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COMMENTARY

The taming of a scramblase

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Eukaryotic cells are separated from their environment by a 30-Å-thick delimitation of lipid and protein—the plasma membrane (PM). Although a greater measure of affection has historically been garnered by their protein machines, cells have devoted ∼5% of their genes to building lipids. In this pursuit of lipidic diversity, cells have developed repertoires of thousands of lipid species (van Meer et al., 2008), many of which fall into the category of phospholipids (PLs), which are largely responsible for the PM's bilayer arrangement. The PLs within the PM bilayer are actively arranged in an asymmetric orientation rendering the exofacial surface largely composed of cylindrical species (e.g., phosphatidylcholine), while the cytofacial leaflet retains most conical and charged species (e.g., phosphatidylethanolamine and phosphatidylserine [PS]). Despite this organization, many of our cells maintain the ability to rapidly abolish PM lipid asymmetry, exposing cytofacial PLs to the exofacial surface as a means of coordinating intercellular processes (Bevers and Williamson, 2016). Bevers et al. (1982) first described this PL "scrambling" in platelet activation, where PS exposure on the exofacial surface propagates coagulation. A few years after Bevers' description, several others noted that platelet activation was accompanied by a rapid alkalization of intracellular pH (pH_i), and that blocking this alkalization inhibited PS exposure (Bucki et al., 2006; Zavoico et al., 1986). In an earlier issue of the *Journal of General Physiology*, Liang and Yang (2021) show us why. In fact, these platelets, and likely other cells that use nonapoptotic scrambling as a signaling module, have tamed their scramblases to fit a distinct biological niche via pH_i.

In an earlier issue of the *Journal of General Physiology*, Liang and Yang (2021) offer an insightful peek into the mechanism by which the TMEM16F scramblase is regulated by pH_i. Using simultaneous monitoring of fluorescent PS probe binding and electrophysiological recordings, Liang and Yang (2021) assess how alterations in pH_i impact both TMEM16F-dependent lipid rearrangements and the ionic current associated with PM scrambling. Under physiological Ca^{2+} concentrations, Liang and Yang (2021) find that TMEM16F is bidirectionally "tamed" by pH_i, both entirely inhibited by cytosolic acidification and potentiated by alkalization. However, the inhibition of TMEM16F via low pH_i can be overcome with super-physiological Ca^{2+} concentrations. pH_i appears to exert its effect on TMEM16F by altering the protonation state of the scramblase's Ca^{2+}-binding sites. To this point, reducing TMEM16F Ca^{2+} affinity also proportionately reduces the sensitivity of TMEM16F to pH_i. Either "reined" in by an acidic cytosol or spurred into a "gallop" through alkaline potentiation, these data demonstrate that pH_i bidirectionally tames the TMEM16 scramblase.

The mechanism described by Liang and Yang (2021) finally illuminates a likely mechanism by which the Na^+/H^+ antiporter NHEI regulates the activation of platelets. Inhibition of NHEI function acidifies platelet pH_i and inhibits the PS exposure and microvesicle release observed in response to Ca^{2+}, as alluded to above; however, the mechanism by which NHEI regulates these platelet functions has remained a mystery for decades (Bucki et al., 2006). A vital tool used to study PS exposure and microvesicle release in platelets has historically been the use of cells from Scott's syndrome patients, which lack both of these activities in platelets and red cells. Previous work has demonstrated that the loss of these platelet/red cell activities stems from a loss of their scramblase, TMEM16F (Suzuki et al., 2010). Based on the work of Liang and Yang (2021), it would appear that physiological alkalization of the platelet cytosol may very well be a mechanism platelets employ to potentiate their scramblase and achieve rapid PS exposure and microvesicle release in response to injury. Moreover, the loss of platelet activation in response to NHEI inhibition likely stems from the acidic environment of the cytosol reining in the platelet's scramblase, TMEM16F.

