Investigating the Molecular Mechanisms of TMEM16F – a Ca\textsuperscript{2+} Activated Phospholipid Scramblase and Ion Channel

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Dong Yan

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate School of Duke University

2021
ABSTRACT

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Abstract

Transmembrane protein 16 (TMEM16) is a novel family of transmembrane proteins that function either as ion channels, lipid scramblases or both. In mammals, the majority of TMEM16 members are Ca\(^{2+}\)-dependent phospholipid scramblases (CaPLSases) that catalyze bidirectional movement of phospholipids across the membrane bilayer. Interestingly, some of these TMEM16 CaPLSases can also conduct ions, making them multifunctional (moonlighting) transporters. These moonlighting TMEM16 members have been linked to various physiological and pathological conditions, such as blood coagulation, ataxia, muscle dystrophy, cell-cell fusion and viral infection.

To further understand their biology and design therapeutics to treat the related diseases, it is urgent to unveil the structures, machineries as well as pharmacological profiles of the multifunctional TMEM16 proteins. However, studying TMEM16 proteins has been challenging due to their unique structural topologies and biophysical properties. Despite the recent progress in the structure and function understanding of the TMEM16 family, how the moonlighting TMEM16s gate and distinguish different permeating substrates remain open questions.

To resolve these unknowns and contribute to a more comprehensive understanding of the multifunctional TMEM16 proteins, this dissertation focuses on investigating the molecular mechanisms of TMEM16F – the first identified moonlighting
member of the TMEM16 family. We first developed a sensitive and reliable fluorescence microscopy-based scrambling assay that can be either used alone to assess TMEM16F CaPLSase activity or combined with electrophysiology to simultaneously examine TMEM16F CaPLSase and ion channel components (Chapter 2). Next, by applying our optimized scrambling assay together with computational simulation, mutagenesis screening and electrophysiology approaches, we uncovered the gating mechanism of TMEM16F and revealed the differences in protein conformation between TMEM16 - CaPLSases and -ion channels (Chapter 3). Furthermore, during our drug screening to identify antagonists for TMEM16F CaPLSase, we made a surprising discovery about the potential pitfalls of using fluorescence-based assay that could cause false positive results and challenge the identification of bona fide inhibitors for the CaPLSases (Chapter 4). Finally, our discovery of Subdued – a TMEM16 fly homolog – as a new moonlighting protein with similar biophysical properties to those of TMEM16F further expands our knowledge about the diversity and relationship among TMEM16 members (Chapter 5).

In summary, this dissertation advances the current understanding of the molecular underpinning and diverse functions of the TMEM16 family in general, and TMEM16F in particular.
Dedication

To my parents, my brothers and my husband for their continuous love and support.
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List of Abbreviations and Symbols

2APB 2-Aminoethyl diphenylborinate
Å Angstrom
a.u. Arbitrary unit
ABC ATP-binding cassette
af Aspergillus fumigatus
Ani9 2-(4-chloro-2-methylphenoxy)-N-[(2-methoxyphenyl)methylideneamino]-acetamide
AnV Annexin V
AnV-CF594 CF594-tagged Annexin V
AnV-CF640R CF640R-tagged Annexin V
ATP Adenosine triphosphate
BK channel Big potassium channel
Ca\(^{2+}\) EC\(_{50}\) Half-maximal activation concentrations of Ca\(^{2+}\)
CaCCinh-A01 6-(1,1-Dimethylethyl)-2-[(2-furanylcarbonyl)amino]-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid
CaCCs Ca\(^{2+}\)-activated Cl- channels
CaPLSase Ca\(^{2+}\)-activated phospholipid scramblase
Cas9 CRISPR associated protein 9
cDNA Complementary DNA
CRISPR Clustered Regularly Interspaced Short Palindromic Repeats
Cryo-EM Cryo-electron microscopy
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>eGFP-mTMEM16F</td>
<td>C-terminally eGFP–tagged mouse TMEM16F</td>
</tr>
<tr>
<td>$E_{rev}$</td>
<td>Reversal potentials</td>
</tr>
<tr>
<td>$F$</td>
<td>Fluorescence signal</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FFA</td>
<td>Flufenamic acid</td>
</tr>
<tr>
<td>$fs$</td>
<td>Femtosecond</td>
</tr>
<tr>
<td>$G/G_{max}$</td>
<td>Normalized conductance</td>
</tr>
<tr>
<td>GHK equation</td>
<td>Goldman-Hodgkin-Katz equation</td>
</tr>
<tr>
<td>$h$</td>
<td>Human</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ Balanced Salt Solution</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293T cells</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>$I$</td>
<td>current</td>
</tr>
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</table>
IgG  Immunoglobulin G
Iono  Ionomycin
m     Mouse/murine
MC    Monte Carlo
MD    Molecular dynamics
min   Minute
MONNA N-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid
n.s.  Non-significant
NFA   Niflumic acid
nh    Nectria haematococca
Non. Scr Non-scrambling
NPPB  5-nitro-2-(3-phenylpropylamino)-benzoate
NPT   Constant particle number, pressure and temperature condition
ns    Nanosecond
OSCA  Osmo-sensitive Ca²⁺ Channel, also known as TMEM63
P/S   Penicillin-Streptomycin
P4-ATPase Type 4 P-type ATPases
pA    picoampere
PBS   Phosphate-buffered saline
PC    Phosphatidylcholine
PCF   Patch clamp fluorometry
PCR  Polymerase chain reaction
PDB  Protein Data Bank
PE   Phosphatidylethanolamine
PFBS Perfluorobutane sulfonic acid
PI   Phosphoinositides
PIP2 Phosphatidylinositol-(4, 5)-bisphosphate
PLL  Poly-L-lysine
PME  Particle Mesh Ewald
PMF  Potential of mean force
P_{Na}/P_{Cl} Na+/Cl− permeability ratio
POPC 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
POPS 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
PS   Phosphatidylserine
pS   Picosiemens
ps   Picosecond
RIPA buffer Radioimmunoprecipitation assay buffer
RMSD Root mean square deviation
RNA  Ribonucleic acid
RNAi RNA interference
ROI  Region of interest
rpm Revolutions per minute
Ru-red Ruthenium red
s, sec second
SCAN Small-conductance, Ca2+-activated nonselective cation channel
SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM Standard error of the mean
sgRNA Single guide RNA
SM Sphingomyelin
STS Staurosporine
t_{1/2(\text{Imax})} The amount of time that each cell takes to reach 50% of its maximum AnV fluorescent intensity within ten minutes after ionomycin treatment
T16Ainh-A01 2-[(5-Ethyl-1,6-dihydro-4-methyl-6-oxo-2-pyrimidinyl)thio]-N-[4-(4-methoxyphenyl)-2-thiazolyl]acetamide
TA Tannic acid
TM Transmembrane
TMC Transmembrane channel-like protein
TMEM16 Transmembrane protein 16
TMEM16F-KO HEK293T CRISPR-Cas9 induced TMEM16F-knockout HEK293T
TRPC4 Transient Receptor Potential Cation Channel, subfamily C, member 4
TRPC5 Transient Receptor Potential Cation Channel, subfamily C, member 5
TRPM4 Transient Receptor Potential Cation Channel, subfamily M, member 4
V  Membrane voltage

$V_{1/2}$  Half-activation voltage

WGA  Wheat germ agglutinin

WT  Wild type

Xkr8  XK-related protein 8

$\tau_{\text{off}}$  Current run-down kinetics
Acknowledgements

First and foremost, I would like to express my gratitude towards my advisor – Dr. Huanghe Yang – whose continuing support and guidance play a significant role in my success in graduate school. Dr. Yang has instilled in me a passion for membrane biology and inspired me to achieve a deep appreciation of membrane transporters and dynamics. Furthermore, Dr. Yang always encourages and provides numerous training opportunities for me as well as other lab members to acquire the skillsets that are beneficial for our research careers. The opportunity to work in the Yang Lab for the past 5 years has helped me prepare for and determine my career goal of becoming an independent researcher.

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activation gate and TA/EGCG projects. Son not only helped me characterize the ion channel properties of TMEM16F, TMEM16A, Subdued and their mutations, but also showed me how to do electrophysiology by myself. I am also very thankful to Dr. Pengfei Liang for his help in characterizing BK channel in the TA/EGCG paper, and Dr. Ping Dong for his inputs in my projects and manuscripts. Additionally, I am grateful to Zoe Shan for her assistance in cell culture and biochemical assays while I was recovering from a hand injury.

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Chapter 1: Introduction

1.1 Phospholipid distribution in plasma membrane

The plasma membrane is built from hundreds of lipid species assembling into a lipid bilayer structure, in which lipids’ hydrophilic headgroups face the cytosolic and extracellular environment and shield their hydrophobic acyl chains from the aqueous surroundings (1-3). In their renowned Fluid-Mosaic model, Singer and Nicholson (4) presented some of the first evidences demonstrating that the lipid bilayer of biological membranes, such as the plasma membrane, consists of not only lipids but also carbohydrates and proteins. The membrane lipid bilayer forms an energetically favorable environment for integral and peripheral proteins to reside and carry out their biological functions (4). Overwhelming evidence has shown that the plasma membrane is a heterogeneous, sophisticated, dynamic, but highly ordered structure that is vital to life: the plasma membrane serves as a selectively permeable barrier regulating chemicals entering and exiting the cells, and as a platform for many biological reactions to occur (5-7). Therefore, plasma membrane is fundamental to cell survival, homeostasis and proper communication between cells in an organism (5-7).

Lipid compositions of biological membranes are extremely diverse: membrane of different tissues, cellular life cycles or organisms could exhibit lipids with different
chemical structures and biophysical properties (2, 7-9). Nonetheless, plasma membrane of
all eukaryotes shares a common blueprint: an asymmetric distribution of phospholipids
between the two membrane leaflets (Figure 1) (1-3, 10-12). This asymmetric arrangement
of plasma membrane is featured by zwitterionic phospholipids, such as
phosphatidylcholine (PC) or sphingomyelin (SM), being distributed mainly to the
extracellular membrane leaflet, whereas negatively charged phospholipids, such as
phosphoinositides (PI) and phosphatidylserine (PS), being confined to the cytosolic leaflet
of the membrane (1-3, 10-12). Interestingly, membrane asymmetry is often derived from
the distribution of lipid headgroups; however, it is unclear whether the fatty acid tails,
which make up the hydrophobic core of the membrane, also contribute to membrane
asymmetric structure. Recently, Lorent et al. (2) elegantly combined lipidomic,
computational simulation, and fluorescence imaging approaches to unveil that fatty acid
tails of lipids indeed participate in forming the membrane asymmetry: the outer
membrane leaflet is mainly packed with saturated acyl chains, while the inner leaflet is
filled with poly-unsaturated lipids.

The asymmetric distribution of phospholipids not only creates unique electrical
properties of the plasma membrane, but also dictates the conformation, location and
functional properties of various integral and peripheral proteins (1, 2, 11, 13, 14).
Disruption of phospholipid asymmetry alters the membrane physicochemical properties,
affecting cellular homeostasis and signaling cascades (1, 11, 15). Hence, it is important to understand the molecular machinery that is involved in maintaining as well as dissipating the phospholipid distribution of plasma membrane.

Figure 1: Schematic illustration of the asymmetric phospholipid distribution of plasma membrane. ATP-dependent flippases and floppases help maintain, whereas ATP-independent scramblases disrupt the asymmetric phospholipid distribution of the plasma membrane. Figure is created with BioRender.com.

1.2 Lipid transporters that are involved in regulating asymmetric phospholipid distribution of plasma membrane

Three key lipid transporters that participate in regulating membrane phospholipid asymmetry are commonly identified as flippases, floppases and scramblases (Figure 1) (1, 5, 11, 16, 17). Flippases and floppases are adenosine triphosphate (ATP) dependent phospholipid transporters that belong to type 4 P-type ATPases (P4-ATPases) and ATP-binding cassette (ABC) families, respectively (5, 17). Flippases specifically transport PS and phosphatidylethanolamine (PE) from the outer to the inner leaflet of the membrane.
(5, 17). On the other hand, floppases have less substrate specificity than flippases and catalyze phospholipids’ movement (mostly PC, but can also transport cholesterol, SM, PE and PS) from the inner to the outer membrane layer (5, 17). By facilitating unidirectional movement of phospholipids against their chemical gradients, flippases and floppases are responsible for establishing the asymmetric distribution of phospholipids (Figure 1) (1, 5, 11, 17).

Scramblases are the third group of lipid transporters. Instead of generating and maintaining the asymmetric phospholipid distribution like flippases and floppases do, scramblases, as passive transporters, facilitate rapid and bidirectional movements of phospholipids across the membrane in an ATP-independent manner (Figure 1) (1, 5, 11, 16, 17). Consequently, the translocation of phospholipids down their chemical gradients destroys the phospholipid asymmetric distribution, changes the physicochemical environment of the membrane and alters various downstream signaling cascades (1, 11, 15). PS – an anionic phospholipid – typically resides at the membrane inner leaflet. However, upon scramblase activation, PS is flipped to the outer leaflet of the membrane (1, 18). The presence of PS on the cell surface has various physiological functions, such as serving as a vital cofactor in the blood coagulation machinery and an ‘eat-me’ signal that recruits macrophages to come and engulf the apoptotic cells (1, 18). Among all the
scramblases identified, TMEM16F has received much of the spotlight and become one of the most well-characterized scramblases so far (1, 11, 15, 19).

1.3 The discovery of TMEM16F

TMEM16F belongs to the TMEM16 family, whose members are expressed throughout eukaryotes (20). Although TMEM16F is one of the most studied members of the TMEM16 family, it is not the first discovered member of the family. TMEM16A and TMEM16B – bona fide Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs) – are the founding members of the TMEM16 family (15, 21-26). Shortly after that, TMEM16F was uncovered and became the first identified Ca\(^{2+}\)-dependent phospholipid scramblase (CaPLSase) of the TMEM16 family (27). More surprisingly, TMEM16F was later found to behave as a Ca\(^{2+}\)-gated ion channel, in addition to its lipid scrambling activity (28). TMEM16F’s unique dual-function renders it a moonlighting protein, which is defined as a protein that can perform different functions in the absence of gene fusions, various RNA splicing or multiple proteolytic fragments (29-32).

Since the discoveries of TMEM16-A, -B and -F, more TMEM16 members were uncovered. Their evolutionary relationships, molecular functions and physiological significance are summarized in Figure 2. Despite the initial prediction that the whole family would function as CaCCs, the majority of TMEM16 proteins are CaPLSases (15, 20, 33-35). Intriguingly, many of these TMEM16 CaPLSases, including the fungal homologs
from *Aspergillus fumigatus* (afTMEM16) and *Nectria haematococca* (nhTMEM16), and mammalian TMEM16D-F,J,K, also behave as ion channels, thus making them moonlighting proteins (34-48). Although many moonlighting TMEM16s play important roles in human health and disease (summarized in Figure 2B) and each TMEM16 member itself is a fascinating transporter, TMEM16F remains the main subject of this dissertation.

**Figure 2: Relationship and functions of TMEM16 proteins.** A, Phylogeny tree depicts the evolutionary relationship between representative members of the TMEM16 family. Murine (m), *Drosophila*, *Caenorhabditis elegans*, yeast and fungal TMEM16s are colored in blue, magenta, black, orange and green, respectively. Protein sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the phylogenetic tree was composed using iTOL v4 (https://itol.embl.de/). B, Summary of mammalian TMEM16 members’ molecular functions and roles in human physiology and pathology. Information in (A) and (B) are based on (15, 20, 34, 49-51).
1.3.1 TMEM16F functions as a Ca\(^{2+}\)-activated phospholipid scramblase

As a CaPLSase, TMEM16F catalyzes rapid flip-flop of phospholipids across the membrane bilayer upon increase of intracellular Ca\(^{2+}\) (1, 27). By using various lipid sensors to detect different phospholipid species such as PC, PS, PE and SM, Suzuki et al. (27) – the first study that discovered TMEM16F as a CaPLSase – demonstrated that TMEM16F could transport all of the tested phospholipids at comparable rates. By using live-cell and cell-free approaches, subsequent studies about TMEM16F and other TMEM16 CaPLSases further confirmed that these lipid transporters could accommodate a wide range of lipid species (35, 38, 44, 52). Hence, TMEM16 CaPLSases are generally considered to be nonselective lipid transporters (15, 27, 33, 35, 38, 53).

Although the identification of TMEM16F as a phospholipid scramblase brings a lot of excitement to the field, much of its molecular mechanism and biophysical properties are subjects of controversy. First, although TMEM16F and other TMEM16 CaPLSases are conventionally considered to be non-selective for lipids, it is impractical to precisely determine lipid selectivity and permeability for these lipid transporters due to the current technical limitations and the highly diverse lipid repertoires (more than 400 lipid species) of the cells (2, 15). Hence, the actual lipid selectivity of TMEM16 CaPLSases remains obscure. Moreover, it is very challenging to detect movement of minor lipids (such as PIs) or highly dynamic lipids (such as cholesterol), so whether TMEM16 CaPLSases transport
these lipid species is still an unsolved question (15, 54-56). Furthermore, it is also undetermined whether TMEM16F and other TMEM16 CaPLSases have preference toward lipids with certain types of acyl chains (15). Lastly, owing to limited structural information and lack of sensitive assays, the regulatory and transporting mechanisms of TMEM16 CaPLSases are largely unknown.

1.3.2 TMEM16F is also a Ca\(^{2+}\)- and voltage-gated ion channel

As an ion channel, TMEM16F displays distinct biophysical properties from its CaCC siblings – TMEM16A and TMEM16B (20). Although TMEM16-A, -B, and -F ion channels are all Ca\(^{2+}\)- and voltage-dependent, the CaCCs, especially TMEM16A, can be opened with low (sub-micromolar range) intracellular Ca\(^{2+}\) level, whereas TMEM16F activation requires much higher (micromolar range) intracellular Ca\(^{2+}\) level (21-26, 28, 52, 57, 58). Moreover, TMEM16A CaCC exhibits Ohmic current at high intracellular Ca\(^{2+}\) level (23, 26, 59), while TMEM16F ion channel can hardly be opened without voltage even with excessive amount of intracellular Ca\(^{2+}\) (28, 57, 58, 60).

TMEM16F also displays a unique ion selectivity and permeability. Instead of being anion selective like TMEM16A and TMEM16B, TMEM16F ion channel is relatively non-selective – similar to its scrambling counterpart (20, 27, 28, 52, 57, 58, 61). Nonetheless, TMEM16F does exhibit higher permeability towards cations than anions with Na\(^+\)/Cl\(^-\) permeability ratio (P\(_{Na}\)/P\(_{Cl}\)) more than two (28, 52). With its low ion selectivity and small
single channel conductance (0.5 pS), TMEM16F is classified as a small-conductance, Ca\textsuperscript{2+}-activated nonselective cation channel (SCAN) (28).

Pharmacological profile of TMEM16F ion channel is also different from that of TMEM16 CaCCs. Common CaCC blockers, such as niflumic acid (NFA) or 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), are ineffective toward TMEM16F, but effectively inhibit TMEM16A and TMEM16B (28, 49, 62). Only antagonists of nonselective cation channels, such as Ruthenium red (Ru-red), 2-Aminoethyl diphenylborinate (2APB) or Cd\textsuperscript{2+}, block TMEM16F ion current from the intracellular side, further supporting that TMEM16F has distinct biophysical properties from TMEM16A and TMEM16B (20, 28, 58).

It is hard to envision how proteins of the same family such as TMEM16F, TMEM16A and TMEM16B share similar structural topologies and sequence homology, but display distinct functions and characteristics (20). Since TMEM16A and TMEM16B were found to be CaCCs (21-26), the subsequent identification of TMEM16F as a CaPLSase and SCAN was unexpected and became matters of debate in the early days of TMEM16 research (20, 27, 28, 57, 58). Thanks to the nonstop efforts to uncover the enigmatic TMEM16F, there are now strong evidences supporting that TMEM16F is a CaPLSase and a Ca\textsuperscript{2+}-gated nonselective ion channel (27, 28, 52, 57, 58, 63).
1.4 The significance of TMEM16F in health and disease

The list of physiological functions of TMEM16F has been expanded rapidly since the protein’s initial identification in 2010 (27). Up to now, TMEM16F has been detected in various tissues throughout human body with its roles ranging from blood coagulation (27, 28, 64-67), bone (68, 69) and vascular (70) development, cell-cell fusion (71) to viral infection (72) (Figure 2B).

1.4.1 TMEM16F in blood coagulation

Although TMEM16F has been linked to many cellular processes in both physiological and pathological conditions, the protein is most well-recognized for its role in blood coagulation (27, 28, 64-67). To prevent continuous bleeding from damaged blood vessels, TMEM16F-mediated PS exposure on the surface of platelets, red blood cells, and lymphocytes serves as an essential co-factor and assembly platform for coagulation cascades such as prothrombinase and tenase complexes (1, 16, 73). Without functioning TMEM16F, the coagulation machinery is malfunctioned, such as in the case of Scott syndrome – a hereditary hemostatic disorder resulted from TMEM16F loss-of-function mutations (16, 27, 64, 74-76). When having major injuries, Scott syndrome patients exhibited prolonged and excessive bleeding condition stemming from the lack of PS exposure, which is important for thrombin generation, on the surface of blood cells (76-79). Likewise, mice with TMEM16F silencing and canines carrying TMEM16F loss-of-
function mutations also exhibit long-lasting bleeding episodes (28, 65-67). Moreover, TMEM16F null mice also exhibited resistance towards thrombotic challenges (28). Together, these evidences not only underscore the importance of TMEM16F in blood clotting, but also suggest that pharmacological molecules targeting TMEM16F CaPLSase could be potential treatments for thrombosis-associated pathologies such as stroke or deep vein thrombosis (80).

1.4.2 TMEM16F in microvesicle shredding

TMEM16F is also known for its roles in microvesicle shredding, which has various physiological implications (65, 68, 81-83). TMEM16F-mediated membrane vesiculation was first described in blood cells, and these microvesicles were suggested to serve as amplifiers for the coagulation signaling cascade by increasing the surface area for coagulation assembly during hemostatic process (76, 79). In fact, blood cells from Scott syndrome patients and TMEM16F-deficient mice not only failed to externalize PS but also lacked microparticle shredding, further underpinning the importance of TMEM16F-induced membrane vesiculation in blood clotting (1, 16, 65, 67, 76, 79). Beside blood cells, TMEM16F-mediated microvesicle release has also been found in other cells and suggested to facilitate membrane repair (83), bone mineralization (1, 68), inflammatory (81) and immune responses (82, 84). Although TMEM16F-induced membrane vesiculation has been widely observed in various cellular processes, the mechanism of TMEM16F-
stimulated membrane shredding remains unknown. Recent studies suggest that TMEM16F-mediated scrambling activity could alter lipid packing and trigger membrane expansion that favor membrane vesicle formation and release (15, 82).

1.4.3 TMEM16F-mediated PS exposure is crucial for cell-cell fusion

TMEM16F is also known to be important for cell-cell fusion, which is a prerequisite cellular process in many physiological events such as muscular development, fertilization or growth (15, 85-87). PS exposure on the cell surface is a critical factor for cell fusion by either priming the fusing site or attracting the PS receptors of other fusing cells (15, 71, 87). Nonetheless, the molecular identity of proteins that transport PS to the extracellular side of the membrane to facilitate cell-cell fusion was undetermined. Recently, our lab identifies TMEM16F CaPLSase as a major lipid transporter that induces externalization of PS for trophoblast cell-cell fusion – an indispensable step in placental development (71). TMEM16F gene ablation in trophoblast cells significantly attenuated the cells’ fusion index (71). Furthermore, TMEM16F knockout mice also displayed severe placental defect, which could stem from the trophoblast cell-cell fusion problem (71). Another lab also reveals that TMEM16F-induced PS exposure plays a major role in HIV-host cell fusion (72). From its initial interaction with the host cells, HIV triggers Ca^{2+} influx to activate TMEM16F CaPLSase (72). TMEM16F-mediated PS exposure on the host cell membrane then serves as a designated site for the virus to dock and fuse with the host, initiating the
infection process (72). These evidences underscore the importance of TMEM16F CaPLSase in membrane fusion not only between cells of the same organism, but also between the host cells and the infectious microbes.

