under different Ca²⁺ concentrations including 5, 100 and 1000 μM. N/S represents no scrambling detected at given time. Error bars represent SEM (n =5). (B) The slopes from logistic equation fitting at different pHᵢ under different Ca²⁺ concentrations including 5, 100 and 1000 μM. Error bars represent SEM (n =5).
Figure S4. The Ca\(^{2+}\) binding site mutation E670Q enhances pH\(_i\) effects on TMEM16F channel activation. (A) Location of the Ca\(^{2+}\) binding residue E670. (B) Representative TMEM16F-E667Q currents recorded from inside-out patches perfused with intracellular solutions containing 1000 µM Ca\(^{2+}\) at different pH\(_i\). Currents were elicited by voltage steps from −100 to +140 mV with 20 mV increments. The holding potential was −60 mV. (C) I-V relationship of E670Q mutant channel recorded at different pH\(_i\). (D) I-pH\(_i\) relationship of E670Q (EQ) under 1000 µM Ca\(^{2+}\) (red, solid line) with averaged slope of 0.32. G-pH\(_i\) relationships of WT TMEM16F at different Ca\(^{2+}\) concentrations were plotted as dashed line as references.
Figure S5. The Ca\(^{2+}\) binding site mutation E730Q enhances pH\(_i\) effects on TMEM16A activation. (A) Location of the Ca\(^{2+}\) binding residue E730 (PDB ID: 6BGI). (B) Representative TMEM16A-E730Q currents recorded from inside-out patches perfused with intracellular solutions containing 100 \(\mu\)M Ca\(^{2+}\) at different pH\(_i\). Currents were elicited by voltage steps from −100 to +160 mV with 20 mV increments. The holding potential was -60 mV. (C) I-V curve of E730Q current recorded at different pH\(_i\). (D) I-pH\(_i\) relationship of TMEM16A E730Q (EQ) under 100 \(\mu\)M Ca\(^{2+}\) (red, solid line) with averaged slope of 0.31. G-pH\(_i\) relationships of WT TMEM16A at different Ca\(^{2+}\) concentrations were plotted as dashed line as references.
Figure S6. TMEM16F knockout (KO) BeWo cells lack TMEM16F-current and lipid scrambling. (A) Whole-cell current in TMEM16F-KO BeWo cells with pipette solution containing 5 µM Ca^{2+} at pH_{i}=7.7. Currents were elicited by voltage steps from -100 to +200 mV with 20 mV increments. The holding potential was -60 mV. Compared with endogenous TMEM16F current in BeWo cells (Fig. 8A), the outward rectifying current of unknown identity in the TMEM16F KO BeWo cells is small in amplitude and lacks tail current. (B) The I-V relationship constructed using tail currents in (A). Error bar represents SEM (n=5 for WT and n=4 for KO). (C) Representative lipid scrambling activity of the TMEM16F-KO BeWo cells at different time points (right, top corner, minutes followed by seconds). None of the cells patched showed PS exposure (n=4).
Captions for Movies S1-S6

Movie S1. Lipid Scrambling activity of HEK293T cells overexpressing TMEM16F with 100 µM Ca²⁺ at pHᵢ=6.1, pHᵢ=7.3 and pHᵢ=8.9 (Related to Fig. 2B).

Movie S2. Lipid scrambling activity of TMEM16F-KO HEK293T cells with 100 µM Ca²⁺ at pHᵢ=8.9 (Related to Fig. S1).

Movie S3. Lipid scrambling activity of HEK293T cells overexpressing TMEM16F with 5 µM Ca²⁺ at pHᵢ=6.1, pHᵢ=7.3 and pHᵢ=8.9 (Related to Fig. 5A).

Movie S4. Lipid scrambling activity of HEK cells overexpressing TMEM16F with 1000 µM Ca²⁺ at pHᵢ=6.1, pHᵢ=7.3 and pHᵢ=8.9 (Related to Fig. 5E).

Movie S5. Lipid scrambling activity of BeWo cells with 5 µM Ca²⁺ at pHᵢ=7.2 and pHᵢ=7.7 (Related to Fig. 8D).

Movie S6. Lipid scrambling activity of TMEM16F knockout (KO) BeWo cells with 5 µM Ca²⁺ at pHᵢ=7.7 (Related to Fig. S6).