For the last 10 yr, we have appreciated that much of the nonapoptotic scrambling that cells employ in intercellular signaling is performed by the TMEM16 family of Ca^{2+}-activated phospholipid scramblases (Ca^{2+}-PLSases). The most abundant PLSase in mammals is TMEM16F (reviewed in Whitlock and Hartzell, 2017). Of note, this family also includes two dearly loved ion channel members that do not scramble PLs but pass ions through the PM (i.e., TMEM16s A and B). Thanks to the work of many, we now know a great deal about which TMEM16s are scramblases, how they ferry lipids between PM leaflets, how they are activated by Ca^{2+}, and their structures. Moreover, we also appreciate that these scramblases are nonselective in

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respect to which lipids they move between leaflets, and that some TMEM16s even scramble lipids in membranes other than the PM (Tsuji et al., 2019). What we are just beginning to appreciate are the mechanisms that cells use to manage these potent remodelers of the PM beyond Ca^{2+} activation.

In an earlier issue of the *Journal of General Physiology*, Liang and Yang (2021) identify a novel mechanism by which cells can tame TMEM16F activities to fit their biological purpose using pH_i. This pH_i regulatory mechanism is one of several newly discovered ways that cells can tame TMEM16 scramblases to fit a particular biological system. Moving forward, it will be vital to better elucidate how pH_i and other means of taming TMEM16 scramblases fit into the ever-growing list of biological processes that are activated by scrambling.

A TMEM16 scramblase module is coming into focus

TMEM16s are unequivocally activated by intracellular Ca^{2+}, but how these Ca^{2+}-activated machines are adapted to fit distinct biological contexts moves beyond cytosolic Ca^{2+}. Because their ion channel cousins were discovered first, our ideas of how TMEM16 scramblases are regulated has largely been colored by the electrophysiological characterization of the Cl^- channel TMEM16A. TMEM16A is activated by Ca^{2+}, but this activity is regulated by membrane voltage, PIP_2, cholesterol, extracellular pH, and pH_i (Yu et al., 2019; Le et al., 2019; De Jesús-Pérez et al., 2018; Xiao et al., 2011; Cruz-Rangel et al., 2017; Chun et al., 2015). Although biological functions differ dramatically between TMEM16 ion channels and scramblases, it appears that many routes of regulation are conserved family traits. In particular, TMEM16F is also regulated by membrane voltage, PIP_2, and, as we learn from Liang and Yang, pH_i (Scudieri et al., 2015; Ye et al., 2018; Liang and Yang, 2021). Moreover, the mechanism by which Liang and Yang (2021) propose pH_i regulates TMEM16F (through the protonation of its Ca^{2+} binding sites) is shared with TMEM16A (Chun et al., 2015). Together, this shared pH_i regulatory mechanism, observed in TMEM16s of differing activities, suggests that most TMEM16s may be regulated by pH_i. However, is it reasonable to assume that TMEM16 sensitivity to pH_i is similar between family members? All TMEM16s are activated by Ca^{2+} binding, and the residues coordinating Ca^{2+} in TMEM16s are highly conserved across the family. However, despite this conservation, TMEM16s differ drastically in their sensitivity to Ca^{2+} (e.g., Ca^{2+} sensitivity differs ∼1,000-fold between TMEM16s A and F). If the mechanism by which pH_i regulates TMEM16s involves these same Ca^{2+}-coordinating residues, how can we trust that pH_i sensitivity would be the same between relatives when Ca^{2+} sensitivity is not? Moreover, Liang and Yang (2021) demonstrate that increased Ca^{2+} can overcome pH_i inhibition. If some TMEM16s are ∼1,000-fold more sensitive to Ca^{2+}, is it reasonable to expect that all would escape pH_i regulation at the same Ca^{2+} concentration? Determining the pH_i sensitivity of different TMEM16s may help further resolve how the same cell can regulate the activities of several TMEM16s at once. Perhaps evolution's differential tuning of these shared TMEM16 regulatory mechanisms has ensured that some TMEM16s never "see" the activating signals of their relatives, even though they are within the same membrane of the same cell.