1.4.4 The importance of understanding the molecular mechanism of TMEM16F

TMEM16F has attracted so much attention due to its importance in human health. However, due to its unique structural architecture and novel moonlighting property, TMEM16F remains a perplexing transporter with many unsolved questions. Therefore, to achieve a more comprehensive understanding about TMEM16F and its clinical implications, there is an urge to uncover the structures, molecular mechanisms as well as pharmacological profiles of the transporter. A solid understanding of TMEM16F molecular mechanisms and structures would pave the way to develop pharmacological tools and therapeutic reagents targeting TMEM16F-related diseases, both of which are critical, yet non-existing at the moment.

1.5 TMEM16F structure

1.5.1 General architecture

Although TMEM16F’s unique moonlighting feature has attracted much attention and been studied extensively, its structures remained unsolved until recently. In 2019, two research groups from the United States and Netherlands – simultaneously unveiled multiple structures potentially representing different conformational states of
mTMEM16F by using cryo-electron microscopy (EM) method (52, 63). Generally, both studies have shown that structures of TMEM16F exhibit common features of the TMEM16 family: a homodimeric architecture with each subunit comprises of ten transmembrane (TM) α-helices; the highly conserved Ca\(^{2+}\) binding site is created by a cluster of negatively charged and polar residues located in TMs 6-8 (Figure 3A, 3C) (52, 63). Across from the dimer interface, which is relatively hydrophobic, TMs 3-7 of each subunit form a hydrophilic groove (also called subunit cavity) at the protein-membrane interface (Figure 3A, 3B) (52, 63). Many computational, functional and structural studies have evinced that the hydrophilic groove is the permeation pathway for many TMEM16 proteins, including TMEM16F (20, 23, 26, 28, 34, 36, 40-45, 52, 58, 59, 88-92). Despite sharing a common design with other TMEM16 members, TMEM16F structures display a unique feature: the hydrophilic groove of TMEM16F is very narrow (52, 63) and its arrangement induces membrane distortion (43, 63). These structural aspects of TMEM16F hold valuable functional implications that will be discussed in the later subsections.
Figure 3: mTMEM16F structure. A, Ca$^{2+}$ bound mTMEM16F structure (PDB: 6QP6 (52)) is depicted using cartoon representation. Each monomer is shown in different colors (green and grey). Brown spheres illustrate Ca$^{2+}$ ions. Hydrophilic groove, dimer interface and Ca$^{2+}$ binding site are marked and labeled with dotted box, line and circle, respectively. B left, Hydrophilic groove of Ca$^{2+}$ bound mTMEM16F structure (PDB: 6QP6). The hydrophilic groove is comprised of TMs 3-7 (numbers are labeled). B right, superposition of hydrophilic grooves of Ca$^{2+}$ bound mTMEM16F (PDB: 6QP6; green) and Ca$^{2+}$ bound nhTMEM16 (PDB: 4WIS (44); raspberry). C, Negatively charged and hydrophilic residues composing Ca$^{2+}$ binding site of mTMEM16F (in A) are shown in sticks. Brown spheres represent Ca$^{2+}$ ions. This figure is generated with the PyMOL Molecular Graphics System, Version 2.4.1 Schrödinger, LLC.

1.5.2 Permeation pathway

TMEM16F structure exhibits very narrow hydrophilic groove, even with Ca$^{2+}$ binding (Figure 3B) (52, 63). The grooves of both Ca$^{2+}$-free and Ca$^{2+}$-bound TMEM16F
structures display ‘hourglass’ shape with water accessible vestibules located at the extracellular and intracellular ends (52, 63). The two aqueous vestibules are connected by a confined neck that is less than 2 Å in diameter, in spite of Ca\textsuperscript{2+} binding (52, 63). Hence, the constricted neck makes the hydrophilic groove impermeable for both ions and lipid headgroups.

This structural arrangement of TMEM16F hydrophilic groove, especially in the Ca\textsuperscript{2+}-bound conformation, is unexpected as previously solved Ca\textsuperscript{2+}-bound structures of other TMEM16 moonlighting proteins, such as human (h) TMEM16K, nhTMEM16 and afTMEM16, exhibit widely open subunit cavities (8-11 Å) (Figure 3B) (34, 41, 42, 44, 45). The widely open subunit cavities of hTMEM16K and the two fungal homologs – nhTMEM16 and afTMEM16 – are lipids accessible and fit the credit-card reader model (93), which has long been envisaged and corroborated to be lipid transporting mechanism of TMEM16 CaPLSases (20, 34, 36, 40-45, 52, 90, 94). In the credit-card reader model, lipids are postulated to dock their hydrophilic headgroups into the hydrophilic grooves of the scramblases. The lipids then slide through the grooves to translocate from one to the other leaflet of the membrane, while their acyl tails remain in the hydrophobic core of the membrane (Figure 4) (93). Therefore, the enclosed and constricted permeation pathway of TMEM16F has caught many people by surprise.
Figure 4: Credit-card reader model. Illustration demonstrates how lipids permeate through hydrophilic grooves of TMEM16 CaPLSases, such as TMEM16F, based on the credit-card reader model. Figure is created with BioRender.com.

1.5.3 Transporting mechanisms derived from TMEM16F structures

To explain this special conformation of TMEM16F permeation pathway, there have been other alternative hypotheses and models generated. Firstly, it has been hypothesized that the recent Ca$^{2+}$-bound structures of TMEM16F may not represent the fully open conformation of the CaPLSase (52). The Ca$^{2+}$-bound structure of TMEM16F is analogous to the structures of Ca$^{2+}$-bound TMEM16A and intermediate state of nhTMEM16 (41, 91). Therefore, it is postulated that the current structures of Ca$^{2+}$-bound TMEM16F might represent the intermediate state or the formation of an enclosed protein pore for ion-conductive-only conformation that were discerned previously in structures of the fungal homologs and TMEM16A (41, 42, 45, 52, 91). Secondly, in a different
explanation, TMEM16F may simply adopt an alternative lipid transporting mechanism (52, 63). For example, in ‘out-of-the groove’ model, it is suggested that lipids slide across the membrane without fully docking their headgroups into the hydrophilic groove (95). Instead, lipid headgroups partially interact and glide on the protein-membrane interface of the hydrophilic groove (63, 95). Thus, the groove does not need to be widely opened for lipids to permeate while it still allows ions to go through (63). Despite these structural information and hypotheses, it is still uncertain how TMEM16F transports lipids across the membrane. Moreover, although multiple lipid transporting mechanisms have been proposed for TMEM16F and other TMEM16 CaPLSases, how these scramblases gate their lipid permeations is unclear. In Chapter 3, I will further discuss the current insights and unknowns about the gating mechanisms of TMEM16 CaPLSases, as well as our efforts to further unravel these challenges.

1.5.4 TMEM16F conformation shapes the surrounding membrane

Despite the ambiguity due to its constricted hydrophilic groove, TMEM16F structures impart some insights on how organization of lipid bilayer could influence the transporter’s function and vice versa. In their TMEM16F structures, Feng et al. (63) showed that membrane surrounding the extracellular and intracellular entrances of the hydrophilic groove was indented, creating a thinner lipid bilayer near the permeation pathway than the average thickness of the membrane. The study speculated that this
membrane thinning phenomenon, which was potentially due to hydrophobic mismatch between the protein and membrane lipids, could shorten the transporting distance for lipids, thus facilitating lipid movement across the membrane bilayer (63). This membrane distortion is dependent on phosphatidylinositol-(4,5)-bisphosphate (PIP₂) (63), which was previously demonstrated to be essential for stability of TMEM16F ion channel activity (60). Although further examination is needed to fully characterize the relationship between membrane thinning and TMEM16F’s functions, similar membrane thinning around the permeation pathways of other TMEM16 CaPLSases, but not that of the CaCCs, have been frequently observed in both structural and computational studies (34, 36, 41, 43, 45, 96). Moreover, Falzone et al. (45) demonstrated that perturbing the membrane thinning by employing lipids with long acyl tails could inhibit CaPLSase activity of aTMEM16. On the other hand, Bushell et al. (34) showed that lipids with short acyl tails could enhance the CaPLSase activity of hTMEM16K. Hence, the membrane thinning phenomenon might be significant and fundamental to scrambling activity of TMEM16 CaPLSases.

1.6 Questions addressed in this dissertation

Being one of the most representative members of the TMEM16 family, TMEM16F is the prime subject for multiple high impact researches. However, TMEM16F structures, functions and activation mechanisms are yet to be comprehensively understood.
TMEM16F mechanistic studies have been challenging, firstly due to its novel architecture with extraordinary proteo-lipidic pore that is not resembling any known ion or lipid transporters. Secondly, the recently solved TMEM16F structures do not provide much mechanistic information, as all those structures likely captured the impermeable state of the protein (52, 63). Lastly, TMEM16F moonlighting property is fascinating, but it also increases the sophistication in studying this protein as well as other dual function TMEM16 members because of current technical limitations. Thus, although various mechanisms and models have been proposed to explain the functional meanings behind TMEM16F’s structures, many of them remain hypothetical and require more works for validation. The gating, the activation mechanisms of TMEM16F and how TMEM16F accommodates two distinct substrates – lipids and ions – are still ambiguous aspects.

Here, to elucidate the functional mechanism of TMEM16F, we will first establish a sensitive imaging based method to reliably detect and quantify activities of TMEM16F and other TMEM16 CaPLSases in live cells (Chapter 2). The method will serve as an essential tool in this study to characterize structure-function of TMEM16F. Next, we will combine the imaging method developed in Chapter 2 with electrophysiology, mutagenesis screening and computational simulation to elucidate the Ca\(^{2+}\) dependent gating mechanism of TMEM16F lipid scramblase (Chapter 3). After having solid understanding of TMEM16F’s molecular mechanism, we will apply our established tools
to investigate the pharmacological profile of TMEM16F (Chapter 4) and characterize other TMEM16 proteins to understand more about the evolution as well as functional diversity of the TMEM16 family (Chapter 5).
Chapter 2: Methods to study moonlighting TMEM16F

Portions of this chapter are reused and adapted from researches originally published in the Journal of Biological Chemistry\(^1\) and Nature Communication\(^2\).

Some data in this chapter were done in collaboration with members of the Yang Lab\(^3\) and Duke RNAi facility\(^4\).

2.1 Background

Deciphering the enigmatic moonlighting mechanisms of dual function TMEM16 members, such as TMEM16F, has not been a straightforward task. Although it is currently well accepted that TMEM16F is a CaPLSase and a non-selective ion channel (20, 27, 28, 52, 57, 58, 61), there used to be a great disagreement regarding the actual functions and biophysical properties of TMEM16F (20). For example, some studies reported that TMEM16F ion channel exhibited Cl\(^-\) channel–like properties (97-102), while others

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\(^1\) Portions of this chapter are reused and adapted from: Le, T., Le, S. C., and Yang, H. Drosophila Subdued is a moonlighting transmembrane protein 16 (TMEM16) that transports ions and phospholipids. J Biol Chem. 2019; 294:4529-4537. © 2019 Le et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.


\(^3\) Dr. Yang Zhang wrote the MATLAB code that enables automatic extraction of the fluorescence signal of AnV in time-lapse imaging data.

\(^4\) Dr. So Young Kim designed sgRNA sequences targeting TMEM16F and generated the original polyclonal population of CRISPR-Cas9 control and TMEM16F-knockout HEK293T cells.
demonstrated that TMEM16F behaved as a cation-permeable channel (28, 52). These variations suggest that TMEM16F could be a highly complex and flexible transporter that adopts multiple conformations, each of which features a unique functional property (20, 52, 61). It is therefore critical to develop robust and quantitative functional assays to study this highly complicated moonlighting transporter. Unfortunately, the currently available methods are only able to capture some, but not all of the functional events, ensuing the inconsistent observations regarding TMEM16F functional properties (20). Moreover, most of the available assays lack the ability to simultaneously assess the scrambling activity and ion channel components of the moonlighting TMEM16s. In this chapter, I will first review the advantages and drawbacks of the commonly used methods in investigating the dual function TMEM16s, and then discuss the approaches that I and my colleagues have developed to overcome the limitations of the existing methods.
2.2 Assays to detect phospholipid scrambling activity

2.2.1 Common approaches in detecting and examining the phospholipid scrambling activity

Table 1: Summary of advantages and limitations of the common methods in studying CaPLSases

<table>
<thead>
<tr>
<th>Methods</th>
<th>Pros</th>
<th>Cons</th>
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<tbody>
<tr>
<td>Flow cytometry</td>
<td>Large sampling size; live cell assays; multiple fluorescence markers can be used at once</td>
<td>Not convenient for adherent cells; lack of single cell and subcellular resolution; potential contamination with endogenous scramblases; no control with intracellular contents/solutions; not compatible for intracellular CaPLSases and combining with electrophysiology</td>
</tr>
<tr>
<td>Liposome reconstitution</td>
<td>Flexibility to incorporate different lipid species; flexibility in controlling the intra- and extra- liposome solutions; minimal contamination with endogenous CaPLSases</td>
<td>The membrane environment is not native to the CaPLSases; the CaPLSases are isolated from their natural cellular contents; labor intensive to purify and reconstitute the scramblases; incompatible with electrophysiology</td>
</tr>
<tr>
<td>Fluorescence microscopy-based assay</td>
<td>Live cell assays; single-cell and subcellular resolution; multiple fluorescence markers can be used at once; compatibility with patch clamp</td>
<td>Potential contamination with endogenous scramblases; no control with intracellular contents/solutions; small sampling size</td>
</tr>
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</table>

2.2.1.1 Flow cytometry

Flow cytometry is a classical approach in studying TMEM16 CaPLSases, especially the mammalian members (35). This technique was employed to detect the deficiency in PS exposure and microvesicle formation of Scott patients’ blood cells a long time ago, even prior to the molecular identity of TMEM16F CaPLSase (76, 79). Flow cytometry has the
advantages of large sampling size and monitoring TMEM16 CaPLSase activities in live cells (27, 35, 65, 88, 103). Furthermore, flow cytometry allows the users to simultaneously utilize different fluorescence wavelengths for different cellular markers. Through this advantage, apoptotic markers, such as propidium iodide, and phospholipid scrambling probes, such as fluorescein taggded Annexin V (AnV) – a small protein that specifically binds to PS in a Ca\(^{2+}\) dependent manner (104) – can be used concurrently to segregate TMEM16-dependent lipid scrambling cells from apoptosis-induced ones (27, 105).

Nonetheless, a major drawback of flow cytometry is that the method is often limited to non-attached cells (27, 35, 65, 88, 103). Applying this method to adherent cells require detaching the cells by mechanical force or enzyme treatment, such as trypsinization, which might interfere with the cell homeostasis and the proteins of interest (106, 107). Besides, flow cytometry can only detect activity of lipid scramblases that are residents of plasma membrane but not those reside in the membrane of intracellular organelles, because the lipid probes that are used in detecting the lipid scrambling activity in live cells are membrane impermeable (35, 57, 58, 88, 103).

2.2.1.2 Liposome reconstitution

Liposome reconstitution is one of the most frequently used methods in examining TMEM16 scramblases (34, 37, 38, 40-42, 44, 45, 52, 95, 108, 109). This method is executed by incorporating purified lipid scramblases into liposomes that consist of fluorescently
tagged lipids evenly distributed to both leaflets of the membrane (38, 109). The membrane impermeable dithionite is added to quench the fluorescent lipids. If there is no active scramblase, only fluorescent lipids of the exofacial surface are quenched, whereas those of the cytofacial surface remain intact, resulting in about 50% reduction of the liposomes’ fluorescence signal. On the other hand, in the presence of active lipid scramblases, lipids at the outer and the inner membrane leaflets of the liposomes are continuously exchanged, allowing all lipids to contact with and be quenched by dithionite. Thus, fluorescence signal of the liposomes will be completely depleted in the presence of lipid scramblases (38, 109).

One major advantage of liposome reconstitution is its flexibility to incorporate different lipid species into the liposomes. Thus, the method is advantageous in assessing lipid permeability, selectivity and behaviors of TMEM16 CaPLSases in different lipid environments (109). Moreover, thanks to liposome reconstitution, elucidating intracellular TMEM16 members, such as TMEM16K, becomes possible (34). Another benefit of liposome reconstitution is the flexibility to control the intra- and extra- liposome solutions, hence ones can monitor the precise amount of Ca\textsuperscript{2+} that they would like to use in activating the CaPLSases (109). This allows a more accurate determination of Ca\textsuperscript{2+} sensitivity for each TMEM16 CaPLSase than the cell-based assays, where controlling intracellular contents is almost unachievable.
Nonetheless, the biggest problem of liposome reconstitution is that it does not recapitulate the actual properties of physiological membrane. Hence, the kinetics and mechanisms of the lipid scramblases that are characterized in liposome reconstitution might not represent how those lipid transporters actually behave in their natural environments. For instance, nhTMEM16 can function as both ion channel and CaPLSase (20, 36, 37, 40, 42). However, in its initial discovery, nhTMEM16 reconstituted in liposome only elicited CaPLSase activity without any detectable ion conductance (44). Only until recently, by using cell-based assay and modifying the lipid components of the liposomes, function of nhTMEM16 as an ion channel was noticed (36, 37, 40, 42). Additionally, both liposome reconstitution and flow cytometry lack the resolution to extract the dynamics and single-cell visualization of the scrambling activity in live cells. It is also challenging to combine flow cytometry or liposome reconstitution with electrophysiology to simultaneously examine scrambling activity and ion conduction of the moonlighting TMEM16 proteins.

2.2.1.3 Fluorescence microscopy-based assay

To address the underlying problems of liposome reconstitution and flow cytometry, the Hartzell Laboratory designed a fluorescence microscopy-based assay to study TMEM16F CaPLSase in live cells (58). The framework of this assay is described as follows: Ca\(^{2+}\)-ionophore (such as ionomycin or A23187) is used to induce Ca\(^{2+}\) influx in
TMEM16F expressing cells to activate the lipid scramblase, which then transports PS from the inner to the outer leaflet of the plasma membrane. The externalized PS will rapidly bind to the fluorescently tagged AnV that is readily available in the extracellular solution (58, 104). As more PS are transported to the outer layer of the membrane, more AnV will bind and fluorescent signal will accumulate on the cell surface indicating the on-going scrambling activity (Figure 5A).

The fluorescence microscopy-based method has many advantages. One of them is that the method allows real-time visualizing and monitoring scrambling activity in live cells at cellular and subcellular resolution (36, 58). The fluorescence microscopy-based assay can also be integrated with patch clamp to assess lipid transport and ion conductance simultaneously (36, 58). Nonetheless, this assay still has certain limitations that require further optimization: firstly, HEK293T cell-line is often used in this assay to transiently express and characterize wild type (WT) TMEM16 CaPLSases and their mutations. However, others’ and our studies have unveiled that HEK293T has a significant amount of endogenous TMEM16F expression and CaPLSase activity, which could complicate the result and data interpretation of the heterologously expressed lipid scramblases (Figure 5B-5D) (97, 110, 111). Besides, A23187, a potent Ca$^{2+}$ ionophore, is commonly used in the assay to increase intracellular Ca$^{2+}$ and subsequently activate TMEM16F CaPLSase (58). Although A23187 is very effective in mobilizing Ca$^{2+}$ into the
cells, it can easily cause Ca\textsuperscript{2+} overload and subsequent cell apoptosis (112), which also triggers lipid scrambling activity and PS exposure in a caspase-dependent manner (19, 105). To accurately characterize TMEM16 CaPLSases, apoptosis-dependent lipid scrambling activity (19, 105) must be controlled and minimized. Thus, to explicitly analyze the lipid transporting activity of TMEM16 CaPLSases that are ectopically expressed in HEK293T cells, we re-designed and optimized the microscopy-based scrambling assay.

2.2.2 Re-designing the microscopy-based scrambling assay

Because of its advantages in live-cell monitoring, high resolution and compatibility with electrophysiology, we decided to re-design and optimize the microscopy-based imaging approach (Figure 5A) to examine the scrambling activity of TMEM16F. The assay optimization is comprised of generating TMEM16F-knockout cell line, modifying the prototypical fluorescence microscopy-based assay (58) and improving data analysis and quantification.

2.2.2.1 Generating TMEM16F-knockout cell-lines

As it has been discussed in subsection 2.2.1, a cell-line with the absence of the endogenous TMEM16F expression and lipid scrambling activity is a prerequisite for developing a reliable and sensitive scrambling assay. Despite its endogenous TMEM16F expression and CaPLSase activity, HEK293T is an efficient and convenient cell system for heterologous protein expression, especially for membrane proteins (113). Hence, we
decided to continue using HEK293T and apply Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated protein 9 (Cas9) to knock-out the endogenous TMEM16F background in HEK293T. With the help of Dr. So Young Kim from Duke RNAi facility and after multiple rounds of single-cell colony screening, we successfully generated CRISPR-Cas9 induced TMEM16F-null HEK293T (TMEM16F-KO HEK293T) cell-line as evidenced by the loss of both TMEM16F protein expression and the endogenous CaPLSase activity (Figure 5C-5E). Reintroduction of C-terminally enhanced green fluorescent protein (eGFP)-tagged mTMEM16F (henceforth referred to as eGFP-mTMEM16F) through transient transfection to TMEM16F-KO HEK293T cell-line rescued its CaPLSase activity (Figure 8A). This data further confirms that TMEM16F is responsible for the background scrambling activity in the WT HEK293T cells and CRISPR-Cas9 has successfully knocked-out the endogenous TMEM16F in the HEK293T cells (Figure 5, 8). Therefore, all of our subsequent functional characterizations of WT TMEM16F, TMEM16F mutants as well as other scramblases will be carried out in the CRISPR-Cas9 generated TMEM16F-KO HEK293T cell-line.
Figure 5: Generation of a TMEM16F deficient HEK293T cell-line to eliminate interference of the endogenous CaPLSase. A, Schematic demonstration of the microscopy-based, live-cell scrambling assay. B, WT HEK293T cells exhibited strong endogenous CaPLSase activity as examined using the assay shown in (A). The CF 594-tagged-AnV (AnV-CF 594) signal representing scrambling activity was recorded by time-lapse imaging for 10 minutes following application of 5 µM ionomycin (0 min). C, Western Blot shows endogenous TMEM16F expression in WT, Cas9 control (Cas9) and TMEM16F-KO HEK293T cells (left panel). Total protein loading was visualized via Ponceau-S staining (right panel). D, The amount of TMEM16F expression from the Western Blot was normalized to the total protein loading from Ponceau-S staining. E, The endogenous CaPLSase activity in HEK293T cells are eliminated in the TMEM16F-KO HEK293T cell line, while the Cas9-HEK293T cells retain robust endogenous CaPLSase activity. The scrambling assay was carried out as described in (A).

2.2.2.2 Optimization of the microscopy-based scrambling assay:

To optimize the preexisting microscopy-based scrambling assay (58), firstly, we chose to use ionomycin instead of A23187 as Ca\(^{2+}\) ionophore in our assay. Ionomycin is more potent in delivering Ca\(^{2+}\) into the cells than A23187 (114, 115), so ionomycin will be
more effective in activating TMEM16F, which has low \( \text{Ca}^{2+} \) sensitivity (28, 58, 116, 117), than A23187.

Secondly, we limited the duration of the assay to avoid contamination of the apoptosis-induced PS exposure. PS exposure is one of the hallmarks of apoptotic cells (118). However, instead of TMEM16F, XK-related (Xkr) proteins – caspase dependent phospholipid scramblases – have been shown to be responsible for apoptosis induced PS exposure (19, 105). Although 5 µM ionomycin potently delivers \( \text{Ca}^{2+} \) into the cells to activate TMEM16F (Figure 5B, 5E, 8A), excessive and prolonged increase of intracellular \( \text{Ca}^{2+} \) can compromise cellular homeostasis and lead to apoptosis (119).

Xkr-dependent PS exposure takes longer time, due to the slow nature of apoptosis, than the rapid TMEM16F-dependent PS exposure (19, 27), so we examined, how long it would take for the cells to start showing signs of apoptosis under the condition of our lipid scrambling assay. At 20-30-minute post ionomycin treatment, both TMEM16F expressing and non-expressing HEK293T cells exhibited ongoing apoptosis events as indicated by strong fluorescent signal of both TF3-DEVD-FMK, a fluorescence indicator of active caspase 3/7, and AnV binding on the cell surface (Figure 6). Nonetheless, at 10-minute after ionomycin stimulation, TMEM16F-overexpressing cells showed significant AnV binding but negligible TF3-DEVD-FMK signal, suggesting apoptosis is nominal at this time point (Figure 6). At 10-minute after ionomycin application, no AnV and TF3-
DEVD-FMK signal was detected in TMEM16F-KO HEK293T, further supporting that the cells experienced minimum apoptotic events at this time point, and the PS-exposure on TMEM16F-expressing cells was mainly ensued from TMEM16F (Figure 6). To further verify that the PS exposure that we observed was mediated by TMEM16F instead of the caspase-dependent lipid scramblases, we conducted the lipid scrambling assay with pan caspase inhibitor (Q-VD-QPh) treated cells. Despite the presence of the caspase inhibitor, TMEM16F expressing cells in these conditions transported PS at comparable rates (Figure 7). Hence, we concluded that the activity of the caspase-dependent lipid scramblases was minimized in our assay. The microscopy-based scrambling assay as well as its associated analyses are performed within 10-minute window of ionomycin application to avoid potential complications associated with Ca$^{2+}$ overload, such as apoptosis and apoptosis-induced phospholipid scramblases (19, 119).
Figure 6: AnV and apoptotic stainings for ionomycin-induced scrambling cells. Representative images of AnV (red) and caspase 3/7 (TF3-DEVD-FMK; green) stainings of TMEM16F-eGFP positive (purple) or negative TMEM16F-KO HEK293T cells after 10-min, 20-min, and 30-min of ionomycin treatment. The images indicate that TMEM16F CaPLSase, but not the caspase-activated phospholipid scramblases is responsible for PS externalization within a ten-minute window after introducing ionomycin.
Figure 7: Pan-caspase inhibitor does not affect TMEM16F CaPLSase activity. No difference in scrambling activity (quantified by $t_{1/2(max)}$) between HEK293T cells stably or transiently expressed TMEM16F with and without Q-VD-OPh, a pan-caspase inhibitor. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test. p-values are 0.98 for Transient vs. Stable and 0.7735 for Transient vs. Q-VD-Oph. n.s denotes not significant. Error bars indicate SEM.

2.2.2.3 Data analysis of the microscopy-based lipid scrambling assay

After optimizing the Ca$^{2+}$ ionophore, cell system and duration of the microscopy-based imaging assay, we next tackled the challenges of analyzing data from the microscopy-based scrambling assay. One of the biggest problems in analyzing data of the microscopy-based scrambling assay is that not all transiently transfected cells have equal TMEM16F expression and membrane trafficking level. This causes a large variation in AnV fluorescent signals among the cells. Hence, it is difficult to plot and interpret AnV-signal-overtime of all the cells on the same graph. Moreover, changes of focal plane or cell morphology after Ca$^{2+}$ ionophore application could also interfere the recording of the AnV signal. Therefore, analyzing and translating the fluorescent AnV signal accumulating on
the cell membrane into a meaningful information regarding CaPLSase kinetics are complicated tasks. To circumvent these obstacles, I collaborated with Dr. Yang Zhang – a postdoctoral fellow in the Yang Lab – to develop a MATLAB code to quantify the fluorescence signal of AnV accumulating on the surface of the scrambling cells. The CaPLSase activity is quantified based on the AnV signal of each cell (within the field of view of the images) in terms of $t_{1/2} (I_{\text{max}})$, which is defined as the time point when each scrambling cell reaches 50% of its maximum AnV fluorescent intensity within ten-minute of ionomycin stimulation.

Next, we applied previously characterized TMEM16F mutations to validate our scrambling assay, TMEM16F-KO HEK293T cell line and data analysis. Using our optimized scrambling assay and a new way of data analysis ($t_{1/2} (I_{\text{max}})$), we confirmed that D409G, a previously identified mTMEM16F gain-of-function mutation (27), displayed an accelerated phospholipid scrambling as seen by its significantly shorter $t_{1/2} (I_{\text{max}})$ in comparison to that of WT mTMEM16F (Figure 8B, 8C). On the other hand, the Ca$^{2+}$-binding site mutation D703R, whose equivalent mutation in TMEM16A completely eliminated Ca$^{2+}$-dependent activation of the CaCC, abolished phospholipid scrambling activity ($t_{1/2} (I_{\text{max}})$ value assigned as infinity and denoted as non-scrambling) (Figure 8A-C) (120). Taken together, our optimized CaPLSase imaging assay, TMEM16F-KO HEK293T
cells and $t_{1/2}(I_{\text{max}})$ quantification approach provide us a faithful method to characterize TMEM16F, its mutations as well as other TMEM16 CaPLSases.

Figure 8: Quantification of CaPLSase activity using an AnV-based imaging assay. A, Representative images of ionomycin-induced scrambling activity of eGFP-tagged mTMEM16F-WT, D409G, or D703R expressed in TMEM16F-KO HEK293T cells. AnV-CF594 signal representing phospholipid scrambling was recorded for 10-minute post 5 µM ionomycin application. B, Representative AnV fluorescence intensity change-over-time of cells in (A), and demonstration of how $t_{1/2}(I_{\text{max}})$ parameter is extracted from the AnV fluorescent intensity. a.u, arbitrary unit. C, Ionomycin-induced CaPLSase activities of
mTMEM16F-WT, D409G, and D703R as quantified by t1/2(Imax). Each data point represents one cell and n is the total number of cells analyzed. Cells that do not exhibit ionomycin-induced CaPLSase activity were denoted as non-scr (non-scrambling) and are excluded from statistical analysis. The pie charts illustrate the percentage of scrambling cells in response to ionomycin stimulation. Statistical analysis was performed using unpaired two-sided t-test for WT and D409G. ****: p < 0.0001. Error bars indicate SEM.

2.3 Assays to simultaneously detect TMEM16F ion channel and lipid scramblase activities

2.3.1 Electrophysiological methods

Many of the characterized TMEM16 members possess ion channel activities despite whether they are scramblases or not (15, 20-26, 28, 33-48). Some studies used Cl\textsuperscript{-} flux assay (37, 38, 40, 42, 121, 122), halide sensors (123-127), or computational simulation (36, 40, 42) to gain more understanding of TMEM16 ion channels. However, patch clamping – a gold-standard method in studying ion channels – is still the most powerful approach to characterize biophysical properties of TMEM16 ion channels (128, 129). Although it is superior in sensitivity and high configurability in monitoring ion flux through ion channels (128), patch clamping does not have the resolution to detect phospholipid scrambling due to lower permeation speed and less charge of phospholipids compared with that of ions. Therefore, we aim to design a method that combines electrophysiology and microscopy-based scrambling assay to simultaneously monitor both ion and lipid permeations activity of the moonlighting TMEM16 members.
2.3.2 Dual recording assay to monitor both ion and lipid permeations

The Hartzell Lab was the first to combine whole-cell patch clamp and microscopy-based scrambling assay to simultaneously monitor the ion channel and lipid scrambling activity of the dual function TMEM16 proteins (36, 58). Nonetheless, the dual recording of Hartzell Lab suffers some technical issues, making their conclusions hard to be interpreted: Firstly, their studies relied on HEK293T cells, which have high endogenous level of TMEM16F expression and CaPLSase activity as discussed in the earlier subsections (36, 58). Secondly, to activate the TMEM16 CaPLSases, the Hartzell Lab infiltrated the cytosol with pipette solution containing 200 µM Ca$^{2+}$ for a long period of time under whole-cell patch clamp configuration (36, 58). This prolonged infusion of extremely high intracellular Ca$^{2+}$ could trigger apoptosis and increase the risk of apoptosis-dependent PS exposure, which can contaminate the actual TMEM16-dependent PS exposure (19, 105, 119). Moreover, nhTMEM16 and TMEM16F ion channel currents recorded by the Hartzell Lab exhibited a long, approximately 5-minute delay (36, 58). This is in stark contrast to the inside-out patch clamp recordings of TMEM16F ion channel, which always give rise to robust and immediate channel activation upon Ca$^{2+}$ supply (28, 52, 60, 61, 130). Although the nature of the long delay has not been fully understood, it is possible that the delay is an artifact derived from abnormal cellular behavior in an extreme Ca$^{2+}$ condition. Hence, this artificially long delay in channel activation makes it hard to
accurately dissect the ion channel and CaPLSase activities of the moonlighting TMEM16s, such as TMEM16F and nhTMEM16.

To improve the current dual recording method, we designed cell-attached patch clamp fluorometry (cell-attached PCF) to simultaneously detect the current and lipid scrambling activity of TMEM16F (Figure 10). In this assay, TMEM16F expressing cells are activated by the mild ionomycin treatment (5 µM) that was optimized in the previous subsection. TMEM16F ion channel is monitored using cell-attached configuration of patch clamp, which is described as follows: glass pipette electrode contacts and forms tight seal with, but does not rupture the cell membrane. The pipette solution contains the fluorescently tagged AnV, which is used to detect the scrambling activity of the patched membrane concurrently with the ion channel recording (Figure 10). By applying this cell-attached PCF and linearly increasing voltage (-80mV to +80mV), we noticed a rapid activation of the CaPLSase activity immediately after ionomycin treatment as evidenced by instant AnV signal accumulating on the recorded patch of membrane (Figure 9). On the other hand, activation of TMEM16F ion channel was not observed until two minutes later (Figure 9). This observation is consistent with the results from the recent TMEM16F structural study, which demonstrates that TMEM16F CaPLSase (EC$_{50}$ = 1 µM) has lower Ca$^{2+}$ EC$_{50}$ than its ion channel counterpart (EC$_{50}$ = 4-7 µM) (52). In our cell-attached PCF assay, ionomycin-induced increase of intracellular Ca$^{2+}$ is time dependent. Therefore, the
small increase of Ca\(^{2+}\) at the beginning of ionomycin treatment is only enough to activate the CaPLSase, but overtime (two minutes in my case), Ca\(^{2+}\) will accumulate and reach appropriate level to trigger the ion channel activation (Figure 9). Hence, PS exposure occurs prior to the current activation might be because TMEM16F CaPLSase has higher Ca\(^{2+}\) sensitivity than its ion channel. This difference in Ca\(^{2+}\) sensitivity between the two functions of TMEM16F suggests that the lipid scrambling and ion permeation can happen in different conditions and are not obligatory to each other.

Figure 9: TMEM16F CaPLSase and ion channel activities were recorded by cell-attached PCF method. A, Representative images of AnV-CF594 (white) binding to the patched membrane within 5 minutes of ionomycin application in the cell-attached PCF. B, Quantification of fluorescent signal of AnV-CF594 (black trace) and ion current (red trace) of the same piece of membrane in the cell-attached PCF. Fluorescent signal and ion current were normalized to their maximum values. Ionomycin was applied at time = 0min.
Although cell-attached PCF can segregate TMEM16F-ion channel from -CaPLSase and minimize the delay of TMEM16F channel’s activation, the method still suffers a number of limitations that hinder further characterization of TMEM16F. Firstly, small area of recording limits AnV fluorescence signals and TMEM16F current amplitude. Secondly, there is always a loss of focus due to an unexplainable upward movement of the patched membrane, which makes it hard to faithfully measure the AnV signal. Thirdly, it is also impossible to exchange different solutions or apply TMEM16F ion channel’s blockers to the cytosolic side of the tested membrane to further dissect the protein’s dual functions under the cell-attached configuration.

Therefore, other patch clamp configurations should be utilized to further develop and improve the PCF assay. The first potential alternative approach is the inside-out PCF. In the inside-out configuration, the piece of membrane is excised out of the cell after a tight seal is formed between the membrane and the glass pipette electrode. This exposes the intracellular membrane leaflet to the bath solution, thus allowing the users to directly perfuse the solutions of choice to the intracellular leaflet of the excised membrane (Figure 10). This is advantageous in controlling the amount of Ca\(^{2+}\) used to activate TMEM16F as well as other TMEM16 members. Moreover, different drugs or molecules can be applied, exchanged and washed away to test their efficiency in inhibiting, activating and dissecting TMEM16F-ion channel and -CaPLSase activities. However, the potential pitfall of this
approach is its high fluorescence background at the patching pipette tip. Since AnV binds to PS in a Ca\(^{2+}\) dependent manner (104), Ca\(^{2+}\) must be present in the patching pipette solution along with AnV at all time. When membrane is excised from the cells, small amount of Ca\(^{2+}\) could leak to the cytosolic side of the patched membrane and activate some small number of TMEM16F, giving rise to the fluorescence background at the glass pipette tip. This issue could be prevented by removing Ca\(^{2+}\) in the pipette solution and replacing the fluorescently tagged AnV with fluorescently tagged Lactadherin-C2 domain, which binds to PS in a Ca\(^{2+}\) independent manner (131). Alternatively, perforated patch PCF can serve as another approach (Figure 10). Perforated patch is formed by applying a monovalent cation selective ionophore, which is Gramicidin in this case (132), into the glass pipette tip. Once a tight seal is formed between the glass pipette electrode and the cell membrane, Gramicidin-generated pores will allow free ion flow for monovalent cations across the membrane (132). Ionomycin is used to facilitate cellular Ca\(^{2+}\) influx, and fluorescently tagged AnV in the bath solution is used to monitor PS exposure from the entire cell. Therefore, the perforated patch PCF has the advantage of the whole-cell PCF to record large TMEM16F ion current and PS exposure from the entire cell without the drawback of disrupting the intracellular contents in the whole-cell PCF. Future work is needed to further optimize these different configurations of the PCF.
Figure 10: Schematic demonstration of different configurations of PCF. Either cell-attached, inside-out or perforated patch clamp configuration can be combined with fluorescence microscope-based scrambling assay to simultaneously monitor both lipid scrambling and ion channel activities of moonlighting TMEM16s such as TMEM16F.

2.4 Experimental procedures

2.4.1 Cell lines and cell culture

All cells were cultured in sterile environment with controlled temperature (37°C) and stable supply of CO₂ (5%).

2.4.1.1 WT HEK293T and mTMEM16F stable HEK293 cell lines

WT HEK293T cells were purchased from Duke Cell Culture Facility and authenticated by Duke University DNA Analysis Facility. HEK293 cells with stable expression of C-terminally eGFP-tagged murine TMEM16F (mTMEM16F stable HEK293) was a generous gift from Dr. Min Li. Both cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, #11995-065) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S) (henceforth referred to as standard DMEM/medium). Homogenous population of mTMEM16F stable HEK293 cells
was maintained and selected by occasional addition of 100 µg ml\(^{-1}\) hygromycin to the standard medium.

2.4.1.2 Generation and maintenance of TMEM16F-KO HEK293T cell line

WT HEK293T cells were transduced with lentiCas9-blast (Addgene #52962) to generate stable Cas9-expressing cells (Cas9-HEK293T). CHOPCHOP ([http://chopchop.cbu.uib.no/index.php](http://chopchop.cbu.uib.no/index.php)) was used to design sgRNA (5’-AATAGTACTCACAACACTCG-3’), which targets exon 2 of ANO6/TMEM16F. The sequence was then cloned into lentiguide-puro (Addgene #52962) to generate TMEM16F-KO HEK293T cells. The lentivectors, psPAX2 and pMD2.g, were co-transfected into HEK293T using TransIT-LT1 (Mirus) to prepare lentiviruses. All transductions were done at a multiplicity of infection (MOI) of <1 in the presence of 4 µg ml\(^{-1}\) polybrene. After twenty-four hours of infection, 10 µg ml\(^{-1}\) blasticidin or 2 µg ml\(^{-1}\) puromycin were applied to select cells for 48-72 hours. The selected cells were then expanded. One week after transduction with sgRNA, genomic DNA was harvested from cells. The presence of deletions was confirmed by PCR amplifying ANO6 exon 2 locus from genomic DNA and subsequently analysis using the Surveyor assay (Integrated DNA Technologies). The forward primer 5’-TTTTCAGTGGTAGACCTTGCCT-3’ and reversed primer 5’-AAGTTTCAGCAACCTATTCCCAA-3’ were used in the PCR.
The initial TMEM16F-KO HEK293T population was serial-diluted into 96-well plates to select for single-cell colonies. After 10-14 days, the single-cell colonies were expanded and screened for the absence of TMEM16F expression and CaPLSase function using Western Blots and phospholipid scrambling assay. Only the TMEM16F-KO HEK293T colonies with complete absence of TMEM16F expression and CaPLSase were used to characterize heterologously expressed TMEM16 scramblases and their mutants in this dissertation. Both Cas9 and TMEM16F-KO HEK293T cell lines were maintained in standard DMEM.

2.4.2 Plasmids used and transient transfection protocol

2.4.2.1 Plasmids

pEGFP-N1 vector carries cDNA of either WT mTMEM16F (Open Biosystems cDNA # 6409332) (28) or their point mutations with C-terminal eGFP tag.

Point mutations of mTMEM16F were created by using QuickChange site-directed mutagenesis kit (Agilent). Primers used to generate point mutations of mTMEM16F are listed in Table 2 (Appendix A). Correct mutations were validated by Genewiz’s Sanger sequencing service. All primers were purchased from IDT DNA Technologies.

2.4.2.2 Transient transfection protocol

Transient transfection was conducted by following manufacturer’s instruction of X-tremeGENE™ 9 DNA Transfection Reagent (Millipore-Sigma). Generally, 24-hour prior
to transfection, appropriate cells (either WT or TMEM16F-KO HEK293T cells) were seeded to poly-L-lysine (PLL; Sigma) coated coverslips in 24-well plate. Appropriate plasmid was mixed with transfection reagent in 1:3 or 1:2 (DNA:reagent) ratio. Each plasmid was added at about 200-250 ng DNA per well of the 24-well plate. Five-hour post transfection, medium of each well was aspirated and changed to standard DMEM. Experiments were often carried out after 24-48 hours of transfection.

2.4.3 Fluorescence microscope-based assays

Appropriate cells were seeded on PLL coated No.0 coverslips. If transient transfection was required, cells were transfected with appropriate plasmids according to transient transfection procedure noted in subsection 2.4.2.2. Extracellular solution in all cell-based imaging assays contains 0.5 µg ml⁻¹ fluorescently tagged AnV, 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4, unless stated otherwise. This extracellular solution is referred to as imaging solution. All experiments were independently repeated at least three times. Stock solution of ionomycin (5mM) was prepared in dimethyl sulfoxide (DMSO). Small aliquots of 5mM ionomycin were stored in -80°C until used to avoid repeated freeze-thaw cycles.

2.4.3.1 Phospholipid scrambling assay

In general, CF 594-tagged AnnexinV (AnV-CF 594, Biotium # 29011) was diluted to 0.5 µg mL⁻¹ by using imaging solution. The diluted AnV-CF 594 and coverslips with the
transfected cells were added subsequently into a homemade imaging chamber. The focal plane was adjusted based on the eGFP signal from the expressing cells. To stimulate CaPLSase activities of TMEM16F, Subdued and their mutations, an equal volume of AnV-CF 594 with 10 µM ionomycin was added into the chamber to reach the final concentration of 5 µM ionomycin unless noted otherwise.

For Q-VD-OPh treatment, after TMEM16F-KO HEK293T cells are transfected with mTMEM16F for 5-hour, culture medium was replaced with the fresh medium containing 10 µM Q-VD-OPh. After 24-hour of transfection and incubation with Q-VD-OPh, the cells were tested for their scrambling activity as described above in the presence of 10 µM Q-VD-OPh.

The increase of the fluorescently tagged AnV on the cell membrane indicates PS being exposed to the exterior of the membrane, hence representing the CaPLSase activity. Fluorescence signal of AnV was captured by time-lapse imaging at 5-s intervals by a Prime 95B Scientific CMOS Camera (Photometrics) connected to an Olympus IX71 inverted epi-fluorescent microscope (Olympus IX73). The total length of the scrambling assay is limited to 10 minutes upon ionomycin application (0min). A 60X oil objectives (NA of 1.35) was used for imaging. Metafluo software (Molecular Devices) was used to monitor and control the image acquisition. Images and data analysis were executed by using ImageJ, MATLAB, Prism (GraphPad) and Excel (Microsoft).
2.4.3.2 Quantifying phospholipid scrambling activity

Only CaPLSase-expressing cells without AnV signal prior to ionomycin treatment were analyzed using $t_{1/2(I_{\text{max}})}$. The term $t_{1/2(I_{\text{max}})}$ indicates the amount of time each CaPLSase-expressing cell takes to reach half of maximum AnV fluorescent intensity ($I_{\text{max}}$) by the course of 10-minute of ionomycin treatment. To determine $t_{1/2(I_{\text{max}})}$, Dr. Yang Zhang wrote a customized MATLAB program (Mathworks) to automate the quantification of AnV fluorescent intensity change overtime of each scrambling cell. In the MATLAB program, a region of interest (ROI) was defined around the scrambling cells, and the AnV fluorescent intensity within the chosen ROI was calculated using the following equation for each frame of the time-lapse imaging:

$$I = \sum_{n=1}^{n=N} i \quad (\text{eq. 1})$$

where $i$ equals the intensity of each pixel and $N$ is the number of the pixels in the ROI. The time reaching half of $I_{\text{max}}$ was defined as $t_{1/2(I_{\text{max}})}$. Each data point in the bar graphs of $t_{1/2(I_{\text{max}})}$ represents value of a single cell. CaPLSase expressing cells with no scrambling activity (as indicated by absence of AnV signal after ionomycin treatment) were omitted from $t_{1/2(I_{\text{max}})}$ analysis and assigned an arbitrary value indicating non-scrambling. Results were analyzed by using ImageJ, MATLAB, Prism (GraphPad) and Excel (Microsoft). The MATLAB code for quantifying the CaPLSase activity is available at Github (yanghuanghe/scrambling_activity).
2.4.3.3 Live cell TF3-DEVD-FMK staining assay

To detect cleaved caspase 3/7 level in live cells, live cell caspase 3/7 binding assay kit (AAT Bioquest, #20101) was used. The 150X stock solution of TF3-DEVD-FMK was prepared and stored as instructed by the kit’s manual (AAT Bioquest). TMEM16F-KO HEK293T were seeded and transfected with WT mTMEM16F plasmid on PLL coated No.0 coverslips. After 24-hour of transfection, the cells were washed with fresh media, and then incubated with 1X TF3-DEVD-FMK (diluted in appropriate culture media) for 1-hour in incubator supplied with 37°C, 5% CO2. TMEM16F positive and negative cells were then treated with 5µM ionomycin in the presence of 1X TF3-DEVD-FMK for 10-min, 20-min and 30-min. After that, the cells were washed twice using the kit’s washing buffer, and then imaged with 0.5 µg mL⁻¹ CF 640R-tagged Annexin-V (AnV-CF 640R; Biotium #29014). The results were collected with a 63X/1.4 NA Oil Plan-Apochromat DIC in Zeiss 780 inverted Confocal microscope, and analyzed with ImageJ and Zeiss.

2.4.4 Western Blot

Cells were grown to 80-90% confluence in 96-mm culture disks. After that, they were trypsinized and pelleted by centifugating at 900rpm, 4°C for 5-minute. After washing twice with cold PBS, cell pellets were incubated with RIPA lysate buffer supplemented with 5mM EDTA and 1X Protease inhibitor cocktail on ice for 30 mins. The lysate was centrifuged at 11000rpm for 20-min at 4°C. Supernatant was collected and
supplemented with 1X Laemml with 112mM DTT at room temperature for 30mins. SDS-PAGE gel was then used to separate the proteins, which were then transferred to PVDF membrane by using Trans-Blot Turbo Transfer system (Bio-Rad). Ponceau-S was used to staining the blot for 15 minutes, following with 5% acetic acids washes (three times). The Ponceau-S staining was imaged by a ChemiDoc XRS+ System (Bio-Rad). The membrane was blocked with PBS supplemented with 5% non-fat milk, 0.1% Tween-20 for 1-hour at room temperature. TMEM16F antibody (Millipore-Sigma, # HPA038958) was diluted to 1:1000 using the blocking buffer. The membrane was incubated with diluted TMEM16F antibody overnight at 4°C. After washing with PBST (PBS supplemented with 0.1% Tween-20) for three times, the membrane was incubated with Anti-Rabbit IgG (whole molecule)–Peroxidase secondary antibody (1:1000 dilution in blocking buffer; Sigma) for 1-hour at room temperature. Chemiluminescence signals from protein bands were detected by the ChemiDoc XRS+ System (Bio-Rad), and band intensity was analysed by the Bio-Rad Imaging software, Prism (GraphPad) and Excel (Microsoft). The TMEM16F bands intensity from the chemiluminescence were normalized to the total protein loading detected by Ponceau-S.