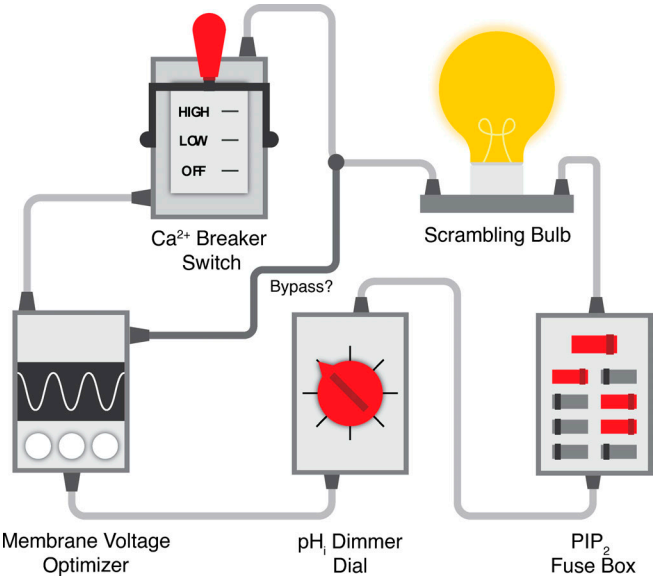

Figure 1. **The TMEM16 scramblase module.** A vastly oversimplified cartoon illustrating how disparate regulators of TMEM16 scramblases come together as a "module" employed in many cells to individually tune the "brightness" of the scrambling. This tuning would undoubtedly alter the speed at which a cell promotes lipid redistribution in response to Ca^{2+}, as well as the extent that scrambling alters the exposure of cytofacial lipid species and the physical characteristics of the PM during scrambling.

Although TMEM16F and other TMEM16 scramblases are expressed in a diverse array of cell types and tissues, we have now begun to appreciate several unique mechanisms that regulate the location, magnitude, and duration of their activities. Together, these disparate regulatory factors come together to create a "TMEM16 scramblase module" employed by many cells and used to uniquely tame, or tune, TMEM16-dependent scrambling to fit a cell's individual biological niche (Fig. 1).

It should be noted that there is an independent PL scrambling pathway associated with apoptotic caspase activity and the Xk-related protein family (Suzuki et al., 2014). Interestingly, this apoptotic scrambling is also modulated by pH_i but in the opposite direction described above, with lower pH_i promoting exofacial PS exposure. Importantly, this response is observed in cells from healthy donors and those from Scott's syndrome patients lacking TMEM16F, demonstrating that this is indeed an alternative scrambling response to that described by Liang and Yang (Stout et al., 1997). Caspase- and Ca^{2+}-activated scrambling pathways are parallel yet independent processes and differ substantially in a number of ways, including the amount of time between activation of the pathway and PS exposure (hours versus minutes), the distribution of the PS each expose in native cells (whole membrane versus patches), and likely the reversibility of PS exposure (caspases cleave PS transporters while Ca^{2+} reversibly inhibits them), and here Liang and Yang (2021) demonstrate that Ca^{2+}-activated PM scrambling differs in its response to pH_i compared with caspase-activated PM scrambling. Moving forward, it will be important to consider the differences between these scramblase pathways that both result in the exposure of cytofacial lipids and altered PM physical properties.