2.4.5 Cell-attached patch-clamp fluorometry

Electrode glass pipettes were prepared using borosilicate capillaries (1.5mm x 0.86mm) and P1000 puller (Sutter Instruments). The electrodes were fire-polished using a
 microforge (Narishge) and had initial resistance of ~2-5 MΩ. All electrophysiology recordings were low-pass filtered at 5kHz (Axopatch 200B), sampled at 10kHz (Axon Digidata 1550A) and digitized by Clampex 10 (Molecular Devices). Offline data analysis was done with Clampfit, Excel (Microsoft), and Prism (GraphPad).

mTMEM16F stable HEK293 cells were seeded on glass-bottom (No.1 glass) 35 mm tissue-culture plate. Prior to the experiment, culture medium was changed to extracellular solution containing 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4. The glass pipette electrode was filled with 0.5 µg ml⁻¹ AnV-CF 594, 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4. After forming tight (gigaohm) seal between the cell membrane and the glass pipette electrode, 100x oil objectives with NA of 1.3 was used to focus to the patched membrane based on the TMEM16F-eGFP signal. After that, the basal signal of AnV-CF 594 of the patched membrane was captured. Then, a repeated ramp protocol from -80mV to +80mV (with 5-sec intervals) was applied to the membrane, and ionomycin (prepared using extracellular solution) was added into the culture plate at the final concentration of 5 µM to stimulate TMEM16F. Immediately upon ionomycin application (0min), AnV signal was recorded by time-lapse imaging (at 5-s intervals) using a Prime 95B Scientific CMOS Camera (Photometrics) connected to an Olympus IX71 inverted epi-fluorescent microscope (Olympus IX73). Image acquisition was controlled by Metafluo software (Molecular Devices). TMEM16F ion channel was also recorded using Clampex 10
(Molecular Devices). Results were analyzed by using Clampfit, ImageJ, MATLAB, Excel (Microsoft), and Prism (GraphPad).

2.4.6 Statistical analysis

All statistical analyses were performed in Prism software (GraphPad). Student’s t test was used for single comparisons between two groups, and one-way ANOVA (Tukey’s multiple comparisons test) was used for multiple comparisons. Comparisons yielding p-values <0.05 are considered to be statistically significant. Data in summary graphs are represented as mean +/- SEM. Each data point represents a single experiment, except $t_{1/2(Imax)}$ summary graphs, in which each point represents a single cell. *, **, *** and **** denote statistical significance corresponding to p-value < 0.05, < 0.01, < 0.001, < 0.0001, respectively.
Chapter 3: Identification of an activation gate controlling TMEM16F CaPLSase

Portions of this chapter are reused and adapted from a research originally published in Nature Communication.(1)

Some data in this chapter were done in collaboration with members of the Yang Lab(2) and the Chen Lab(3).

3.1 Background

The CaPLSases from the TMEM16 family have been found to be major phospholipid transporters that shuffle lipids across membrane bilayer in response to intracellular Ca\textsuperscript{2+} elevation (1, 15, 27, 35, 38, 39, 44, 89). Beside TMEM16F’s extensive roles in physiological processes discussed in Chapter 1, other mammalian TMEM16 scramblases also play significant roles in health and disease (Figure 2). For example, mutations of TMEM16E have been implicated in a number of inherited diseases including gnathodiaphyseal dysplasia (133), limb-girdle muscular dystrophy (39, 134-136), and

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2 Son C. Le performed the electrophysiology experiments and generated mTMEM16A point mutations: L543K, S588K and I637K.
3 Dr. Jianhan Chen and Dr. Zhiguang Jia from the Chen Lab (University of Massachusetts, Amherst) performed and collected data for the computational experiments.
Miyoshi myopathy (134-136). Mutations of hTMEM16K have been linked to ataxia (34, 137, 138), or TMEM16G has been found to be associated to and could serve as a biomarker for prostate cancer (35, 139). Considering their importance in health and disease, a comprehensive understanding of the structures and functions of the mammalian TMEM16 CaPLSases would facilitate drug discovery for these therapeutic targets.

There have been substantial efforts to uncover the structures of the TMEM16 family. The first look at TMEM16 structures was revealed by an X-ray structure of the fungal nhTMEM16 captured in an activated Ca\(^{2+}\)-bound state (44). This structure displays a homo-dimeric assembly with each monomer containing ten TM helices (Figure 13A) (44). Recent structural studies on mTMEM16A-CaCC (91, 140, 141), mTMEM16F-CaPLSase (52, 63) and hTMEM16K-CaPLSase (34) indicate that the mammalian TMEM16 proteins also adopt a double-barreled homo-dimeric architecture. Moreover, all of these structures exhibit a highly conserved Ca\(^{2+}\)-binding site in TMs 6-8 (34, 44, 52, 63, 91, 120, 140-142). Ca\(^{2+}\)-binding triggers ion or phospholipid permeation through a hydrophilic pathway/groove comprising of TMs 3-8 (34, 41, 44, 45, 52, 63, 91, 140, 141). Interestingly, the hydrophilic grooves in the Ca\(^{2+}\)-bound structures of nhTMEM16 (41, 44), afTMEM16 (45) and hTMEM16K (34) are exposed to the lipid environment owning to the physical separation of TM4 and TM6, both of which are located at the periphery of the proteins. This intricate architecture thus suggests that phospholipid headgroups can enter and
move along the permeation pathway while maintaining their hydrophobic acyl tails in the hydrocarbon core of the membrane (34, 36, 40, 41, 43-45), consistent with the widely accepted credit-card reader model for phospholipid permeation (Figure 4) (93). Nevertheless, the credit-card reader model does not implicate a gating control mechanism, which is required to regulate passive phospholipid permeation through TMEM16 CaPLSases in response to Ca$^{2+}$ binding.

Recent computational and functional studies guided by the Ca$^{2+}$-bound nhTMEM16 structure have proposed three critical sites for controlling lipid scrambling (36, 40, 43): an extracellular $S_E$ site formed by charged residues from TM3 and TM6, a central constriction site in the middle of the hydrophilic groove and a cytosolic $S_C$ site composed of charged residues in TM4 (Figure 13A). Although the exact roles of the $S_C$ site and the central constriction site in nhTMEM16 gating are unclear, molecular dynamic (MD) simulations and mutagenesis studies have suggested that the network of charged amino acids within the $S_E$ site (Figure 13A) can serve as a putative extracellular gate to control lipid permeation (40, 43). In this model, Ca$^{2+}$ binding to nhTMEM16 triggers rearrangements of these charged amino acids at the $S_E$ gate allowing the hydrophilic pathway to accommodate and permeate lipids. The nhTMEM16 structure has provided an invaluable structural template for computational and functional studies to unravel the molecular mechanism of lipid scrambling and regulation of the fungal CaPLSases (36, 40,
43, 44). However, there was little structural information about the mammalian TMEM16 scramblases. This limitation has precluded a comprehensive mechanistic understanding of lipid scrambling, and casted questions on how mammalian TMEM16 CaPLSases gate their lipid permeation pathways in a tightly controlled fashion.

In this Chapter, combining various approaches ranging from structure-guided mutagenesis, quantitative measurements of CaPLSase activity to atomistic MD simulations, we identify three hydrophobic residues (F518, Y563, and I612), which are located in the middle of the TMEM16F lipid permeation pathway, form an inner activation gate to control lipid translocation. Amino acid substitutions at these inner gate residues profoundly affect TMEM16F scrambling activity. Remarkably, we find that F518K and Y563K mutations lead to constitutively active CaPLSases independent of Ca\(^{2+}\) activation. Moreover, TMEM16A-L543K, a corresponding mutation of TMEM16F-F518K, confers the CaCC the capability to constitutively permeate lipids. Our identification of an inner activation gate in controlling TMEM16F-CaPLSase lipid permeation thus provides an important framework for the mechanistic understanding of the gating mechanism of mammalian TMEM16 CaPLSases.
3.2 Results

3.2.1 Homology models of TMEM16F predict an inner activation gate

At the time of this study was initiated, structural information regarding the TMEM16 family was very limited. Therefore, we employed atomistic MD simulation to build homology models for TMEM16F based on structures of TMEM16A and nhTMEM16 – the only two TMEM16 proteins that have available structures at that time. In order to study TMEM16F-CaPLSase gating, a closed state structural model is necessary. Given the high sequence conservation between mammalian TMEM16F and TMEM16A, and low sequence conservation between mammalian TMEM16F and fungal nhTMEM16 (Figure 11), we hypothesized that the Ca$^{2+}$-free structure of a mTMEM16A (PDB: 5OYG) (91) would be a more suitable template for modeling the closed state of TMEM16F (Figure 12). The Ca$^{2+}$-free TMEM16A-derived homology model of TMEM16F clearly stays in a closed conformation with its hydrophilic groove constricted, which results in high free energy barriers for both water and phosphate permeation during atomistic simulations (Figure 13C).
Figure 11: Sequence alignment of mTMEM16A, mTMEM16F and nhTMEM16. TM domains and the inner gate residues are highlighted in cyan and red, respectively.
Figure 12: Comparison between the permeation pathways of mTMEM16A and nhTMEM16. A, Superposition of Ca\(^{2+}\)-free (PDB: 5OYG; green) and Ca\(^{2+}\)-bound (PDB: 5OYB; magenta) mTMEM16A structures (91) shows the movement of TM6 around the glycine hinge upon Ca\(^{2+}\) binding. B, Superposition of Ca\(^{2+}\)-bound nhTMEM16 (PDB: 4WIS (44); yellow) and Ca\(^{2+}\)-bound mTMEM16A structures (PDB: 5OYB; magenta). Only TMs 4-6 of the hydrophilic groove are highlighted using green, magenta or yellow colors, whereas other supporting TMs are colored as light blue. Ca\(^{2+}\) are illustrated as red spheres.

To build an open state TMEM16F homology model, we first attempted to build a structural model (Figure 13B) based on the Ca\(^{2+}\)-bound structure of TMEM16A (PDB: 5OYB) (91), which shows that Ca\(^{2+}\) binding straightens TM6 around the glycine hinge to trigger TMEM16A activation (Figure 12A). Due to this Ca\(^{2+}\)-induced conformational change, the pore in the Ca\(^{2+}\)-bound TMEM16F model becomes more accessible to water permeation in comparison to the Ca\(^{2+}\)-free closed state (Figure 13C, 13D). However, there remains a major constriction for phosphates near the center of the groove (Figure 13C, 13E), suggesting that the 5OYB-derived TMEM16F model may instead represent an intermediate, partially open or inactivated state. Hence, we assign this 5OYB-derived
TMEM16F model to an intermediate state to distinguish it from the Ca\(^{2+}\)-free closed state and the Ca\(^{2+}\)-bound fully open state.

Figure 13: F518, Y563, and I612 form a putative inner activation gate of TMEM16F-CaPLSase. A, X-ray structure of nhTMEM16 (PDB: 4WIS (44)). The two monomers are colored in grey and brown, respectively. The TMs 4-6 lining the interior of the hydrophilic groove are highlighted in green. The bound Ca\(^{2+}\) ions are represented as red spheres. Previously proposed S_E and S_C sites (43) are marked with magenta circles. B, Superposition of TMs 4-6 in the predicted intermediate and open state models of TMEM16F. Side chains of the putative inner activation gate residues are shown in blue sticks and numbered as 1, 2, and 3, respectively. C, Effective free energy profiles of water (left) and phosphates (right) along the hydrophilic groove. The profiles are derived from the average densities from the last 100 ns of the 400 ns atomistic simulations of TMEM16F in open (black), intermediate (magenta), and closed (green) states. The y-axis is the relative distance from E604, a residue at the outer leaflet surface. The standard errors were estimated from the calculated averages over three independent simulations. D, Snapshots from the atomistic simulations of the open or intermediate TMEM16F homology models. The protein is represented as silver surfaces and all polar residues in TMs 4-6 are colored in yellow.
Waters within 15 Å of the protein are represented as stick-and-balls. E, Phosphate densities near TMs 4-6 (green cartoons) derived from atomistic simulations. The phosphate accessible region (probability > 0.005) is represented as orange grid. Three putative gate residues (F518, Y563, I612) are represented as blue sticks.

To obtain a fully open TMEM16F structural model, we sought to utilize the Ca\(^{2+}\)-bound nhTMEM16 structure (PDB: 4WIS) (44), whose hydrophilic groove is widely opened (Figure 12B-yellow). However, owing to the low sequence homology between nhTMEM16 and TMEM16F, particularly outside of the TM region (Figure 11), we started with the 5OYB-derived TMEM16F intermediate model and used steered MD to gradually move its TMs 3, 4 and 6 (Figure 13B-magenta), whose conformations are most distinct between the TMEM16A and the nhTMEM16 structures (91), to mimic the positions observed in the open nhTMEM16 structure (PDB: 4WIS) (see Methods for details). The final TMEM16F model thus harbors a widened hydrophilic groove, which we believe would represent an open conformation (Figure 13B-open). Subsequent atomistic simulations of the open structural model verify that the final TMEM16F model is energetically stable (Figure 14A, 14B). More importantly, this hydrophilic groove becomes readily accessible to both water and especially phosphates (Figure 13C-13E), supporting the notion that this open state model could represent an active TMEM16F CaPLSase. Interestingly, despite the limited simulation timeframe of 400 ns, we observed a total of 3 phosphatidylcholine externalization events (one in each run) via the widened hydrophilic
groove, closely recapitulating the well-known credit-card reader transport mechanism (Figure 15) (93).

Figure 14: MD simulation of an open state TMEM16F-CaPLSase homology model. A, Atomistic simulations show the stability of the open state model of TMEM16F. Backbone RMSD values of the entire TM domains (black trace) and TMs 4-6 (red trace) in one of the TMEM16F monomers in a 400-ns simulation with respect to the initial structure. B, Superposition of the 0 ns (cyan), 100 ns (yellow) and 400 ns (red) structures from the same simulation in (A).

After carefully scrutinizing the closed, intermediate and fully open models of TMEM16F and their capabilities to permeate water and phospholipids (Figure 13C-13E), we find that the constriction impeding the permeation of phospholipid headgroups mainly originates from three hydrophobic residues, F518, Y563 and I612, whose sidechains point toward the central axis of the hydrophilic groove (Figure 13C, 13E). Indeed, in the closed and intermediate states, the side chains of these residues stay in close proximity to create a physical barrier restricting phospholipid permeation; whereas in the Ca\(^{2+}\) bound fully open state, they separate to widen the hydrophilic groove, thus
permitting phospholipid permeation (Figure 13C-13E and Figure 15). Based on these observations, we hypothesize that F518, Y563 and I612 constitute an inner activation gate of the mammalian TMEM16F-CaPLSase.

Figure 15: Atomistic simulation of a POPC phospholipid permeating through the putative inner gate of TMEM16F. Inner gate residues are illustrated as purple spheres.
The head group of the POPC molecule (orange spheres with long cyan tails) initially resides at the inner leaflet (0-14 ns), then spontaneously crosses the inner gate region (15-25 ns), and eventually reaches the outer leaflet of the membrane (43 ns and onward). TMs 4-6 are highlighted in green. The phosphate groups of POPC within 15 Å of the protein are represented using orange transparent van der Waals surfaces.

3.2.2 Alanine mutations at the inner gate promote TMEM16F-CaPLSase

To examine the roles of the putative gate residues in regulating phospholipid movement across the hydrophilic groove, we first replaced these bulky residues with Ala. We hypothesized that the smaller and less hydrophobic sidechain from Ala would widen the inner gate to alleviate the constrictive effect, thereby facilitating phospholipid permeation. Using the optimized CaPLSase assay (developed in Chapter 2), we found that all three Ala mutations of F518, Y563, and I612 exhibited strong gain-of-function phenotypes (Figure 16A, 16B). When quantified with $t_{1/2(t_{\text{max}})}$ (Chapter 2), F518A and I612A showed significantly enhanced CaPLSase activity compared to WT-TMEM16F. Consistent with our observation, a recent report also shows an enhanced TMEM16F scrambling activity by the I612A mutation (143).
Figure 16: Alanine mutations of the inner gate residues promote activation of TMEM16F CaPLSase. A, Representative images of ionomycin-induced scrambling activity of TMEM16F-KO HEK293T cells expressing eGFP-tagged TMEM16F-F518A, Y563A or I612A. AnV-CF 594 signal indicates lipid scrambling activity after ionomycin application. Scale bars, 25 µm. B, Ionomycin-induced CaPLSase activities of WT and the mutant TMEM16F were quantified by $t_{1/2}(\text{Imax})$. Each data point represents one cell and n denotes the total number of cells measured. The pie charts illustrate the percentage of ionomycin-induced scrambling cells. The cells that did not exhibit ionomycin-induced CaPLSase activity were denoted as non-scr (non-scrambling) and were excluded from statistical analysis. Y563A expressing cells were cultured in Ca$^{2+}$-free medium to minimize cytotoxicity (labeled as Ca$^{2+}$ free). Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test. ****: p < 0.0001. Error bars indicate SEM. # denotes mutations with spontaneous CaPLSase activity.

Strikingly, we found that Y563A expression in regular Ca$^{2+}$-containing medium resulted in constitutively exposed PS even in the absence of ionomycin stimulation (Figure 17A). Rounded cell morphology and strong caspase3/7 activity staining (TF3-DEVD-FMK signal) further suggest that overexpressing Y563A likely induces cytotoxicity (Figure 17A). Interestingly, a rounded cell morphology has also been observed when a gain-of-
function mutation of TMEM16E is heterologously expressed in HEK293 cells, suggesting that gain-of-function CaPLSases tend to induce cytotoxicity (46). On the other hand, overexpression of TMEM16F D409G gain-of-function mutation (27) in the TMEM16F deficient HEK293T cells only resulted in accelerated ionomycin-induced phospholipid scrambling (Figure 8) without apparent changes in the cell morphology and constitutive PS exposure in regular cell culture medium (Figure 8A-D409G at 0min). This result is different from the observation of constitutive PS exposure when D409G was expressed in the lymphoma cells (144). We speculate that this discrepancy may be due to the differences in basal Ca\(^{2+}\) activities and cellular regulations in the two different cell types. Thus, Y563A-induced cytotoxicity in Ca\(^{2+}\) containing medium and constitutive PS exposure in Ca\(^{2+}\) free medium suggest that Y563A might be a more potent gain-of-function mutation compared with D409G.
Figure 17: Constitutive scrambling activity of Y563A single mutation and F518A-Y563A-I612A triple mutation. A, Representative images of TMEM16F-KO HEK293T cells expressing either eGFP-tagged Y563A- or F518A-Y563A-I612A without ionomycin stimulation. AnV-CF 640R labels PS positive cells. White asterisk labels apoptotic cells with positive AnV, strong cytosolic TF3-DEVD-FMK (indicative of cleaved caspases 3/7) staining, and rounded morphology. Scale bars, 20 µm. B, Percentage of cells with spontaneous CaPLSase activities without ionomycin stimulation. Each data point represents the percentage of PS positive cells from the total TMEM16F-expressing cells in one coverslip. Cells expressing Y563A and F518A-Y563A-I612A were cultured in Ca\(^{2+}\)-free medium to avoid cytotoxicity. Statistical analysis was performed using unpaired two-sided Student’s t-test. **: p=0.0047 (<0.01). Error bars indicate SEM.

To test whether this cytotoxicity was due to the excessive Ca\(^{2+}\)-dependent activation of TMEM16F, we cultured Y563A-expressing cells in a Ca\(^{2+}\)-free medium to suppress the basal Ca\(^{2+}\) activity. In this condition, we observed about 42% of the Y563A-expressing cells still exhibited spontaneous PS surface exposure (Figure 17). Notably, these PS positive cells possessed healthy, polarized morphology and displayed minimal caspase activity, all of which indicate that they were viable and that TMEM16F-Y563A expression promotes spontaneous PS externalization. While the remaining (58%) Y563A-
expressing cells did not show spontaneous CaPLSase activity, likely due to lower expression levels, they did exhibit gain-of-function phenotype upon ionomycin stimulation as evidenced by a smaller $t_{1/2(max)}$ value in comparison to that of TMEM16F-WT (Figure 16). These observations suggest that, distinct from D409G, which is not spontaneously active (Figure 8A-D409G at 0min), Y563A is a potent gain-of-function mutation that can facilitate strong CaPLSase activity under resting intracellular Ca$^{2+}$. Consistent with its increased CaPLSase activity, we found that Y563A also significantly enhanced its ion channel activation by Ca$^{2+}$ (Figure 18A-18D). In addition, Y563A completely abolishes TMEM16F channel desensitization or rundown in addition to altering ion selectivity (Figure 18E, 18F). All Taken together, these results further support that Y563 plays a critical role in gating substrate permeation of TMEM16F.
Figure 18: Ion channel properties of WT TMEM16F and its Y563A mutant. A, B, Representative current traces from inside-out patches excised from HEK293T cells expressing WT TMEM16F (A) and TMEM16F-Y563A (B) when exposed to 0.39, 2.26 and 100 µM Ca\(^{2+}\). Testing potentials were from -120 mV to +140 mV at a 20 mV increment. Both holding and repolarizing potentials were -60 mV. The red dotted lines mark zero current. C, D, G-V relations of WT TMEM16F (C) and TMEM16F-Y563A (D) channels under 2.26 µM and 100 µM Ca\(^{2+}\). Relative conductance was determined by measuring the amplitudes of tail currents measured at the -60 mV repolarization following each test voltage step. Error bars represent SEM. E, F, Whereas WT TMEM16F exhibited pronounced rundown
under 100 µM intracellular Ca$^{2+}$ (E), Y563A abolished channel rundown (F). G, H, Measurements of the $E_{\text{rev}}$ for WT TMEM16F (G) and Y563A (H). Black traces denote currents at symmetric 140 mM NaCl; red traces denote currents upon switching to an intracellular solution with low 14 mM NaCl. I, J, Changes in the $E_{\text{rev}}$ of WT TMEM16F and Y563A (I) and their permeability ratio $P_{\text{Na}}/P_{\text{Cl}}$ (J). Two-tailed unpaired Student’s t-tests: p-values are both <0.0001 in I and J.

As F518A, Y563A, and I612A all exhibited pronounced gain-of-function phenotypes in their scrambling activity, we tested whether their effects on phospholipid permeation could be additive. We therefore generated a triple Ala mutation, F518A-Y563A-I612A, and interrogated its Ca$^{2+}$-dependent phospholipid scrambling. Interestingly, we found that this triple mutation displayed more enhanced gain-of-function phenotype than the single Ala mutations (Figure 17). Specifically, more than 90% of the triple mutation-expressing cells exhibited constitutive CaPLSase activity in the absence of ionomycin treatment. The observed additive effect of the triple Ala mutation suggests a possibility that the three putative inner gate residues work synergistically to control phospholipid movement across the hydrophilic groove (Figure 13C, 13E).

### 3.2.3 Bulky mutations at the inner gate hinder TMEM16F-CaPLSase

Having established the critical roles of the putative inner gate residues in phospholipid gating, we next tested whether replacing these residues with a bulkier amino acid could disrupt the physical constriction and affect CaPLSase activity. A recent study identifies residues that are important for lipid scrambling in nhTMEM16 via Trp substitutions (40). Taking a similar approach, we found that Trp substitutions of Y563 and
I612, Y563W and I612W, markedly impaired phospholipid scrambling of TMEM16F, giving rise to loss-of-function scramblases (Figure 19A, 19B). Almost all Y563W-expressing cells and ~52% of I612W-expressing cells showed no ionomycin-induced CaPLSase activity. Paradoxically, F518W slightly enhanced TMEM16F phospholipid permeation (Figure 19A, 19B). We suggest that this enhancement in scrambling of F518W is likely due to the higher hydrophilicity of Trp compared with that of Phe (the hydropathy indexes for Trp and Phe are -0.9 and 2.8, respectively (145)). This mild gain-of-function phenotype of F518W is further corroborated by a similar observation that an equivalent mutation in nhTMEM16 also enhances phospholipid scrambling (40). To examine the significance of the hydrophobicity at F518 for phospholipid permeation, we substituted F518 with Leu, a more hydrophobic residue with the hydropathy index of 3.8 (145). In contrast to F518W, F518L exhibited a loss-of-function phenotype with a reduced scrambling activity (Figure 19A, 19B). Furthermore, we found that a large population of F518L-expressing cells showed no CaPLSase activity whereas cells that did scramble lipids exhibited significantly prolonged $t_{1/2}^{(l_{max})}$ values compared with that of WT-TMEM16F (Figure 19B). Taken together, our findings highlight the importance of both size and hydrophobicity at the inner gate, both of which are critical in controlling phospholipid permeation.
Figure 19: Bulky hydrophobic mutations at the inner gate hinder TMEM16F lipid permeation. A, Representative images of ionomycin-induced scrambling activity of TMEM16F-KO HEK293T cells expressing eGFP-tagged TMEM16F-F518L, F518W, Y563W or I612W. AnV-CF 594 signal represents lipid scrambling was recorded by time-lapsed imaging for 10-minute post 5 μM ionomycin application (0 min) at a 5-s acquisition interval. Scale bar, 25 μm. B, Ionomycin-induced CaPLSase activities for WT- and mutant-TMEM16F were quantified by $t_{1/2(\text{max})}$. Each data point represents one cell and n is the total number of cells measured. The pie charts illustrate the percentages of ionomycin-induced scrambling cells. The cells that did not exhibit ionomycin-induced CaPLSase activity were denoted as non-scr (non-scrambling) and are excluded from statistical analysis. As a majority of Y563W expressing cells does not scramble, mean and SEM were not assigned for this mutation. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test. ***: p = 0.0006; **: p=0.0013; ns: not significant (p=0.9167). Error bars indicate SEM.

3.2.4 Hydrophilic and charged mutations at the inner gate

To further dissect the role of the inner gate in controlling phospholipid permeation, we disrupted the hydrophobicity at the inner gate by substituting the inner gate residues with either hydrophilic or charged amino acids. Surprisingly, all of these
mutations turned out to be gain-of-function (Figure 20A, 20B, 21). Furthermore, with the exception of I612Q, which showed the modest gain-of-function phenotype, all other tested mutations exhibited constitutive scrambling activities (Figure 20A, 21A). Similar to Y563A and the triple Ala mutation (Figure 17), all of the spontaneous scrambling mutations from the hydrophilic/charged substitutions, except I612Q, induced cell stress and cytotoxicity when cultured in regular Ca\(^{2+}\) medium. To verify that the cytotoxicity was due to Ca\(^{2+}\)-dependent activation of TMEM16F, we cultured the transfected cells in the Ca\(^{2+}\)-free medium. Removal of extracellular Ca\(^{2+}\) alleviated the cytotoxicity from most of the spontaneous gain-of-function mutations, except for the two most potent ones, F518K and Y563K (Figure 20A, 21A). In fact, these two mutations were highly active that all of the expressing cells were PS-positive with apparent signs of apoptosis (rounded morphology and strong caspase 3/7 activities) even when cultured in Ca\(^{2+}\)-free medium (Figure 21A). Cells expressing other spontaneous gain-of-function mutations were healthy in the Ca\(^{2+}\)-free medium and exhibited various levels of spontaneous and ionomycin-induced CaPLSase activities (Figure 20A, 20B, 21B). Interestingly, negatively charged substitution to the inner gate residues, especially F518E and I612E, also caused a large population of transfected cells to display spontaneous PS externalization (Figure 20A, 21A). Thus, these results suggest that increasing hydrophilicity or introducing charges at the inner activation gate promotes phospholipid permeation.
Figure 20: Hydrophilic or charged mutations at the inner gate make TMEM16F constitutively open. A, Percentage of cells with spontaneous CaPLSase activities without ionomycin stimulation. B, Ionomycin-induced CaPLSase activities for WT and mutant TMEM16F were quantified by t(2)(Imax). The constitutively activated TMEM16F mutants that caused >80% of spontaneous PS exposure (##) were excluded from this analysis. For the modest gain-of-function mutations (<80% spontaneous PS exposure, #), only the cells that did not show spontaneous PS exposure were analyzed for their t(2)(Imax) values. Each dot represents one cell, and n is the total number of cells measured. The pie charts illustrate the percentages of ionomycin-induced scrambling cells, and non-scr denotes non-scrambling. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test. **: p = 0.0093; ****: p < 0.0001. Error bars indicate SEM. C, Percentage of cells with spontaneous CaPLSase activities without ionomycin stimulation. D, Representative AnV-CF 640R and TF3-DEVD-FMK stainings of TMEM16F-KO HEK293T cells expressing eGFP-tagged F518K-D703R and Y563K-D703R in the absence of ionomycin stimulation. In A and C, each dot represents the percentage of PS-positive cells over the total expressing cells (either WT-TMEM16F or mutants) in one coverslip. All constitutively active TMEM16F mutants were expressed in Ca^{2+}-free medium as indicated in the figure to suppress their excessive scrambling activity and cytotoxicity. Scale bar, 20 µm.
Besides lowering the permeation energy barrier by facilitating phospholipid headgroups interaction, the hydrophilic and charged residues introduced at the inner gate may also shift the equilibrium between the open and closed states of TMEM16F-CaPLSase by destabilizing the closed state and/or stabilizing the open state. Other than directly affecting the inner gate itself, the mutations at the putative inner gate may allosterically enhance Ca\(^{2+}\)-binding to give rise to the gain-of-function phenotype. To examine this possibility, we couple our polar and charged inner gate mutations with the Ca\(^{2+}\)-binding site mutation D703R (Figure 8). Interestingly, abrogating Ca\(^{2+}\) binding successfully suppresses the gain-of-function phenotypes for most of the polar and charged inner gate mutations (except F518K and Y653K) even in the regular Ca\(^{2+}\)-containing medium (Figure 20C, 22). Since all of the double mutations with D703R have suffice cell surface expression (Figure 22), membrane targeting is unlikely to be the reason why these double mutations lost their spontaneous CaPLSase activities. We speculate that the resting, basal Ca\(^{2+}\) level (about 100 nM) (146) is sufficient to trigger the opening of the activation gate in the corresponding single gain-of-function mutations to induce spontaneous CaPLSase activity (Figure 20A).

Notably, we found that disrupting Ca\(^{2+}\) binding by the D703R mutation did not prevent spontaneous scrambling of F518K and Y563K (Figure 20C, 20D). In fact, about 34% of F518K-D703R and nearly 93% Y563K-D703R-expressing cells still exhibit
spontaneous PS exposure in Ca\(^{2+}\)-free medium. The constitutively active F518K-D703R and Y563K-D703R mutations further support that the positive charges at these two inner gate residues destabilize the closed state of the phospholipid pathway independent of Ca\(^{2+}\) binding. I612K in TM6, however, when coupled with D703R, failed to spontaneously expose PS (Figure 20C), suggesting that the charge on this residue imposes a weaker effect in destabilizing the activation gate than those of F518K and Y563K in TM4 and TM5, respectively. The results also illustrate that positive charges introduced at these two positions allow TMEM16F phospholipid pathway to stay constitutively open without Ca\(^{2+}\) binding. Although we could not investigate the Ca\(^{2+}\) binding affinity of TMEM16F mutations to completely rule out the possibility that these gain-of-function mutations allosterically enhance Ca\(^{2+}\) binding, the constitutive scrambling observed in F518K-D703R and Y563K-D703R mutations, whose Ca\(^{2+}\) binding is severely impaired, strongly indicates that these charged residues introduced at the inner activation gate directly open the phospholipid pathway without much facilitation from Ca\(^{2+}\) binding.
Figure 21: Representative images show lipid scrambling activities of charged and polar mutations of TMEM16F inner gate residues. A, TMEM16F inner gate mutations with charged or polar sidechains are constitutively activated under basal Ca\(^{2+}\) level. Images were taken in the absence of ionomycin stimulation. Protein expression of TMEM16F mutants is visualized by their C-terminally tagged eGFP in TMEM16F-KO HEK293T cells. AnV-CF 640R staining labels spontaneously exposed PS on the cell surface. Strong and punctuated TF3-DEVD-FMK staining of cleaved caspases 3/7 indicates apoptotic cells. B, Representative images of ionomycin-induced scrambling activity of TMEM16F-KO HEK293T cells expressing eGFP-tagged TMEM16F- F518Q, Y563Q, Y563E, I612Q and I612K. AnV-CF 594 signal represents lipid scrambling activity. Scale bars, 20 µm in (A) and 25 µm in (B).
Figure 22: Ionomycin-induced CaPLSase activities of TMEM16F gain-of-function mutations coupling with D703R. Representative images of ionomycin-induced scrambling activity of TMEM16F-KO HEK293T cells expressing eGFP-tagged TMEM16F
gain-of-function mutations coupling with D703R. AnV-CF 594 signal indicates lipid scrambling.

3.2.5 An equivalent inner gate controls TMEM16A gating

As TMEM16f-CaPLSase and TMEM16A-CaCC share a high degree of sequence similarity in their TMs (Figure 11), we tested whether equivalent residues in TMEM16A could also serve as an inner activation gate. Interestingly, unlike WT-TMEM16A (120), our inside-out patch clamp recordings show that both TMEM16A-L543K and I637K mutant channels could be activated by membrane depolarization in the absence of intracellular Ca\textsuperscript{2+} (Figure 23A, 23B), suggesting that introducing positive charges to these two hydrophobic residues in TM4 and TM6 could unlatch the gate and promote TMEM16A channel opening. Among all three mutations, S588K showed the weakest effect on TMEM16A voltage-dependent activation despite being activated by Ca\textsuperscript{2+} (Figure 25A).
Figure 23: Lysine mutations at TMEM16A inner gate potentiate the CaCC. A, Representative current traces of inside-out patches excised from TMEM16A-WT or mutations-expressing HEK293T cells in the absence of intracellular Ca\(^{2+}\). Testing potentials were from −100 mV to +140 mV with 20 mV increments. Both holding and repolarizing potentials were −60 mV. B, Current–voltage (I–V) relationship of the equivalent inner gate mutations in TMEM16A. Error bars indicate SEM.

More strikingly, similar to TMEM16F-F518K (Figure 20A), the corresponding mutation TMEM16A-L543K induced cytotoxicity when the transfected cells were cultured in regular Ca\(^{2+}\)-containing medium (Figure 25B). However, culturing these cells in a Ca\(^{2+}\)-free medium alleviated cytotoxicity; yet about 44% of the L543K-TMEM16A-expressing cells still exhibited constitutive PS exposure as indicated by the positive AnV signal (Figure 24A, 24C). Their morphology as well as activated caspase 3/7 level were similar to the healthy non-transfected cells (Figure 24C). For the L543K-expressing cells that did not show spontaneous PS exposure, application of a lower concentration of ionomycin (2.5
μM) rapidly induced PS exposure (Figure 24B, 25C), consistent with TMEM16A having a higher Ca$^{2+}$ sensitivity than TMEM16F (28). These results clearly demonstrate that L543K, a single point mutation at the inner activation gate, is capable of converting TMEM16A-CaCC into a CaPLSase. In addition, a potent TMEM16A-CaCC blocker Ani9 (147) effectively blocked ionomycin-induced phospholipid scrambling through L543K (Figure 24B), confirming that this mutation rendered TMEM16A the capability to permeate phospholipids. Interestingly, TMEM16A-L543K also has altered ion selectivity (Figure 25D-25F), further supporting the critical role of L543 residue in controlling ion permeation.
Figure 24: Lysine mutations at TMEM16A inner gate convert the CaCC into a phospholipid scramblase. A, TMEM16A-L543K expression induces spontaneous PS exposure (in the absence of ionomycin treatment) even when cultured in Ca^{2+}-free medium. Each dot represents the percentage of PS-positive cells over the total expressing cells of one coverslip. B, Ionomycin-induced CaPLSase activity for WT and the mutant TMEM16A quantified by t_{1/2(max)}. Treatment with 10 µM Ani9 inhibits TMEM16A-L54K scrambling activity as indicated by its t_{1/2(max)}. Each dot represents one cell and n is the total number of cells measured. The pie charts illustrate the percentages of ionomycin-induced scrambling cells. Non-scr denotes non-scrambling and are excluded from statistical analysis. As a majority of TMEM16A-WT, -L543K (Ani9), S588K, and I637K expressing cells did not scramble, mean and SEM were not assigned for those data. Error bars indicate SEM. C, Representative images of eGFP-tagged TMEM16A-L543K expressing TMEM16F-KO HEK293T cells without ionomycin stimulation. AnV-CF 640R labels PS-positive cells. TF3-DEVD-FMK stains for activated caspases 3/7. Scale bar, 25 µm.
In contrast to L543K, neither S588K nor I637K expression caused spontaneous or ionomycin-induced PS exposure through TMEM16A (Figure 24A, 24B, 25C). As I637K shows a gain-of-function in its channel activation (Figure 23A, 23B), the lack of CaPLSase activity indicates that while the positive charge at I637 can enhance ion permeation, its activation gate might not be opened widely enough to permeate phospholipids. This is also consistent with the milder gain-of-function phenotype observed in the equivalent I612K-TMEM16F-CaPLSase (Figure 20A, 20B). In summary, our observation that the conversion of TMEM16A-CaCC to a constitutively activated CaPLSase by L543K further strengthens the importance of positive charges at the inner activation gate in promoting gating of TMEM16 proteins.
Figure 25: Measurement of ionomycin-induced CaPLSase activity of the TMEM16A inner gate mutants. A, Representative recording shows activation of S588K when 0 µM Ca$^{2+}$ (black trace) or 100 µM Ca$^{2+}$ (red trace) is applied to the cytosolic side of an inside-out patch configuration. B, Representative images of TMEM16A L543K-expressing TMEM16F-KO HEK293T cells in normal medium without ionomycin stimulation. AnV-CF 640R labels PS positive cells. TF3-DEVD-FMK staining indicates cleaved caspases 3/7. C, Representative images of ionomycin-induced scrambling activity of TMEM16F-KO HEK293T cells expressing eGFP-tagged TMEM16A-WT, -L543K, -S588K and -I637K. AnV-CF594 signal representing lipid scrambling was recorded after 2.5 µM ionomycin application (0 min). D, E, Measurements of ion selectivity of TMEM16A-WT (D), -L543K (E). Black traces denote currents in symmetric 140 mM NaCl. Red traces denote currents upon switching to an intracellular solution with low 14 mM NaCl. F, The permeability ratios $P_{Cl}$/$P_{Na}$ of TMEM16A-WT and -L543K. Two-tailed unpaired Student’s t-test: p-value is 0.0191. Scale bars, 20 µm in (B) and 25 µm in (C).
3.3 Discussion

In this study, we identified an inner activation gate formed by three hydrophobic residues, F518, Y563, and I612, near the middle of the phospholipid permeation pathway of TMEM16F-CaPLSase. Side chain properties of the inner gate residues have profound effects on TMEM16F-CaPLSase activities. In particular, introducing positive charge to F518 and Y563 constitutively opens the activation gate to allow spontaneous phospholipid permeation even in the absence of Ca\(^{2+}\) binding. Moreover, the positive charge at the putative inner activation gate not only promotes phospholipid scrambling in TMEM16F but also enhances channel activation in TMEM16A, consistent with a possibility that they share a similar mechanism of activation. More strikingly, we found that a single point mutation, L543K, at the inner activation gate could convert the non-scrambling CaCC TMEM16A into a constitutively activated CaPLSase. This observation stands in contrast to a previous study, which shows that substitution of a long stretch of 34 amino acids at TMs 4–5 from TMEM16F is required to transform TMEM16A CaCC into a CaPLSase (58).

Based on our functional and computational studies, here we propose a clam shell model to describe the gating mechanism of TMEM16F-CaPLSase (Figure 26). We speculate that the interface between TM4 and TM6 can open and close like a clam shell to control the accessibility of phospholipids to the interior of the hydrophilic groove, where phospholipid permeation is catalyzed (Figure 15). F518 in TM4 and I612 in TM6 likely
serve as gate-keepers for the opening of this interface. In the absence of Ca\textsuperscript{2+}, they stay close to each other to block the accessibility of phospholipid headgroups to the hydrophilic groove. Upon Ca\textsuperscript{2+} binding, large-scale conformational changes, likely including the movement of TM6 (Figure 12A) around a conserved glycine hinge (59, 91) and the rearrangement of TMs 3–5, separate TM4 and TM6 (Figure 13B). These conformational rearrangements likely culminate in the opening of the TM4–TM6 interface as well as the inner gate residues. Subsequently, the interior of the hydrophilic groove becomes exposed to the surrounding phospholipids such that their phospholipid headgroups can enter and translocate via the credit-card reader mechanism. By projecting its bulky sidechain into the center of the hydrophilic groove, Y563 in TM5 likely serves as a cap that stabilizes the inner gate and further obstructs phospholipid permeation in the closed state. Dilation of the inner gate following TM4 and TM6 separation allows the Y563 hydroxyl group to interact with phospholipid headgroups and facilitate phospholipid permeation. Introducing smaller, polar or charged residues to these critical locations tends to remove steric hindrance, likely via destabilizing the TM4–TM6 interface to promote spontaneous opening transitions, resulting in enhanced phospholipid permeation. In the case of F518K and Y563K, their inner activation gates become so severely disrupted that phospholipids can freely go through the constitutively open gate in the absence of Ca\textsuperscript{2+} binding.
Recently, four independent structural characterizations of TMEM16 CaPLSases were released, reporting the structures of the fungal nhTMEM16, afTMEM16, mTMEM16F and hTMEM16K, respectively (34, 41, 45, 52, 63). Interestingly, the Ca\textsuperscript{2+}-free mTMEM16F structure exhibits high similarities to the mTMEM16A structures (91, 140), especially in the TM region including TM4 and TM6. This is consistent with our hypothesis that mammalian TMEM16F and TMEM16A share more structural similarities than fungal nhTMEM16 (Figure 11, 12). While the structures of mTMEM16F provide valuable insights into the mammalian CaPLSases, minimal conformational changes were observed between the Ca\textsuperscript{2+}-bound and Ca\textsuperscript{2+}-free mTMEM16F or between mTMEM16F and the non-scrambling mTMEM16A. It thus remains unclear how TMEM16F CaPLSase regulates phospholipid permeation based on these structural studies. Moreover, the extracellular segments of TM4 and TM6 in the Ca\textsuperscript{2+}-bound mTMEM16F structure are packed against each other preventing phospholipids from accessing the interior of the hydrophilic groove (52, 63), suggesting that this structure may represent an intermediate conformation rather than a fully open and active conformation (Figure 13D, 13E). On the other hand, the structures of the Ca\textsuperscript{2+}-bound fungal afTMEM16 (45), nhTMEM16 (41, 44) and hTMEM16K (34) all adopt a widened TM4-TM6 interface upon Ca\textsuperscript{2+} binding, which is consistent with our proposed clam shell gating model in TMEM16F.
Identification of the inner activation gate for TMEM16F-CaPLSase also offers insights into our understanding of the gating mechanism of TMEM16-CaCCs. We suggest that, instead of utilizing the Ca\(^{2+}\)-dependent clam shell activation mechanism for TMEM16-CaPLSases, Ca\(^{2+}\) binding to TMEM16-CaCCs may induce coordinated movement of TM4 and TM6 to dilate the ion permeation pathway, thereby allowing ions to go through while still maintaining a tight barrier at the TM4-6 interface, which excludes phospholipid permeation. This gating mechanism is supported by our observation that I637K-TMEM16A, which is a gain-of-function CaCC, does not facilitate phospholipid permeation (Figure 23, 24A, 24B). On the other hand, when a positive charge is introduced to L543 in TM4, the interaction between TM4 and TM6 might be dramatically weakened so that they may open like a clam shell to allow spontaneous permeation of phospholipids. We therefore postulate that TMEM16 CaPLSases and CaCCs may share evolutionarily conserved Ca\(^{2+}\)-dependent gating mechanism and overall similar design in their inner activation gates. Their distinct substrate selectivity and permeation may be partially derived from the differences on how widely the putative inner activation gate can open. We hope that our current findings can inspire future structural, computational and functional studies to further establish the molecular mechanisms underlying Ca\(^{2+}\)-dependent substrate permeation of TMEM16 proteins.
Figure 26: A clam shell model for TMEM16F-CaPLSase gating. In each subunit, TMs 3-8 enclose a hydrophilic phospholipid permeation pathway. The interface between TM4 and TM6 guards the inner activation gate, precluding phospholipid access to the hydrophilic pathway when Ca$^{2+}$ is absent. Ca$^{2+}$ binding triggers the separation of TM4 and TM6, leading to the opening of the inner activation gate and subsequent phospholipid permeation. Reducing size or increasing hydrophilicity of the inner activation gate residues weakens the TM4-TM6 interface, thereby enhances phospholipid permeation.

3.4 Experimental procedures

3.4.1 Cell lines and cell culture

Please refer to subsection 2.4 for cell-culture procedure, list of cell-lines and method of generating TMEM16f-KO HEK293T cell line.

3.4.2 Plasmids used and transient transfection protocol

3.4.2.1 Plasmids

Plasmids used in this chapter includes pEGFP-N1 vector carries cDNAs of either mTMEM16F (Open Biosystems cDNA # 6409332) (28), mTMEM16A (Open Biosystems cDNA # 30547439) (92, 120, 140), or their point mutations with C-terminal eGFP tag.
Point mutations of mTMEM16F and mTMEM16A were created and validated as described in subsection 2.4. Primers used to generate point mutations of mTMEM16F and mTMEM16A are listed in Table 2 and Table 3 (Appendix A), respectively. Double and triple mutations were constructed by performing multiple single point mutations using the same cDNA template.

3.4.2.2 Transient transfection

Transient transfection was carried out as described in subsection 2.4. Most of experiments were often performed after 24-48 hours of transfection. However, in the transfection with constitutively active scramblases, the culture medium was changed to Ca\(^{2+}\)-free DMEM (Gibco, #21068-028) instead of standard DMEM, unless otherwise noted, 5-hour post transfection and experiments were strictly done within 22-24 hours after transfection.

3.4.3 Phospholipid scrambling assay

The microscope-based phospholipid scrambling assay was performed as detailed in subsection 2.4, except the followings:

To assess the CaPLSase activity of TMEM16A and its mutants, a final concentration of 2.5 \(\mu\)M of ionomycin was used. In the experiments with Ani9, TMEM16A-L543K expressing cells were pre-treated with 10 \(\mu\)M Ani9, and then activated with 2.5 \(\mu\)M ionomycin in the presence of 10 \(\mu\)M Ani9.
3.4.4 Quantifying phospholipid scrambling activity

The quantification of phospholipid scrambling activity was performed as detailed in subsection 2.4 with the following modifications: CaPLSase expressing cells with no scrambling activity (as indicated by absence of AnV signal after ionomycin treatment) and CaPLSase expressing cells with AnV signal (constitutively active CaPLSases) were omitted from $t_{1/2}/I_{\text{max}}$ analysis.

For the mutations that induce constitutive scrambling activity prior to ionomycin treatment, we quantified the percentage of spontaneous PS-positive cells by manually counting the number of cells that are both eGFP and AnV positive over all expressing cells (eGFP positive) in 10-20 random fields of view per coverslip in the absence of ionomycin. Each data point represents the percentage of spontaneous PS-positive expressing cells in one coverslip. Results were analyzed by using ImageJ, Prism (GraphPad) and Excel (Microsoft).

3.4.5 Live cell caspase 3/7 staining assay

Similar to the procedure detailed in subsection 2.4, here, TMEM16F-KO 293T were seeded and transfected with appropriate plasmid constructs on PLL coated No.0 coverslips. For caspase 3/7 staining of the spontaneously scrambling TMEM16F and TMEM16A mutations, TF3-DEVD-FMK dye was diluted to 1X in either standard or Ca$^{2+}$-free DMEM depending on the mutations that were transfected to the cells. After
incubating with 1X dye for 1 hour, the cells were washed twice with the kit’s washing buffer. 0.5 µg mL\(^{-1}\) AnV-CF 640R was added into the imaging chamber to monitor the cleaved caspase 3/7 and spontaneous PS-exposure simultaneously. The results were collected with a 63X/1.4 NA Oil Plan-Apochromat DIC in Zeiss 780 inverted Confocal microscope. The results were analyzed with ImageJ and Zeiss.