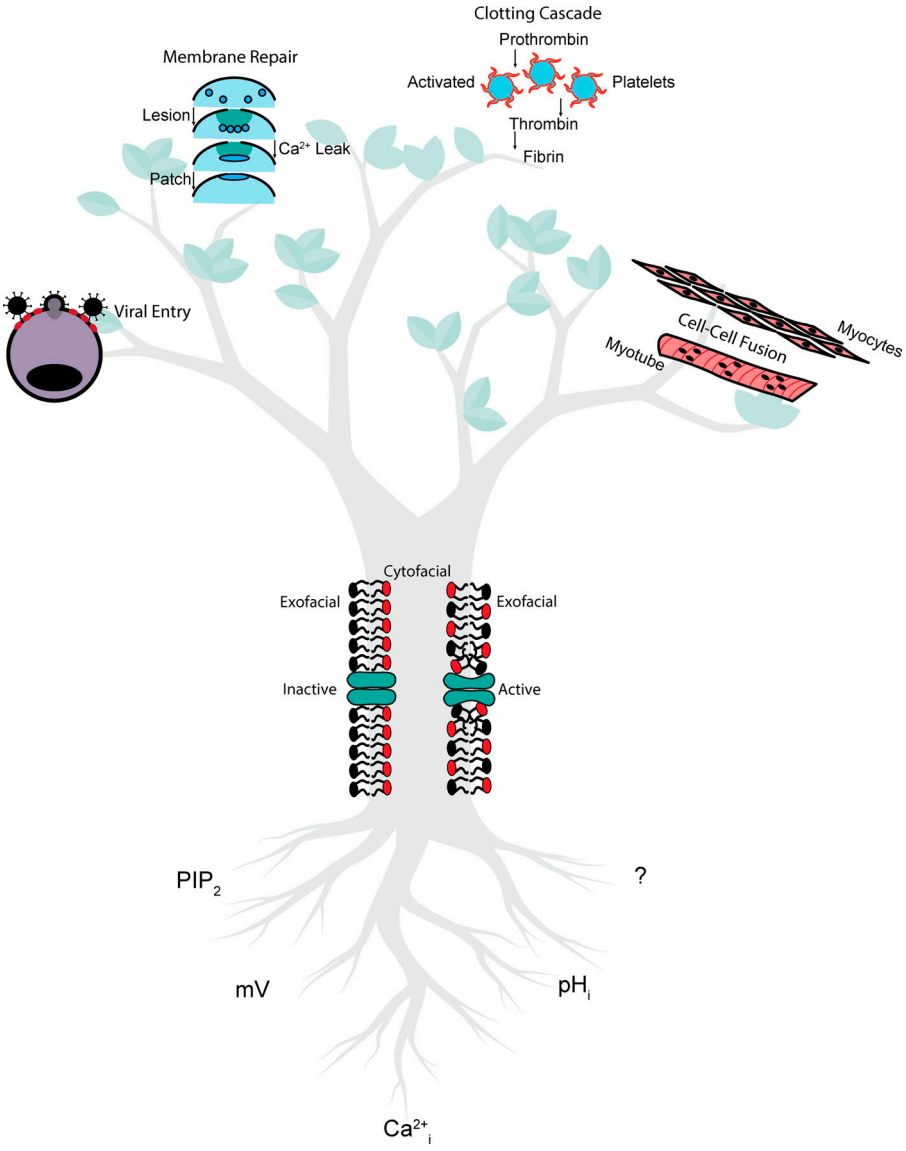

Figure 2. **The TMEM16F scramblase function in cellular processes.** An illustration highlighting how the regulated activity of TMEM16 scramblases permeates biological processes. TMEM16 scramblases are activated by increases in intracellular calcium (Ca^{2+}_i); however, scramblase activity is regulated by a host of other cellular factors including membrane voltage (mV), phosphatidylinositol 4,5-bisphosphate (PIP_2), intracellular pH (pH_i), and likely others (?; see roots). Together, these regulatory factors modulate the exofacial exposure of cytofacial lipids (trunk) that coordinate a variety cellular processes in diverse systems including cell–cell fusion, platelet facilitated blood clotting, membrane patch repair, and enveloped viral entry (see branches). To appreciate how these scramblase regulators fit into distinct, biologically relevant processes, we must begin watching the branches as we tickle the roots.

And now for the most important question: how do cells, tissues, and systems employ TMEM16 scramblase regulation for biological purposes (Fig. 2)?

How does the taming of a scramblase affect biological processes?

As the list of methods available to cells for taming TMEM16 scramblase function grow, we are left with a major question: how are these regulatory mechanisms employed in the local domestication of scramblases in biologically relevant contexts? In light of this latest contribution by Liang and Yang (2021), a few biological processes come to mind.

In addition to platelet activation, nonapoptotic PS exposure is a reoccurring signal employed in the coordination of cell–cell fusion processes (Whitlock and Chernomordik, 2021). The role of TMEM16 scramblases has been linked to the fusion of gametes, skeletal muscle, osteoclasts, placental trophoblasts, and virus–cell fusion. These could be ideal systems to dissect the mechanistic contribution of pH_i in the exposure of PS required for these fusion events. In particular, during capacitation and/or the acrosome reaction, PS is exposed on the heads of sperm and is required for fertilization (Rival et al., 2019). Interestingly, during capacitation and/or the acrosome reaction, sperm both alkalinize their cytosol and increase its Ca^{2+} concentration simultaneously (Chávez et al., 2018). TMEM16E, a close scramblase relative of TMEM16F, is highly expressed in sperm, and its loss perturbs murine fertility. Characterization of pH_i in fertilization and its impact on TMEM16E function may offer insight into how mammalian gametes prepare for fusion and better inform our understanding of the regulatory processes surrounding human fertility.