### 3.4.6 Electrophysiology

TMEM16F-KO HEK293T cells were used for expression and recordings of mTMEM16F current. Normal HEK293T cells were used for expression and recordings of mTMEM16A current. HEK293T cells grown on PLL and laminin (Sigma)-coated coverslips placed in a 24-well plate reaching 40-60% confluency were transiently transfected following protocol described above. All recordings were carried out at room temperature after 24-48 h following transfection. Inside-out recordings were performed on patches excised from cells expressing eGFP-tagged TMEM16A or TMEM16F constructs. Pipette electrodes were made from borosilicate glass capillaries (1.5 mm x 0.86 mm) with a puller (Sutter Instruments), fire-polished with a microforge (Narishge), and had a resistance of ~2-3 MΩ when filled with recording solutions. The pipette (external) solutions contain 140 mM NaCl, 5 mM EGTA, 10 mM HEPES, 2 mM MgCl\(_2\), adjusted to pH 7.3 (with NaOH). The perfusion (internal) solution contain 140 mM NaCl, 10 mM HEPES, 5 mM EGTA, adjusted to pH 7.3 (with NaOH). The internal solution with 100 µM
Ca\textsuperscript{2+} was made by directly adding CaCl\textsubscript{2} into a solution containing 140 mM NaCl and 10 mM HEPES, adjusted to pH 7.3 (with NaOH). The internal solution with 2.26 µM Ca\textsuperscript{2+} contains 140 mM NaCl, 10 mM HEPES, 5 mM EGTA, and 4.64 mM CaCl\textsubscript{2}, adjusted to pH 7.3 (with NaOH). The amount of added CaCl\textsubscript{2} was calculated using WEBMAXC (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/). Free Ca\textsuperscript{2+} concentration of EGTA- buffered solution was further verified using the ratiometric Ca\textsuperscript{2+} dye Fura-2 (ATT Bioquest) and plotted against a standard curve from a calibration kit (Biotium).

For the current-voltage relationship (I-V) recordings, the membrane was held at −60 mV and 250-ms test voltage steps ranging from −120 mV to +140 mV were applied at a 20 mV increment. Due to the small amplitudes and rapid deactivation of the tail currents at the −60 mV repolarization step of TMEM16A in the absence of Ca\textsuperscript{2+}, steady-state peak currents from the test voltage steps were used to construct the I-V plot. For TMEM16F’s I-V recordings in the presence of 2.26 µM and 100 µM Ca\textsuperscript{2+}, peak tail currents measured at −60 mV repolarization steps were normalized to the max tail current to generate the G-V curves.

For the reversal potential (E\textsubscript{rev}) measurements, TMEM16A and TMEM16F channels were first activated by perfusion of internal solution containing 100 µM Ca\textsuperscript{2+}. Both the pipette (external) and bath (internal) are symmetric and contain 140 mM NaCl, 5
mM EGTA, 10 mM HEPES, adjusted to pH 7.3 (NaOH). For TMEM16A, a ramp protocol from −100 mV to +100 mV was used to elicit currents. For TMEM16F, an inverted V-shaped protocol in which the membrane was ramped from −120 mV to +120 mV and back to −120 mV was used. The second phase of the ramp (from −120 mV to −120 mV) was used to construct the I-V plots for measuring the $E_{\text{rev}}$. For both TMEM16A and TMEM16F, the changes in $E_{\text{rev}}$ were triggered by switching internal solution with a low NaCl solution containing 14 mM NaCl, 5 mM EGTA, 10 mM HEPES, and 100 µM Ca$^{2+}$ adjusted to pH 7.3. The $E_{\text{rev}}$ was determined as the membrane potential at which the current was zero, and the shifts in the $E_{\text{rev}}$ ($\Delta E_{\text{rev}}$) are the differences between the $E_{\text{rev}}$ measured in symmetric NaCl and low internal NaCl. The permeability ratio $P_{\text{Cl}}/P_{\text{Na}}$ was calculated using the Goldman-Hodgkin-Katz equation:

$$V_m = \frac{RT}{F} \ln \left( \frac{P_{\text{Na}}[\text{Na}]_o + P_{\text{Cl}}[\text{Cl}]_i}{P_{\text{Na}}[\text{Na}]_i + P_{\text{Cl}}[\text{Cl}]_o} \right)$$

(eq. 2)

in which $V_m$ is the measured $E_{\text{rev}}$ shift ($\Delta E_{\text{rev}}$); $P_{\text{Na}}$ and $P_{\text{Cl}}$ are the relative permeabilities of Na$^+$ and Cl$^-$; [Na]$_o$ and [Na]$_i$ are external and internal Na$^+$ concentrations; [Cl]$_o$ and [Cl]$_i$ are external and internal Cl$^-$ concentrations; $F$ is the Faraday’s constant (96485 C mol$^{-1}$); $R$ is the gas constant (8.314 J mol$^{-1}$), and $T$ is the absolute temperature (298.15 K or 25 °C).

For time-course monitoring of TMEM16F channel activity, a voltage step protocol in which the membrane was stepped to +80 mV and then −80 mV lasting 200 ms each. The membrane was held at 0 mV. Channel opening was elicited by perfusion of internal
solution containing 100 µM Ca\textsuperscript{2+}. Peak currents measured at +80 mV steps were used for
time-course monitoring of channel opening.

All electrophysiology recordings were low-pass filtered at 5 kHz (Axopatch 200B)
and sampled at 10 kHz (Axon Digidata 1550 A) and digitized by Clampex 10 (Molecular
Devices). Offline data analysis was performed in Clampfit, Excel (Microsoft), and Prism
(GraphPad).

### 3.4.7 Protein sequence alignment

Protein sequences of nhTMEM16, mTMEM16A (UniProtKB: Q8BHY3) and
mTMEM16F (UniProtKB: Q6P9J9) were aligned using Clustal Omega
(https://www.ebi.ac.uk/Tools/msa/clustalo/).

### 3.4.8 Homology modeling of TMEM16F in open and closed states

Homology models of the closed (without Ca\textsuperscript{2+}) and intermediate (with Ca\textsuperscript{2+}) states
of mTMEM16F were derived directly using Ca\textsuperscript{2+}-free (PDB: 5OYG) and Ca\textsuperscript{2+}-bound (PDB:
5OYB) mTMEM16A structures (91) with the sequence alignment shown in Figure 11 using
the Swiss-pdb server (148). Even though the Ca\textsuperscript{2+}-bound structure of nhTMEM16 (PDB:
4WIS) (44) is believed to provide a good model of the open conformation of the TM region
of mTMEM16F, the sequence conservation in the extracellular domain and loops is poor.
To derive a model of mTMEM16F in the open state, steered MD was initiated from the
intermediate state model derived from 5OYB to reposition TMs3-6 to the configuration
observed in the Ca\textsuperscript{2+}-bound nhTMEM16 structure (PDB: 4WIS) using the CHARMM software (149, 150). We note that, even though TM5 is similarly positioned between PDBs 5OYB and 4WIS, it lines the putative phospholipid permeation pathway and thus was included in the steered MD to ensure the structural integrity. For this, a homology model of mTMEM16F TM region alone was first derived from nhTMEM16 using the sequence alignment derived from Clustal Omega (see above). This model was then used as the target (open state TM conformation) during the steered MD simulation, where root mean square deviation (RMSD) restraint was applied on TMs3-6 with increasing force constants ranging from 5 to 75 kcal mol\textsuperscript{-1} Å\textsuperscript{2}. In addition, all heavy atoms of adjacent 5 residues of loops before and after TMs 3-6 were harmonically restrained using a weak force constant of 0.1 kcal mol\textsuperscript{-1} Å\textsuperscript{2}. This allows appropriate, well-controlled loop reconfiguration to accommodate the movement of TMs 3-6. The rest of the whole protein were restrained by harmonic positional restraints with a force constant of 100 kcal mol\textsuperscript{-1} Å\textsuperscript{2} to avoid unnecessary structure disruption. The simulation was performed in vacuum with a distance-dependent dielectric constant. Timestep was set to 1 fs. The final model was only ~0.14 Å backbone RMSD from 4WIS for TMs 3-6.

3.4.9 MD simulations of mTMEM16F

The mTMEM16F structures (in open, intermediate or closed state) were first inserted in model lipid bilayers and then solvated in TIP3P water (151) using the
CHARMM-GUI web server (152). To mimic mammal plasma membranes, pure POPC and 2:1 POPC/POPS mixture were used for upper and lower leaflets, respectively. The solvated systems were then neutralized and 150 mM KCl was added. The final simulation boxes contain about ~660 lipid molecules and ~73,000 water molecules, with a total of ~335,000 atoms and dimensions of ~155 × 155 × 140 Å$^3$. The CHARMM36m all-atom force field (153) and the CHARMM36 lipid force field (154) were used. All simulations were performed using CUDA-enabled Amber14 (155). Long-range electrostatic interactions were described by the Particle Mesh Ewald (PME) algorithm (156) with a cutoff of 12 Å. Van der Waals interactions were cutoff at 12 Å with a smooth switching function starting at 10 Å. The lengths of hydrogen-containing covalent bonds were constrained using SHAKE (157) and the MD time step was set at 2 fs. The temperature was maintained at 298 K using the Langevin dynamics with a friction coefficient of 1 ps$^{-1}$. The pressure was maintained semi-isotopically at 1 bar at both x and y (membrane lateral) directions using the Monte Carlo (MC) barostat method. Several segments were absent in the cryo-EM structures of TMEM16A; they were considered dynamic and thus not included in simulations. These include the N- and C-terminal segments (M1-N86 and S882-E911) and a long loop in the cytosolic domain (Y101-V134). The residues before and after the missing part were capped with either an acetyl group (for N-termini) or a N-methyl amide (for C-termini). To minimize the effects of missing residues on the cytosolic domain, the
backbone of structured parts of the cytosolic domain (E92-A100, L135-F182, S190-D194, P206-V220, G232-L248, R420-V431, K862-E881) were harmonically restrained with a modest force constant of 1 kcal mol\(^{-1}\) Å\(^{-2}\) during all simulations.

All systems were first minimized for 5000 steps using the steepest descent algorithm, followed by a series of equilibration steps where the positions of heavy atoms of the protein/lipid were harmonically restrained with restrained force constants gradually decreased from 10 to 0.1 kcal mol\(^{-1}\) Å\(^{-2}\). In the last equilibration step, only protein heavy atoms were harmonically restrained and the system was equilibrated 10 ns in under NPT (constant particle number, pressure and temperature) conditions. All production simulations were performed under NPT conditions. For state (open, closed with Ca\(^{2+}\), and closed), three independent 400-ns simulations were performed. Snapshots were saved every 50 ps. Only snapshots from 100-400 ns of each trajectory were included for analysis. The PMFs of water and phosphate group of lipids were calculated directly from the corresponding probability distributions along the membrane normal. The standard errors were estimated from the calculated averages over three independent simulations. The 3D phosphate density distribution near the channel was calculated using a cubic grid with a resolution of 0.5 Å.

3.4.10 Statistical analysis

Statistical analysis is the same as subsection 2.4.6.
Chapter 4: Examining the pharmacological profile of TMEM16F

Portions of this chapter are reproduced and adapted from a research originally published in the Journal of Biological Chemistry.\(^{(1)}\)

Some data in this chapter were done in collaboration with members of the Yang Lab.\(^{(2)}\)

### 4.1 Background

With its physiological functions ranging from blood coagulation (27, 28, 64), cell-cell fusion (71), bone and vascular development (68-70) to viral infection (72), TMEM16F is unquestionably an important element in human health and disease. Interestingly, Scott syndrome patients with TMEM16F loss-of-function mutations and TMEM16F knockout mice exhibited an anticoagulant trait (27, 28, 64, 65), suggesting that TMEM16F CaPLSase might be a promising therapeutic target for preventing thrombosis-related diseases such

\(^{1}\) Portions of this chapter are reproduced and adapted from: Le, T., Le, S. C., Zhang, Y., Liang, P., and Yang, H. Evidence that polyphenols do not inhibit the phospholipid scramblase TMEM16F. J Biol Chem. 2020; 295:12537-12544. © 2020 Le et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.

\(^{2}\) Dr. Yang Zhang performed the cell-free (spectrometry) fluorophores quenching experiments. Son C. Le performed the electrophysiology experiments to examine effects of TA and EGCG on TMEM16F ion channel. Dr. Pengfei Liang characterized the effects of TA on BK channel.
as stroke or deep vein thrombosis (80). Although TMEM16F CaPLSase is important in physiology and a potential therapeutic target, its pharmacological profile remains elusive.

In an effort to identify pharmacological tools that can specifically target TMEM16F, we employed our optimized microscopy-based scrambling assay approach (Chapter 2) to screen various molecules ranging from common ion channel blockers (e.g. Ru-red, CdCl$_2$, 2APB, SKF96365) (158), TMEM16A’s potent antagonists (e.g. Benzbromarone, T16Ainh-A01, CaCCinh-A01, Ani9, MONNA) (123, 147, 159-161) to molecules that alter cellular signaling (e.g. Idebenone, Ibrutinib, Imatinib) or membrane lipid composition (e.g. Wortmannin) (162-165). However, these molecules, including R5421 – a molecule that was formerly reported to be lipid scramblase inhibitor (166) – or molecules (Ru-red, CdCl$_2$, 2APB) that were previously shown to suppress TMEM16F SCAN from the cytosolic side (28), did not exhibit inhibitory effects toward TMEM16F CaPLSase from the extracellular side (Figure 27).
Figure 27: Effects of different pharmacological reagents on TMEM16F CaPLSase. mTMEM16F stable HEK293 cells were pre-treated with pharmacological molecules. 5 µM ionomycin was then used to trigger Ca$^{2+}$ influx and activate TMEM16F CaPLSase. AnV-CF 594 was used to detect TMEM16F scrambling activity. Fluorescence signal of AnV-CF 594 was recorded for 10 minutes after ionomycin application (t = 0min).
Despite our previous unsuccessful attempt to identify the inhibitors for TMEM16F CaPLSase, recent reports have demonstrated that tannic acid (TA) and epigallocatechin gallate (EGCG) are promising antagonists for the CaPLSase (103, 167). TA and EGCG are natural polyphenolic compounds (Figure 28) that are abundantly present in plant-based foods and drinks such as fruits, vegetables, green tea and red wine (168-170). Recently, these polyphenolic compounds were described to potently inhibit TMEM16F CaPLSase based on the observations that TA and EGCG could efficiently prevent fluorescently tagged phospholipids and AnV probes from reporting TMEM16F-mediated lipid scrambling (103, 167). In agreement with numerous reported beneficial effects of TA and EGCG on human health (168-171), the findings that TA and EGCG could potently inhibit TMEM16F CaPLSase thus raised the enthusiasm to apply these natural polyphenols as research and therapeutic tools to target TMEM16 CaPLSases.

![Chemical structures of TA and EGCG](image)

**Figure 28: Chemical structures of TA and EGCG.** Chemical structures of TA and EGCG were drawn using ChemDraw 18.2 (PerkinElmer).
To further explore the inhibitory effects of polyphenolic compounds on TMEM16F CaPLSase, we expanded our molecule screening pools to include other natural polyphenols, such as ellagic acid and (-)-epigallocatechin (172, 173). Unfortunately, ellagic acid and (-)-epigallocatechin treatments did not block TMEM16F CaPLSase (Figure 27). As TA and EGCG were the only molecules displaying the inhibitory effects toward TMEM16F CaPLSase, we attempted to further assess the inhibitory effects of TA and EGCG on TMEM16F CaPLSase and ion channel activities by fluorescence imaging and electrophysiology. To our surprise, TA and EGCG displayed minimal effects on inhibiting TMEM16F ion channel activity. This prompted us to re-examine the effects of these polyphenols on TMEM16F lipid scramblase activity.

Here, we found that TA and EGCG indeed quenched various fluorophores including those that are tagged to the AnV probes. We further demonstrated that the previous inhibitory effects of TA and EGCG on TMEM16F were mainly due to the fluorescence suppressing effects of the polyphenols on the fluorophores conjugated to the extracellular AnV probes, thus prevent AnV from reporting the scrambling activity. We, therefore, conclude that TA and EGCG do not function as TMEM16 CaPLSase inhibitors. These results also suggest that precautions need to be taken in developing inhibitors targeting TMEM16 CaPLSases.
4.2 Potential pitfalls in identifying inhibitors for TMEM16 CaPLSases: the case of TA and EGCG

4.2.1 Extracellular fluorescently tagged AnV cannot report TMEM16F CaPLSase activity in the presence of TA and EGCG

We applied our established fluorescence imaging-based scrambling assay (Chapter 2) (58, 111) to characterize the effects of TA and EGCG on TMEM16F CaPLSase. As described in the previous Chapter, fluorescently tagged AnV (henceforth referred to as AnV) was included in the extracellular solution to monitor scramblase activity of mTMEM16F stable HEK293 cells. Upon its activation by ionomycin-induced Ca\(^{2+}\) influx, TMEM16F CaPLSase rapidly translocates PS from the inner to outer leaflet of the plasma membrane. Because the externalized PS recruits AnV to the cell surface, the gradual accumulation of fluorescence signal from AnV on the cell membrane is indicative of TMEM16F scrambling activity (Figure 29A, 29B). Consistent with previous reports (103, 167), there was no AnV fluorescence signal on the surface of TMEM16F expressing HEK293 cells when TA (20 µM) and EGCG (20 µM) were present (Figure 29C-29F). On the other hand, robust AnV signal was observed on the cell surface in the control experiment without TA or EGCG (Figure 29A, 29B). Furthermore, Ca\(^{2+}\) influx via ionomycin was not affected by the polyphenols as intracellular Ca\(^{2+}\) was instantly mobilized following Ca\(^{2+}\) ionophore stimulation regardless of the presence of TA or EGCG (Figure 29A-29F). All of these observations imply that the polyphenols could impair
TMEM16F lipid scrambling activity. It is worth noting that 20 µM TA completely abolishes AnV surface signal, whereas 20 µM EGCG still allows weak AnV accumulating on the cell membrane, suggesting that EGCG may have a weaker inhibitory effect than TA (Figure 29C-29F).

Figure 29: TA and EGCG diminish fluorescently tagged AnV-CF640R signal. A, C and E, Representative images show that TA (C) and EGCG (E) treatments do not affect ionomycin-induced intracellular Ca\textsuperscript{2+} influx (labeled by Calbryte (magenta)), but the polyphenols completely suppress AnV-CF640R signal (yellow) on mTMEM16F stable HEK293 cells (green) after 10 min of ionomycin treatment. Scale bars, 25 µm. Without TA and EGCG, the cells have normal AnV signal accumulating on their membrane after ionomycin stimulation (A). B, D and F show the fluorescence intensity changes of Calbryte (magenta traces) and AnV (yellow traces) over 10 min of ionomycin treatment for the cells in A, C and E, respectively. n = 3-5 cells. Error bars indicate SEM. a.u., arbitrary unit.

4.2.2 TA and EGCG do not specifically block TMEM16F ion channel activity

TMEM16F is a dual functional moonlighting protein with both CaPLSase and ion channel activities (27, 28, 52, 57, 58, 63). Several lines of structural and functional evidences
have suggested that in TMEM16F, phospholipids and ions share the same activation gates and Ca$^{2+}$-dependent activation mechanism (28, 52, 58, 63, 90). As Ca$^{2+}$-dependent activation of scrambling activity accompanies ion channel activity (58), we tested the effects of extracellular TA and EGCG on TMEM16F ion channel function using whole cell patch clamp. Surprisingly, high concentration of TA (20 µM) only inhibited about 50% of TMEM16F current and 20 µM EGCG had a negligible effect on TMEM16F current (Figure 30C, 30D, 30G, 30H, 30I and 30J). Neither 1 µM TA nor EGCG affected TMEM16F ion channel function (Figure 30A, 30B, 30E, 30F, 30I and 30J). As TA is a large molecule and previously known to have nonspecific effects on various proteins (123, 174-177), we tested if the inhibitory effect of 20 µM TA on TMEM16F’s current could stem from its nonspecific effects by applying TA to the large conductance, Ca$^{2+}$-activated BK type K$^{+}$ channel, which does not belong to the TMEM16 family. Our outside-out patch clamp recording revealed that extracellular application of 20 µM TA significantly promoted BK channel activation as seen by the left shift in the conductance-voltage (G-V) relationship toward negative voltages and prolonged deactivation kinetics (Figure 31). Taken together, our electrophysiology results demonstrate that EGCG and low concentration of TA do not inhibit TMEM16F ion channel activity. High concentration of TA could partially inhibit TMEM16F ion channel. Given the massive chemical structure of TA (Figure 28), we speculate that its inhibitory effect at high concentration is likely indirect and nonspecific.
Figure 30: Effects of TA and EGCG on TMEM16F ion channel activity. A,C,E and G, Representative whole-cell recordings show the inhibitory effects on TMEM16F ion channel by extracellular application of 1 µM TA (A), 20 µM TA (C), 1 µM EGCG (E), 20 µM EGCG (G). The cells are mTMEM16F stable HEK293 cells. B,D,F and H, Quantifications of TMEM16F’s peak currents in control, 1 µM TA (B), 20 µM TA (D), 1 µM EGCG (F), 20 µM EGCG (H), and after TA/EGCG washout. Numbers in parentheses denote the number of inter-individual recordings. I, The inhibitory effects of 1 and 20 µM TA on TMEM16F current. J, The inhibitory effects of 1 and 20 µM EGCG on TMEM16F current. Un-paired two-tailed student’s t-test: p-values are <0.0001 in (I) and 0.9912 in (J) (n.s.: non-significant). Data are presented as mean ± SEM (error bars).
Figure 31: TA activates BK channels. A, Representative BK currents recorded from HEK293T cells overexpressed BK-α subunit using outside-out configuration. B, Representative BK currents recorded from outside-out patches exposing to 100 µM intracellular Ca\textsuperscript{2+} and 20 µM extracellular TA. C, Mean G-V relations of the BK channels with and without TA. Relative conductance was determined by measuring the amplitude of tail currents 400 µs after repolarization to a fixed membrane potential (~60 mV). Error bars represent SEM (n=5). D, V\textsubscript{1/2} obtained from (C). V\textsubscript{1/2} in the absence and presence of TA are -1.4 ± 7.9 mV and -24.0 ± 1.9 mV, respectively. Student’s t-test was performed and *** represents p<0.001 (n=5). E, τ\textsubscript{off} obtained from single exponential fitting of the tail currents for both control and TA treatment. Student’s t-test was performed and ** represents p<0.01(n=5).

4.2.3 In vitro assay shows that TA and EGCG quench fluorescent signal of PS probes and other dyes

TA and EGCG are known to be able to quench various fluorophores (171, 178-181). Therefore, we suspected that the polyphenols might hinder the ability of the extracellular AnV to correctly report TMEM16F CaPLSase activity through their quenching effects on the fluorophores conjugated to AnV. To test this hypothesis, we measured the
fluorescence signals of the AnV probes tagged with various fluorophores (CF488A, CF594, and CF640R) in the presence or absence of polyphenols in vitro using the spectrometry mode of a SpectraMax plate reader. In the presence of 20 µM TA or EGCG, the fluorescence signal of all AnV probes was significantly attenuated, indicating that the polyphenols indeed quench the PS probes in aqueous solution (Figure 32A-32C). Furthermore, our in vitro experiments also corroborated that TA and EGCG could suppress the fluorescence intensities of various fluorescence probes/dyes, including the Alexa Fluor-conjugated immunoglobulin G (IgG) (Alexa Fluor 488, 594 and 640, Figure 32D-32F), nucleic acid dyes (Hoechst and Nuclear Green DCS1, Figure 32G and 32H), and CF488A conjugated wheat germ agglutinin (WGA) (Figure 32I).
Figure 32: Effects of TA and EGCG on fluorescence signal of different fluorophores and fluorescent probes in aqueous solution. 20 µM EGCG (blue traces) and 20 µM TA (red traces) were applied to various fluorescent probes/fluorophores and the intensities of the emission spectra of the fluorophores were measured by the spectrometry function of a plate reader. Controls (black traces) are fluorescence probes/fluorophores in the absence of TA and EGCG. A, 0.75 µg/ml AnV-CF488A; B, 0.75 µg/ml AnV-CF594; C, 0.75 µg/ml AnV-CF640R; D, 4 µg/ml IgG-Alexa Fluor 488; E, 4 µg/ml IgG-Alexa Fluor 594; F, 4 µg/ml IgG-Alexa Fluor 647; G, 16 µM Hoechst in the presence of 10 µM nucleotide; H, 1:500 Nuclear Green DCS1 in the presence of 10 µM nucleotide; I, 4 mg/ml WGA-CF488A. Data are presented as mean ± SEM (error bars). a.u., arbitrary units.
4.2.4 TA and EGCG quench fluorescence signal of the extracellular PS probes

As the cell-free results evinced that TA and EGCG could quench the AnV probes in aqueous solution, we verified if this was the case on our cell-based assays. First, we allowed AnV to accumulate on the surface of TMEM16F expressing HEK293 cells after ionomycin stimulation (Figure 33A, 33C and 33E, middle rows). Next, we introduced TA and EGCG to the medium after significant amount of AnV had accumulated on the cell surface (2.5 minutes after ionomycin application). AnV fluorescence signal on the cell membrane was instantaneously decreased upon polyphenol applications (Figure 33B, 33D and 33F). 20 µM TA completely abolished AnV signal while 1 µM TA and 20 µM EGCG eliminated more than 80% of AnV signal.
Figure 33: TA and EGCG suppress fluorescence signal of fluorescently tagged AnV instead of inhibiting TMEM16F CaPLSase. A, C and E, Representative images showing 20 µM TA (A), 1 µM TA (C), and 20 µM EGCG (E) immediately deplete fluorescence signal of AnV-CF594 (magenta) of scrambling cells (mTMEM16F stable HEK293 cells; green). Scale bars = 25 µm. B, D and F, Mean fluorescence intensity change of AnV fluorescence over 10-minute of ionomycin treatment for each cell in (A), (C) and (E), respectively. Applications of ionomycin, TA and EGCG are marked by a downward arrow and chemical abbreviations. n = 3-8 cells. Error bars indicate SEM. a.u., arbitrary units.

To further validate the fluorescence quenching effects of TA and EGCG on the AnV probes and verify that loss of AnV signal was not due to defects in TMEM16F functions, we tested if the polyphenols also quenched the fluorescence signal of the AnV probes binding to apoptotic cells, which also have PS-exposed cell surface but are independent of TMEM16F-CaPLSase (19). Staurosporine (STS) was used to induce apoptosis, which resulted in PS being exposed to the cell surface through caspase-
dependent phospholipid scramblases (105), as evidenced by AnV signal on cell membrane and strong cytosolic caspase dye (TF3-DEVD-FMK) staining (Figure 34, before treatment). Application of TA and EGCG immediately abolished the AnV signal on the apoptotic cell surface (Figure 34C-34H), suggesting that the polyphenols indeed quenched the fluorophores of the AnV probes. Interestingly, the intensity of the intracellular caspase dye was also instantly reduced upon TA and EGCG addition, suggesting that the compromised membrane, as a result of apoptosis, allowed TA and EGCG to leak into the cytosol and partially quench the caspase dye (Figure 34C-34H). Based on the above observations, we conclude that TA and EGCG can rapidly quench the extracellular fluorescence signal from fluorophore-conjugated AnV.

Figure 34: TA and EGCG instantaneously suppress fluorescence signal of AnV-CF640R binding to apoptotic cells. A, C, E and G, Representative images show the effects of control (A), 20 µM TA (C), 1 µM TA (E), and 20 µM EGCG (G) on AnV-CF640R signal (yellow) binding to the membrane surface of STS-induced apoptotic cells, which are

![Figure 34](image-url)
labeled by caspase 3/7 dye (magenta). Scale bars, 25 µm. B, D, F and H, Plots show the quantification of AnV and caspase dye fluorescence intensity before and after treatments of control (B), 20 µM TA (D), 1 µM TA (F) and 20 µM EGCG (H) of each cell in A, C, E and G, respectively.

Additionally, to further corroborate that TA and EGCG treatments have minimal effects on TMEM16F scrambling activity, we first treated the mTMEM16F stable HEK293 cells with ionomycin and then re-probed the stimulated cells with AnV-CF594. Prior to ionomycin treatment, the cells were stained with AnV-CF594 to verify that the cell surface was PS-negative (Figure 35, left). The cells were first treated with either ionomycin alone or ionomycin in combination with TA or EGCG. After 10-min treatment and extensive washes to eliminate ionomycin, TA, and EGCG, the cells were re-probed with AnV-CF594 to observe surface-exposed PS. We found that the polyphenol-treated cells did not show obvious inhibitory effect on AnV-CF594 binding to the cell surface (Figure 35, right). This observation is consistent with the lack of polyphenol inhibitory effect on TMEM16F current (Figure 30). As TMEM16F is a moonlighting protein with both CaPLSase and ion channel activities (27, 28, 52, 57, 58, 63), and its activation leads to simultaneous phospholipid and ion permeation (58), our electrophysiology and fluorescence lipid scrambling results explicitly demonstrate that TA and EGCG do not inhibit TMEM16F.
Figure 35: TA and EGCG do not inhibit TMEM16F scrambling activity. A, B, and C, Representative images demonstrate AnV-CF594 binding of mTMEM16F stable HEK293 cells before and after treatment with either 5 µM ionomycin (iono) alone (A), 5 µM iono with 1 µM TA (B), or 5 µM iono with 20 µM EGCG (C). After incubating the cells in each treatment for 10-minute, the cells were washed rigorously with Hanks’ Balanced Salt Solution (HBSS) before reapplying AnV-CF594 to stain the scrambling cells.

4.3 Discussion

In this chapter, we carefully examined the potential inhibitory effects of TA and EGCG on TMEM16F. Through a series of experiments, including patch clamp, cell-free and cell-based fluorescence assays, we failed to find evidence to support that the
Polyphenols would be specific inhibitors for TMEM16F. Instead, these polyphenols quench the fluorophores of the AnV probes, as well as other fluorescence dyes/probes. The quenching artifacts prevent the AnV probes from reporting TMEM16F-mediated PS exposure on cell surface, thereby gives the impression that TA and EGCG ‘inhibit’ TMEM16F activation.

Polyphenols TA and EGCG have been reported as potent inhibitors of TMEM16F scramblase (103, 167). Due to their well-known beneficial effects on health (168-171), TA and EGCG are seemingly promising candidates to treat TMEM16F scramblase-related diseases such as stroke and heart attack. They also have been increasingly used as pharmacological tools to understand the biology of TMEM16 scramblases over the past several years (182-184). Our current study casts cautions on utilizing these polyphenols as research and therapeutic tools to study and target TMEM16 CaPLSases. Furthermore, precautions need to be taken when developing inhibitors for TMEM16 CaPLSases using fluorescence-based assays, which are commonly used in the field. Given the dual functionality of TMEM16F as both phospholipid scramblase and ion channel, we recommend combining fluorescence imaging-based scrambling assays with electrophysiology when trying to identify bona fide inhibitors for TMEM16 scramblases.

It is worth noting that higher concentration of TA can exert non-specific effects on TMEM16F as evidenced by the partial inhibitory effect on TMEM16F current (Figure 30I)
and stimulating effect on different types of ion channels, such as BK channel shown in this study (Figure 31) or TRPM4, TRPC4 and TRPC5 channels in a previous report (177). Therefore, the non-specific nature of TA prevents it from serving as an ideal pharmacological tool for mechanistic studies of TMEM16F.

4.4 Experimental procedures

4.4.1 Cell culture and transfection

Cell lines used in this chapter are mTMEM16F-stable HEK293 (a generous gift from Dr. Min Li) and WT HEK293T (obtained from Duke Cell Culture Facility). Details about the cell lines as well as their culture procedure were described in subsections 2.4.1.

Plasmid used in this chapter is pCDNA3 vector carries encoding sequences of mouse BK channel alpha subunit (Addgene #113566) (185). Please refer to subsection 2.4.2 for transient transfection protocol.

4.4.2 Pharmacological reagents

Benzbromarone, Ti6Ainh-A01, CaCCinh-A01, MONNA, CdCl₂, 2APB, Ru-red, SKF96365, R5421, menthol, orthovanadate, ellagic acid, (-)-epigallocatechin, wortmannin, ibedenone, imatinib, ibrutinib, cytosine β-D-arabinofuranoside, PFBS, salicylic acid, STS, TA and EGCG were dissolved in either DMSO or ultrapure water. Stock solutions of these molecules were prepared, aliquoted and stored at appropriate temperature until used to avoid repeated freeze-thaw cycles. Only fresh aliquots of molecules were used in each
experiment. Working concentrations of each molecule were established by further diluting the stock solution in the indicated solution of each experiment.

4.4.3 Fluorescence microscopy assays

Murine-TMEM16F-stable HEK293 cells were seeded on PLL coated No.0 coverslips. Extracellular solution in all cell-based imaging assays contains 0.5 µg ml\(^{-1}\) fluorescently tagged AnV, 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl\(_2\), pH 7.4, unless stated otherwise. This extracellular solution is referred to as imaging solution. All experiments were independently repeated at least three times.

4.4.3.1 Phospholipid scrambling assay with pharmacological reagents

Microscope-based scrambling assay is proceeded as detailed in subsection 2.4.3.1, except the following modification: in drug screening for TMEM16F-CaPLSase, coverslips containing mTMEM16F-stable HEK293 cells were inserted into the imaging chamber containing a volume of 0.5 µg mL\(^{-1}\) AnV-CF 594 with an appropriate concentration of each testing molecule or drug. Cells were briefly incubated with each molecule for about 5 minutes. Then, an equal volume of the same solution containing 10 µM ionomycin was added into the chamber to reach the final concentration of 5 µM ionomycin to activate TMEM16F.
4.4.3.2 Ca\textsuperscript{2+} and phospholipid scrambling co-imaging assay

2mM stock solution of Ca\textsuperscript{2+} indicator Calbryte\textsuperscript{TM} 590 AM (AAT Bioquest, #20701) was prepared by using DMSO and stored at -20\degree C. The stock solution was diluted to working concentration of 1 \mu M by using HBSS (Gibco, #14025-092). The mTMEM16F stable HEK293 cells were incubated with 1 \mu M Calbryte\textsuperscript{TM} 590 AM for 20-30 minutes in the incubator supplied with 5\% CO\textsubscript{2} at 37\degree C. After washing the cells with HBSS, the coverslips were mounted to our customized imaging chamber containing the imaging solution that has AnV-CF 640R and proceeded with our scrambling assay as described in section 6.4.1 in the presence of 5 \mu M ionomycin. In TA or EGCG treatment, 20 \mu M of either TA or EGCG was added together with ionomycin into the imaging chamber. Changes in fluorescence intensity of Calbryte and AnV-CF640R were recorded using time-lapse imaging (5-s intervals) with a x63/1.4 NA Oil Plan-Apochromat DIC of Zeiss 780 inverted Confocal microscope. Results were analyzed using ImageJ, Zeiss, MATLAB, Prism (GraphPad) and Excel (Microsoft).

4.4.3.3 Cell-based AnV quenching assay with TA and EGCG

mTMEM16F stable HEK293 cells were stimulated with 5\mu M ionomycin and the scrambling activity was monitored using AnV-CF 594 for 2-3 minutes. After that, either 1 \mu M TA, 20 \mu M TA or 20 \mu M EGCG was added into the imaging chamber. Changes in the fluorescence intensity of AnV-CF594 was recorded using time-lapse imaging with 5-s
intervals. This experiment was executed using a x63/1.4 NA Oil Plan-Apochromat DIC in Zeiss 780 inverted Confocal microscope. Results were analyzed using ImageJ, Zeiss, MATLAB, Prism (GraphPad) and Excel (Microsoft).

4.4.3.4 Reprobing the scrambling cells with fluorescently tagged AnV after TA or EGCG treatment

mTMEM16F stable HEK293 cells were seeded on PLL coated #0 coverslips for 48-hour. The cells were first stained and imaged with 0.5 µg ml⁻¹ AnV-CF594 prior to any treatments. The coverslips were then transferred to a chamber containing either 5 µM ionomycin alone, 5 µM ionomycin with 1 µM TA, or 5 µM ionomycin with 20 µM EGCG. After 10 minutes, the ionomycin solutions with or without TA or EGCG in the chamber were removed, and the cells were washed rigorously with large volume of HBSS. After washing, the cells were briefly incubated with AnV-CF594 solution for 3-5 minutes. AnV-CF594 signal on the cell membrane was then captured by a Prime 95B Scientific CMOS Camera (Photometrics) connected to an Olympus IX71 inverted epi-fluorescent microscope (Olympus IX73). A x40 objective with NA of 0.75 was used for imaging. The image acquisition was managed by MetaFluor software (Molecular Devices). At least 3 fields of view were imaged per sample. The results were analyzed by using ImageJ.

4.4.3.5 STS induced apoptosis and active caspase 3/7 staining

STS was diluted in culture medium and applied to the cells at the final concentration of 10 µM. The cells were incubated with STS for 4-hour. Next, medium
containing STS was replaced with fresh medium containing 1x TF3-DEVD-FMK (caspase 3/7 dye) from Live cell Caspase 3/7 binding assay kit (AAT Bioquest, # 20101). The detailed staining procedure was described in subsection 2.4.3.3. Only cells that were positive with TF3-DEVD-FMK and AnV-CF640R were focused for imaging. The control, TA or EGCG solutions at appropriate concentrations were added to the apoptotic cells. Fluorescence changes of AnV-CF640R and caspase 3/7 dye were captured with confocal time-lapse imaging at 1-s interval. This experiment was executed using a x63/1.4 NA Oil Plan-Apochromat DIC in Zeiss 780 inverted Confocal microscope. The results were analyzed using ImageJ, Zeiss, Origin, Excel (Microsoft) and MATLAB.

4.4.4 In vitro fluorescence quenching assay using TA and EGCG

To examine the effects of TA and EGCG on different fluorophores, aqueous solution of 0.75 µg/ml fluorescently tagged AnV (either CF488A, CF594 or CF640R) (Biotium), 4 µg/ml IgG (Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647) (ThermoFisher Sci.), 16 µM Hoechst (Life Technologies), 1:500 Nuclear Green DCS1 (AAT Bioquest), and 4 mg/ml WGA-CF488A (Biotium) were prepared using phosphate-buffered saline (PBS). Hoechst and Nuclear Green DCS1 solutions were supplemented with 10 µM nucleotide to boost the fluorescence signals of these two nucleic dyes. TA and EGCG were added into each of these fluorophores at the final concentration of 20 µM. After mixing using pipette, 100 µL of each of these fluorophores containing 20 µM TA or
EGCG were added to a clear bottom 96-well plate. The emissions of the fluorophores were measured using the spectrometry mode of a Spectramax M5 plate reader (Molecular Devices) with a 5 nm increment. The same set of fluorophore solutions without TA and EGCG was also examined as controls. Each sample was done in three replicates. The results were analyzed using Prism (GraphPad) and Excel (Microsoft).

4.4.5 Electrophysiology

Voltage-clamp recordings were low-pass filtered at 5 kHz (Axopatch 200B) and digitally sampled at 10 kHz (Axon Digidata 1550A) and digitized by Clampex 10 (Molecular Devices, Molecular Devices, CA, USA). Electrodes were pulled from borosilicate capillaries (Sutter Instruments) and had initial resistances of 2–5 MΩ. Pipette electrodes were made from borosilicate capillaries (Sutter Instruments) and fire-polished with a microforge (Narishige). All experiments were performed at room temperature.

TA and EGCG were diluted into bath buffer at desired concentrations using aqueous stock solutions of 20 mM and 10 mM, respectively. External application of TA or EGCG was performed via local focal perfusion using a pressurized perfusion apparatus (ALA-VM8, ALA Scientific Instruments). For whole-cell recording, the perfusion outlet was positioned close to the patched cell before forming whole-cell configuration for recordings. For outside-out recording, the patches were moved next to the perfusion outlet before recording.
Whole-cell TMEM16F channel recordings were performed on the mTMEM16F stable HEK293 cells. The cells were trypsinized and plated on PLL-coated coverslips 1-2 hours before electrophysiology. Bath solution contains: 140 mM NaCl, 10 mM HEPES, 2 mM MgCl₂, and pH 7.3 (adjusted with NaOH). Pipette solution contains: 140 mM CsCl, 10 mM HEPES, 1 mM CaCl₂, and pH 7.3 (adjusted with NaOH). Upon formation of whole-cell configuration, TMEM16F channels were activated by a current-voltage (I-V) protocol in which the membrane was held at -60 mV and test voltage steps from -100 mV to +140 mV. Typically, with the presence of 1 mM intracellular Ca²⁺, considerable TMEM16F currents were observed within 30-60 seconds after breaking in. Following acquisition of a control I-V recording, TA or EGCG at desired concentrations was focally perfused to the cell for 2-3 seconds before recording acquisition. Next, the cell was followed by focal perfusion of a control bath solution to wash off TA or EGCG for 3-4 seconds before another recording acquisition.

To test TA and EGCG effects on BK channels, outside-out configuration was used. The pipette solution contains 140 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 0.1 mM free Ca²⁺, pH=7.3 (adjusted with KOH). Extracellular solution contains: 140 mM KCl, 10 mM HEPES, 1 mM MgCl₂, pH=7.3 (adjusted with KOH). The smooth curves represent Boltzmann fits $G/G_{\text{max}}=1/(1+\exp(-ze(V-V_{1/2}))/kT)$. $G_{\text{max}}$ represents tail current amplitude in response to depolarization to +200 mV; z is the number of equivalent gating charges; V is
the membrane voltage; $V_{1/2}$ is the half-activation voltage; $F$ is the Faraday’s constant (96485 C mol$^{-1}$), $R$ is the gas constant (8.314 J mol$^{-1}$), and $T$ is the absolute temperature (298.15 K at 25 °C).

### 4.4.6 Data analysis

Statistical analysis was performed as described in subsection 2.4.6. All fluorescence imaging data analysis was performed in Zeiss, MetaFluor, ImageJ, MATLAB, Microsoft Excel, Origin and GraphPad Prism. All electrophysiology data analysis was performed in Clampfit, Microsoft Excel, and GraphPad Prism.
Chapter 5: Discovery of Drosophila Subdued as a novel moonlighting TMEM16 member

Portions of this chapter are reproduced and adapted from a research originally published in the Journal of Biological Chemistry.(1)

Some data in this chapter were done in collaboration with Son C. Le from the Yang Lab.(2)

5.1 Background

The ground-breaking discoveries of TMEM16A and TMEM16B as the long-sought CaCCs (21, 23, 26) advanced the understanding of a novel membrane protein superfamily that includes the TMEM16 family and its closely related OSCA, TMEM63 and TMC membrane protein families (186, 187). TMEM16 proteins have been found in fungi (38, 44), amoeboids (188), insects (189) and vertebrates (15, 35, 49). The unexpected findings of mammalian TMEM16F as a moonlighting protein (27, 28) expanded our understanding of the enigmatic TMEM16 family (15, 33, 35, 49, 50). Serving as a bona fide CaPLSase (27) and a SCAN channel (28), TMEM16F has evolved the capability to passively transport

1 Portions of this chapter are reproduced and adapted from: Le, T., Le, S. C., and Yang, H. Drosophila Subdued is a moonlighting transmembrane protein 16 (TMEM16) that transports ions and phospholipids. J Biol Chem. 2019; 294:4529-4537. © 2019 Le et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.
2 Son C. Le performed the electrophysiology experiments for TMEM16F, TMEM16A and Subdued.
phospholipids and ions, two structurally distinct classes of permeants, down their chemical gradients.

Recent structural and functional studies elegantly reveal that the fungal nhTMEM16, afTMEM16, mammalian TMEM16D-K, except TMEM16G and TMEM16H, are also moonlighting proteins with CaPLSase and ion channel activities (34-48). Interestingly, the mammalian TMEM16A and TMEM16B CaCCs only displayed ion channel activities (35, 39, 58), while an amoebozoa TMEM16 homolog from Dictyostelium discoideum only showed CaPLSase activity when heterologously expressed in HEK293 cells (188). In order to understand the biological functions of TMEM16 moonlighting, there is an urgent need to have an in-depth understanding of TMEM16 evolution and function in different kingdoms ranging from Protozoa, Fungi to Animalia.

TMEM16 moonlighting proteins have not been identified thus far in insects, despite a recent study that clearly demonstrated the physiological importance of CaPLSase in the degeneration of Drosophila sensory neurons (190). However, the molecular identity of the Drosophila CaPLSase responsible for the observed scramblase activities remains elusive. Among the five Drosophila TMEM16 homologs, Subdued is the only protein that has been thoroughly characterized using electrophysiological tools (189). When heterologously expressed in HEK293T cells, whole-cell patch clamp recordings suggested that Subdued was a CaCC. Interestingly, Subdued-deficient Drosophila
exhibited severe defects in host defense when challenged with the pathogenic bacterium *Serratia marcescens* (189). It remains, however, unclear how Subdued CaCC function is involved in *Drosophila*'s immunity.

Combining the fluorescence microscopy-based scrambling assay with inside-out patch clamping technique, we have discovered that Subdued is also a moonlighting TMEM16 protein in *Drosophila*. Notably, we have also found that Subdued harbors biophysical features that strikingly resemble those of the mammalian TMEM16F, which has been unambiguously shown to function as a CaPLSase and a SCAN channel (27, 28). Our results thus support the notion that TMEM16 moonlighting could be an ancient feature of the TMEM16 family, which is conserved in fungi, insects and vertebrates. We hope our findings can provide new insights into understanding the evolution of the TMEM16 family, the molecular mechanisms of their ion and phospholipid permeations, as well as TMEM16 physiological functions in *Drosophila*.

### 5.2 Results

**5.2.1 Subdued ion channel differs from TMEM16A-CaCC in Ca\(^{2+}\)- and voltage-dependence**

Consistent with a previous report using whole-cell patch clamp recording (189), our inside-out patch clamp characterization of Subdued expression in HEK293T cells also robustly elicited Ca\(^{2+}\)- and voltage-dependent current (Figure 36B, 36C). Similar to TMEM16A-CaCC and TMEM16F-SCAN (Figure 37), Subdued channel opening requires
the synergistic action of both intracellular Ca\textsuperscript{2+} and membrane depolarization. However, one hallmark of TMEM16A-CaCC is that it adopts two distinct modes of Ca\textsuperscript{2+}-dependent activation (Figure 37A, 37B). On the one hand, under sub-micromolar intracellular Ca\textsuperscript{2+} level (e.g. 0.39 µM), TMEM16A-CaCC is partially opened and exhibits time- and voltage-dependent activation and deactivation kinetics. On the other hand, when exposed to saturating Ca\textsuperscript{2+} level (e.g. 100 µM), TMEM16A-CaCC becomes constitutively open to give rise to a linear current-voltage (I-V) relationship (Figure 37A, 37B). However, this fingerprint feature was not observed in Subdued (Figure 36B, 36C) (189), as well as TMEM16F-SCAN (Figure 37C, 37D). Without depolarization, Subdued and TMEM16F channels could barely open even in the presence of 100 µM Ca\textsuperscript{2+} (Figure 36B, 36C, 37C, 37D). In addition, both Subdued and TMEM16F channels are less sensitive to Ca\textsuperscript{2+} than TMEM16A-CaCC, as evidenced by the rightward shift of their Ca\textsuperscript{2+} dose response curves compared to TMEM16A-CaCC (Figure 36D, 36E), in addition to minimal activation under 0.39 µM Ca\textsuperscript{2+} (Figure 36B, 36C, 37C, 37D). Therefore, Subdued’s channel activation bears more similarities with TMEM16F-SCAN than with TMEM16A-CaCC.
Figure 36: Subdued encodes a Ca\textsuperscript{2+}- and voltage-activated ion channel that is different from TMEM16A-CaCC. A, Phylogenic tree shows the evolutionary relationship between Subdued and other well-characterized members of the TMEM16 family. B, Subdued current traces elicited by different voltages and various intracellular Ca\textsuperscript{2+} concentrations from inside-out patch recordings. Voltage protocol used was shown on top left. C, Current-voltage (I-V) relationship of Subdued current measured at 0, 0.39 µM and 100 µM Ca\textsuperscript{2+}. D, Ca\textsuperscript{2+} dose-response curves of mTMEM1A, mTMEM16F and Subdued. E, Half-maximal activation concentrations of Ca\textsuperscript{2+} (EC\textsubscript{50}) of mTMEM16A, mTMEM16F and Subdued channels. One-way ANOVA with Tukey’s multiple comparisons test: in E, p-values are <0.0001 (TMEM16A vs. TMEM16F), 0.0001 (TMEM16A vs. Subdued), 0.0181 (TMEM16F vs. Subdued). Error bars indicate SEM.
Figure 37: Voltage and Ca\textsuperscript{2+}-dependent activation of mTMEM16A-CaCC and mTMEM16F-SCAN. A, C, Current-voltage (I-V) recordings of mTMEM16A (A) and mTMEM16F (C) at different intracellular Ca\textsuperscript{2+} concentrations from inside-out patch recordings. Voltage protocol used was shown on top left. B, D, I-V curves of mTMEM16A (B) and mTMEM16F (D) measured at 0, 0.39 µM and 100 µM Ca\textsuperscript{2+}. Error bars indicate SEM.