Platelets, red cells, and sperm each exhibit high steady-state levels of a particular TMEM16 scramblase, but many cell types exhibit abundant levels of several TMEM16 scramblases simultaneously. For example, we previously demonstrated that skeletal muscle exhibits high levels of both TMEM16s E and F (Whitlock et al., 2018). What advantage would one cell gain by maintaining multiple scramblases that are each activated by a

single, shared signal? Upon increases in cytosolic Ca^{2+}, each of these TMEM16 scramblases should be activated simultaneously. However, we found that loss of TMEM16E perturbed Ca^{2+}-activated PS exposure in primary muscle cells despite high levels of TMEM16F, suggesting that the two play distinct roles in the same cell type. At the time, we assumed TMEM16s E and F were functionally separated in muscle by differences in Ca^{2+} sensitivity and/or trafficking, but could the function of these scramblase cousins be separated by pH_i? pH_i is rather distinct in skeletal muscle as compared with other, nonexcitable cell types with a pH_i of ∼6 at resting membrane potential (Carter et al., 1967). Depending on the Ca^{2+} concentration, the observations of Liang and Yang (2021) would suggest that TMEM16F could be entirely inhibited in resting skeletal muscle fibers. This idea is of course speculative but serves to illustrate how nodes within the TMEM16 scramblase module (Fig. 1) may serve to functionally separate TMEM16s in the same cell. One can easily assert that because the Ca^{2+} binding sites of TMEM16s F and E are so highly conserved, their pH_i sensitivity should be similar. After all, these are the same residues where pH_i likely exerts its regulatory effect in both. However, we found that TMEM16E appears to be much more sensitive to Ca^{2+} than TMEM16F (∼50% of cells expressing TMEM16E scramble in response to 1 µM Ca^{2+}, while >100 µM Ca^{2+} was required to achieve a similar response in TMEM16F expressing cells; Whitlock et al., 2018). If these relatives, with highly conserved Ca^{2+} binding sites, can differ dramatically in their sensitivity to Ca^{2+}, how can we assume they will not differ in their sensitivity to pH_i? By moving past Ca^{2+}, TMEM16 machines may have afforded themselves greater utility and promoted the diversity of their family in many cells.

Apart from healthy, physiological processes, pathogens may also use pH_i to tame TMEM16 scramblase function. PD-1 is an immunoregulatory protein that, when exposed on the surface of a variety of different cell types, dampens T cell activation and prevents self-reactive, pathogenic T cells. Perturbed, excessive exposure of PD-1 at the cell surface is a major contributor to T cell exhaustion in chronic HIV-1 infection (Porichis and Kaufmann, 2012). Interestingly, mice lacking TMEM16F also experience T cell exhaustion, and TMEM16F activity appears to play an important role in regulating the trafficking of PD-1 to the PM (Hu et al., 2016; Bricogne et al., 2019). Our laboratory has demonstrated that HIV activates TMEM16F and utilizes exposed PS as a cofactor in the fusion of the viral envelope with the PM (Zaitseva et al., 2017). After HIV gains entry into the cell, the HIV protein Vpr specifically down-regulates the expression of NHEI and acidifies the cytosol of infected cells (Janket et al., 2007). If the reader will permit some additional speculation, acidification may be a mechanism by which HIV inhibits TMEM16F in infected cells by downregulating NHEI and acidifying the cytosol. Inhibiting TMEM16F would likely contribute to T cell exhaustion, just as observed in the TMEM16F knockout model, and facilitate the ability of HIV-infected cells to avoid detection by the immune system, likely through increased trafficking of PD-1 to the PM. Perhaps HIV is a master scramblase tamer, both activating TMEM16F to gain cell entry and silencing the scramblase once in the cell?

In this reader's view, Liang and Yang's observations in the earlier issue of the *Journal of General Physiology* are tightly focused, but the consequences of these observations are broadly reaching (Liang and Yang, 2021). For what purpose do we tame something if not to leverage it for our goals? Over the last decade, we have learned much about the business of taming a scramblase. In the next, let us resolve what that taming is for (Fig. 2).

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