5.2.2 Biophysical properties of Subdued is similar to that of TMEM16F

To further characterize Subdued channel, we measured its ion selectivity using inside-out patches by switching intracellular NaCl concentration from symmetric 140 mM to asymmetric 14 mM. The ten-fold change of ion gradients would result in a large shift of the reversible potentials. For TMEM16A-CaCC, we observed a -37.6±1.0 mV leftward shift of reversible potential, which was close to the predicted -58 mV shift for a strict Cl\textsuperscript{-} permeable channel (Figure 38A, 38B). The small Na\textsuperscript{+} permeability (Na\textsuperscript{+}/Cl\textsuperscript{-} permeability
ratio $P_{Na}/P_{Cl}$ of 0.14±0.05) of TMEM16A-CaCC (Figure 38C) is consistent with previous reports (21, 23, 26, 28, 58). On the contrary, we observed a +29.1±5.8 mV rightward shift for Subdued when switching intracellular NaCl from 140 mM to 14 mM in the presence of 100 µM intracellular Ca$^{2+}$ (Figure 38A, 38B). The shift is strikingly similar to the reversible potential change for TMEM16F-SCAN (+30.2±0.84 mV) (28). The $P_{Na}/P_{Cl}$ ratios of Subdued and TMEM16F are 5.83±0.42 and 4.79±0.24, respectively (Figure 38C). Our inside-out patch clamp recordings thus demonstrate that Subdued is not a CaCC. Instead, similar to TMEM16F-SCAN, it has higher permeability towards cations than anions in 100 µM intracellular Ca$^{2+}$. 
Figure 38: Subdued is a non-selective ion channel with higher cation permeability. A, Measurements of the reversal potentials ($E_{\text{rev}}$) for mTMEM16A, mTMEM16F and Subdued. Blue traces denote currents at symmetric 140 mM NaCl. Red traces denote currents upon switching to an intracellular solution with low 14 mM NaCl. B, Changes in the reversal potential ($\Delta E_{\text{rev}}$) of mTMEM16A, mTMEM16F and Subdued. C, Permeability ratio $P_{\text{Na}}/P_{\text{Cl}}$ mTMEM16A, mTMEM16F and Subdued calculated based on $\Delta E_{\text{rev}}$ in (B) using the GHK equation. One-way ANOVA with Tukey’s multiple comparisons tests: in B, p-values are <0.0001 (TMEM16A vs. TMEM16F), <0.0001 (TMEM16A vs. Subdued), 0.8393 (TMEM16F vs. Subdued); in C, p-values are <0.0001 (TMEM16A vs. TMEM16F), <0.0001 (TMEM16A vs. Subdued), 0.1792 (TMEM16F vs. Subdued). Error bars indicate SEM. n.s. denotes non-significant.

5.2.3 Subdued is a CaPLSase

With the optimized CaPLSase assay (Figure 5, 8), we tested whether Subdued could serve as a CaPLSase. C-terminally eGFP-tagged Subdued was readily expressed in
the TMEM16F-KO HEK293T cells following 24 hours of transfection (Figure 39B). Treatment with 5 µM ionomycin induced rapid and time-dependent accumulation of AnV fluorescent signal on the surface of Subdued-expressing cells (Figure 39B, 39C). The $t_{1/2(I_{\text{max}})}$ value of Subdued is 313±12 seconds, which is slightly shorter than that of TMEM16F (378±10 seconds) (Figure 39D). Our results on the heterologous expression of Subdued in TMEM16F-KO HEK293T cells thus suggest that Subdued can function as a CaPLSase.
Figure 39: Subdued expression induces CaPLSase activity. A, B, Exogenous expression of mTMEM16F (A) and Subdued (B) in TMEM16F-KO HEK293T (16F-KO 293T) cells induced strong CaPLSase activity. The C-terminal eGFP tagged (green) was used to identify TMEM16F- or Subdued-expressing cells. The AnV-CF594 signal indicates phospholipid scrambling. C, Representative AnV fluorescent intensity changes overtime and its extracted $t_{1/2(I_{\text{max}})}$ values for WT TMEM16F and Subdued expressing cells in (A) and (B). TMEM16F-KO HEK293T cells lacking CaPLSase activity served as a negative control. a.u., arbitrary units. D, Ionomycin-induced CaPLSase activity for WT TMEM16F and Subdued as quantified by their $t_{1/2(I_{\text{max}})}$. Each data point represents one single cell and n
denotes the total number of expressing cells analyzed. The pie charts represent the percentages of the WT TMEM16F- and Subdued- expressing cells that scrambled after ionomycin application. Statistical analysis was performed using unpaired two-sided t-test. ***p-value = 0.0002. Error bars indicate SEM.

To further validate Subdued as a bona fide CaPLSase, we mutated two well-characterized and conserved residues. First, the residue corresponding to Subdued-E716 has been proposed to serve as an extracellular entrance controlling phospholipid permeation through nhTMEM16-CaPLSase (43). Mutating this residue in fungal nhTMEM16- and TMEM16F- CaPLSases abolished phospholipid scrambling (36, 40, 88). Consistent with the importance of this residue in controlling phospholipid permeation, Subdued-E716A also abrogated ionomycin-induced phospholipid scrambling and PS externalization (Figure 40A, 40B). Second, we characterized Subdued-D824, which is equivalent to D703 in TMEM16F and D734 in TMEM16A, a highly conserved Ca\(^{2+}\) binding Aspartate. Introducing a positively charged mutation to this residue likely disrupted Ca\(^{2+}\) binding as evidenced by the lack of TMEM16A channel activation (120) and absence of TMEM16F CaPLSase activity (Figure 8) upon Ca\(^{2+}\) activation. Consistent with its importance in Ca\(^{2+}\) binding, a majority of the Subdued-D824R-expressing cells exhibited no detectable PS externalization (Figure 40A, 40B). Only a small population (5 out of 47) of Subdued-D824R expressing cells displayed weak AnV surface binding towards the end of our 10-minute recording with significantly prolonged t\(_{1/2(\text{max})}\) values (Figure 40B). The residual CaPLSase activity from this small percentage of cells may reflect residual
activities from Subdued-D824R mutant or emerging apoptosis-induced phospholipid scrambling in these cells. In summary, the mutagenesis studies using our optimized CaPLSase assay demonstrate that Subdued is a bona fide CaPLSase.

![Figure 40: Mutations of Subdued alter its CaPLSase activity.](image)

**A**
- Representative images of ionomycin-induced scrambling activity of TMEM16F-KO HEK293T cells transiently transfected with plasmids encoding eGFP-tagged Subdued mutations, E716A and D824R. Expression was detected by the C-terminus tagged eGFP signal (green). The AnV-CF594 signal represents lipid scrambling.

**B**
- Ionomycin-induced CaPLSase activity for WT and mutant Subdued as quantified by their t_{1/2(Imax)}. Each data point represents one single cell and n denotes the total number of expressing cells that were analyzed. The pie charts represent the percentages of the WT and mutant Subdued-expressing cells that scrambled lipids after ionomycin application. Error bars indicate SEM. non. scr denotes non-scrambling.

### 5.3 Discussion

Protein moonlighting as both ion channels and phospholipid scramblases has been observed in mammalian and fungal TMEM16 proteins (27, 28, 34-42, 44, 45, 52, 63). By using patch clamp electrophysiology and an improved phospholipid scrambling assay,
our studies reveal that *Drosophila* Subdued, an insect TMEM16, is also a moonlighting protein that can serve as both a CAN channel and a CaPLSase.

By expressing Subdued into our TMEM16F KO HEK293T cell line, we observed robust Subdued-mediated CaPLSase activity (Figure 39B-39D). Disrupting a key conserved residue at the extracellular entrance of Subdued abolished its CaPLSase activity. In addition, when one of the conserved Ca$^{2+}$-binding residues was replaced with a positively charged Arg residue, the mutant Subdued failed to scramble phospholipids (Figure 40). Collectively, our data show that Subdued is a bona fide CaPLSase.

Our inside-out patch clamp recordings demonstrate that Subdued is a CAN channel with higher cation permeability than chloride ($P_{Na}/P_{Cl} = 5.83$) in 100 µM intracellular Ca$^{2+}$. This conclusion stands in stark contrast to a previous study, which reported that Subdued functioned as a CaCC ($P_{Na}/P_{Cl} = 0.16$) based on whole-cell patch recordings (189). We postulate that this discrepancy might be derived from the inherent differences between the two patch clamp configurations. First, infusion of pipette solution with high micromolar Ca$^{2+}$ into cytosol could disrupt intracellular environment, which might subsequently alter channel activity. In the case of Subdued, channel current run-up was observed in whole-cell recording (189). When whole-cell recording was used to measure TMEM16F current, a 5 to 15-minute delay of channel activation has been frequently observed after membrane break-in (58). Under inside-out configuration, both
Subdued (Figure 36, 38) and TMEM16F current (28) can be immediately recorded after membrane excision. Without the long delay to obtain stable current, the reversal potential measured using inside-out configuration may reflect the intrinsic channel selectivity. Second, whole-cell patch clamp may suffer from larger leak current during recording, especially when infusing with high micromolar Ca\(^{2+}\) into the cytosol. The potential leak current could confound the reversible potential measurement. Third, measuring the reversal potential requires exchanging solutions with drastically different ionic concentrations. Whole-cell recording usually requires whole-chamber solution exchange, which can induce large liquid junction potential to complicate reversal potential measurement. In our inside-out patch clamp experiments, we used a pressurized focal perfusion system to achieve rapid solution exchange directly to the excised patch membrane. As this process is fast and only requires a small volume of solution, the impact of liquid junction potential is negligible.

We also find that Subdued ion permeability resembles that of the mammalian TMEM16F-SCAN. Similar to TMEM16F-SCAN (28), common CaCC blockers such as NFA, flufenamic acid (FFA) and NPPB could not block Subdued current (189), further supporting that Subdued channel is different from TMEM16A-CaCC. Interestingly, the fungal afTMEM16 and nhTMEM16 channels also exhibit substantial cation permeability (36-38). The non-selective nature of the moonlighting TMEM16 proteins towards
phospholipids and ions suggests that their ancestors may have experienced low selective pressure during evolution, so that they could mediate simultaneous permeation of different ions and phospholipids without expanding the genome size. Interestingly, TMEM16A and TMEM16B are more selective to anions and lack CaPLSase function (15, 20). We hope our current findings can shine new lights on understanding TMEM16 evolution and molecular mechanisms for substrate selectivity.

Our study also provides new insights into understanding the physiological functions of TMEM16 moonlighting proteins. Previous studies have shown that Subdued knocked-out or knocked-down Drosophila strains harbored defects in their host defense and exhibited lethality upon ingestion of the pathogenic bacteria *S. marcescens* (189, 191). It is thus far not clear whether Subdued’s CaPLSase function and/or ion channel function play a major role in participating host defense. Interestingly, the moonlighting protein TMEM16F has also been reported to express in immune cells and play an important role in immune responses (84, 184, 192). Considering the fact that channel activation of both Subdued and TMEM16F SCAN current (28) requires both high Ca\(^{2+}\) and membrane depolarization (Figure 36C, 37D), both of which conditions are unlikely to be achieved under physiological conditions, it is likely that their CaPLSase functions might play the major role in immunity. A recent study suggested that PS externalization induced by overexpressing mammalian TMEM16F-CaPLSase played an important role in controlling
neurite degeneration in *Drosophila* sensory neurons (190). Our current finding that Subdued as a bona fide CaPLSase might help identify the CaPLSase that is responsible for *Drosophila* neuronal degeneration.

### 5.4 Experimental procedures

#### 5.4.1 Cell culture and transfection

Cell lines used in this chapter are WT HEK293T (obtained from Duke Cell Culture Facility), Cas9 control and TMEM16F-KO HEK293T cells. Details about the cell lines as well as their culture procedure were described in subsection 2.4.1.

Plasmids used in this chapter includes pEGFP-N1 vector carries cDNAs of either mTMEM16F (Open Biosystems cDNA # 6409332) (28), mTMEM16A (Open Biosystems cDNA # 30547439) (92, 120, 140), Subdued (FlyBase ID# FBcl0155770) (189), or their point mutations with C-terminal eGFP tag. Point mutations of mTMEM16F and Subdued were carried out and validated as described in subsection 2.4.2. Primers used in generating mTMEM16F and Subdued mutations are listed in table 2 and table 4 (Appendix A), respectively. Please also refer to subsection 2.4.2 for transient transfection protocol.

#### 5.4.2 Microscope-based phospholipid scrambling assay and its quantification

The assay and data analysis were detailed in subsections 2.4.3.1 and 2.4.3.2, respectively.
5.4.3 Electrophysiology

The inside-out configuration was employed to examine ion channel properties of mTMEM16A, mTMEM16F and Subdued in this chapter. The procedure and its data analysis are same as those described in subsection 3.4.6, except the followings:

For the current-voltage (I-V) relationship recordings, the membrane was held at -60mV (or -80mV), and 250ms-long voltage steps ranging from -100mV to +140mV with a 20mV increment were applied. Currents were elicited by perfusion of different intracellular solutions of desired free Ca\(^{2+}\).

For Ca\(^{2+}\) concentration-dependent recordings of TMEM16A, the membrane was constantly held at +60mV, and the dose-dependent currents were elicited by perfusion of intracellular solutions with various free Ca\(^{2+}\) concentrations. Quasi-steady state current amplitudes were measured for each Ca\(^{2+}\) puff and normalized to the peak current elicited by 100 µM Ca\(^{2+}\). For TMEM16F and Subdued, a repeated voltage step protocol (-80mV and +80mV, holding at 0mV) was used to avoid rapid rundown of channel activity. Different Ca\(^{2+}\)-containing solutions were perfused to obtain currents for each voltage step. Peak currents at +80mV were used to construct the Ca\(^{2+}\)-dose response curves.

For reversal potential (E\(_{\text{rev}}\)) measurements, inside-out configuration was used for rapid exchange of the intracellular solutions. First, the channels were activated in which the pipette solution and perfusion solution (symmetric 140 mM Cl\(^{-}\)) both contained: 140
mM NaCl, 5 mM EGTA, 10 mM HEPES, adjusted to pH 7.3 (NaOH) and osmolarity of ~300 (D-mannitol). The perfusion solution also contained 100 µM Ca$^{2+}$. For TMEM16A, inward and outward currents were elicited by a repeated ramp protocol from -120mV to +120mV, or -100mV to +100mV. For TMEM16F and Subdued, due to the stringent requirement for depolarization during activation, an inverted V-shaped ramp protocol in which the membrane was ramped from -120mV to +120mV and back to -120mV. The second ramp phase (+120mV to -120mV) was used to construct the I-V plots used for $E_{rev}$ measurements of TMEM16F and Subdued. Changes in $E_{rev}$ were triggered by replacing intracellular solution with a solution (low 14mM Cl$^-$) containing: 14 mM NaCl, 5 mM EGTA, 10 mM HEPES, adjusted to pH 7.3 (NaOH), 100 µM Ca$^{2+}$ and osmolarity of ~300 (adjusted with D-mannitol). The $E_{rev}$ was determined as the membrane potential at which the current was zero. The $\Delta E_{rev}$ was calculated as the difference between the $E_{rev}$ of intracellular 14mM NaCl and the $E_{rev}$ measured in symmetric 140mM NaCl. The permeability ratio $P_{Cl}/P_{Na}$ was calculated based on GHK equation (eq.2).

Offline data analysis was performed in Clampfit, Microsoft Excel, and Prism (GraphPad). For quantification of Ca$^{2+}$ dose-dependent concentrations ($EC_{50}$), Ca$^{2+}$-induced currents were normalized to the max current elicited by 100 µM Ca$^{2+}$ and fit into a non-linear regression curve fit with the equation:
\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \left( \frac{[\text{Ca}^{2+}]}{[\text{EC}_{50}]^{H}} \right)} \quad (\text{eq.3})
\]

where \( \frac{I}{I_{\text{max}}} \) denotes current normalized to the max current elicited by 100 µM \( \text{Ca}^{2+} \), \([\text{Ca}^{2+}]\) denotes free \( \text{Ca}^{2+} \) concentration, \( H \) denotes Hill coefficient, and \( \text{EC}_{50} \) denotes the half-maximal activation concentration of \( \text{Ca}^{2+} \).

### 5.4.4 Protein sequence alignment and analysis

Protein sequences of nhTMEM16, afTMEM16, mTMEM16A (UniProtKB: Q8BHY3), mTMEM16B (UniProtKB: Q8CFW1), mTMEM16E (UniProtKB: Q75UR0), mTMEM16F (UniProtKB: Q6P9J9), and Subdued (FlyBase ID# FBcl0155770) were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree was visualized and plotted using iTOL v4 (https://itol.embl.de/).

### 5.4.5 Data and statistical analysis

Data and statistical analysis are identical to details in subsection 2.4.6.
Chapter 6: Conclusions

By identifying an inner activation gate of TMEM16F, this dissertation has advanced the understanding of the gating mechanisms of moonlighting TMEM16s (90). Using both computational and our optimized functional approaches, we found that there are three inner gate residues (F518, Y563 and I612) – located at the middle of the hydrophilic groove. Without Ca$^{2+}$, these residues collapsed onto each other creating a steric hindrance that occluded charged and polar molecules (i.e. lipid headgroups and ions) from passing through the permeation pathway of TMEM16F (90). Upon Ca$^{2+}$ binding, TM4 and TM6, which are parts of permeation pathway, separate from each other. This not only dilates the constriction but also alleviates the steric hindrance created by the three inner gate residues. We named this activation mechanism ‘clam shell model’ for its analogy to the open and closed appearances of the clams (90). Molecular and structural analyses of other TMEM16 CaPLSases have also revealed similar hydrophobic residue clusters at the middle of the permeation pathways and separation of TM4-TM6 upon Ca$^{2+}$ binding (34, 36, 40-42, 45). This suggests that our proposed ‘clam shell’ gating model could be shared among the TMEM16 CaPLSases.

Furthermore, our study of TMEM16F inner gate also provides mechanistic insight into the differences between TMEM16 -CaPLSases and -ion channels (90). Our data show
that the inner gate residues are also conserved in TMEM16A-CaCC indicating that the CaCCs might also employ the hydrophobic gating mechanism resembling to TMEM16-CaPLSases (90). Nonetheless, instead of completely splitting apart and exposing the permeation pathway’s lumen to the membrane environment as in the case of the CaPLSases, TM4 and TM6 of the CaCCs maintain their interactions and the inner gate residues dilate to provide enough space for only ions to go through (90). This dilated, enclosed proteinaceous pore for ion conduction of TMEM16 proteins has also recently been demonstrated for both nhTMEM16-CaPLSase and TMEM16A-CaCC (42, 193). Thus, the ion channel component of TMEM16 proteins, with or without CaPLSase activity, retains a proteinaceous pore (as opposing to the proteo-lipidic pores of the CaPLSases) that grants accessibility for only ions but not lipid headgroups throughout its open state.

For characterizing the TMEM16 proteins in live cells, current techniques are limited to only those that can be trafficked to the plasma membrane. Only a few moonlighting TMEM16s are reliably trafficked to the plasma membrane, whereas most of other members are trapped in the intracellular membrane (51, 194-196). It is therefore challenging to investigate and unveil the similarities and differences among all moonlighting TMEM16s. Hence, it requires either improvement in the current techniques or discovery of new multifunctional TMEM16s that inhabit plasma membrane to achieve the desired knowledge about the moonlighting TMEM16s. In this dissertation, by
discovering Subdued as a new plasma membrane moonlighting TMEM16 protein, we facilitate studying the mechanism and cellular functions of the multifunctional TMEM16s (111). *Drosophila* Subdued (Chapter 5), fungal nhTMEM16 (36, 37, 40-44), fungal afTMEM16 (38, 45) and human TMEM16K (34) behave similarly to TMEM16F, suggesting that biophysical properties of moonlighting TMEM16s across the eukaryote might be conserved. Future studies can look into the sequence and structural homologies and disparities between the moonlighting TMEM16s, CaPLSase-only TMEM16s and CaCC-only TMEM16s to establish the comprehensive understanding of the evolution and diversity of TMEM16 members.

Our studies not only unraveled the biophysical and regulatory properties of multifunctional TMEM16 proteins but also raised new research questions regarding these moonlighting transporters. First, what is the relationship between the hydrophobic inner gate and other activation gates, such as the voltage gate or the extracellular gate (Sε site) that has been identified in the fungal and TMEM16F CaPLSases (40, 43, 52, 88)? Or in other words, how do these gates synergistically work with each other to control ion and lipid permeations? Second, how do the inner gates of the moonlighting TMEM16 proteins, such as TMEM16F, distinguish ions from lipids and regulate the permeation for each substrate? Even though there are ample evidences supporting lipids and ions sharing the same permeation pathway (20, 23, 26, 28, 34, 36, 40-45, 52, 58, 59, 88-92), the exact mechanism of
dual transporting remains poorly characterized. The CaPLSases were shown to adopt a widely open hydrophilic grooves for lipid permeation upon Ca\(^{2+}\) binding (34, 36, 40-45, 90). However, ion channel activation of some moonlighting CaPLSases, such as TMEM16F, requires strong membrane depolarization, in addition to Ca\(^{2+}\) binding (28, 57, 58). Thus, how the wide permeation pathway of the CaPLSases in the presence of Ca\(^{2+}\) only allows permeation of lipids but not ions at the resting membrane potential is intriguing and yet to be understood.

Apart from their molecular mechanisms, pharmacological properties of the moonlighting TMEM16s have been attractive research areas owing to their benefits and potential clinical applications. Discovery of molecules that specifically target each TMEM16 member could facilitate the mechanistic and structural studies of that protein. Moreover, specific agonists and antagonists of the moonlighting TMEM16s would also be valuable pharmacological tools in examining their cellular functions. Third, pharmacological molecules targeting moonlighting TMEM16 proteins might have therapeutic potentials and/or provide insights on drug design to treat their related diseases, such as muscular dystrophy (TMEM16E) (39, 134-136), ataxia (TMEM16K) (34, 137, 138) or thrombosis (TMEM16F) (27, 28, 64-67).

Although proven to have potential clinical significance, specific molecules targeting moonlighting TMEM16s, especially their CaPLSase activities, have not been
established. The unorthodox structures and perplexing functional features of the moonlighting TMEM16s have greatly challenged the task of characterizing their pharmacological profiles. Pharmacological characterization of TMEM16 CaPLSases has been especially difficult as most of lipid scrambling assays rely on fluorescence sensors for indirect reporting of the lipid scramblase activity (27, 58, 109, 111, 167). Since pharmacological molecules might influence the fluorescent lipid reporters or Ca\textsuperscript{2+} influx instead of affecting the CaPLSase functions (121), limitations in the current lipid scrambling assays not only complicate the identification of CaPLSase-specific molecules but may also cause false positive hits. Indeed, our study of TA and EGCG – the formerly reported inhibitors of TMEM16F CaPLSase (103, 167) – has demonstrated how non-specificity of molecules could affect the fluorescence probes in the lipid scrambling assay and lead to false identification of the CaPLSase inhibitors (197). Therefore, careful controls and multi-approaches should be employed in future pharmacological studies of TMEM16 CaPLSases to prevent similar artifacts as in the case of TA/EGCG.

Although identifying bona fide agonists or antagonists for the moonlighting TMEM16 proteins is challenging, two potential approaches could be employed to discover the pharmacological molecules targeting the moonlighting TMEM16s. First, with rapid advances in structural and functional understanding of TMEM16 proteins, *in silico* rational design could be a promising approach for future pharmacological studies (198).
For example, in the case of CaPLSases, we can design the inhibitory molecules that mimic the structures of lipid headgroups, but rather than sliding through the permeation groove, the molecules could be designed to stably occupy and obstruct the groove, preventing any lipids or ions from going through the permeation pathways. On the other hand, we can also design molecules that affect the membrane properties. Based on the recent structural studies, TMEM16 CaPLSases cause membrane thinning around their permeation pathways (34, 36, 41-43, 45, 63, 96). Falzone et al. also demonstrated that application of lipids with long acyl tails into the liposome reconstitution assay to interrupt the membrane thinning could inhibit aTMEM16 CaPLSase activity (45). Therefore, designing molecules that alter membrane properties might be another potential approach in developing blockers for the CaPLSases. Second, high throughput screening could be another potential approach to identify inhibitors for TMEM16 CaPLSases, but with cautions. The technique has been successfully applied to identify antagonists for TMEM16A (121, 123, 199). However, to transfer this approach to identify inhibitors for the CaPLSases, it requires additional controls and validation steps. The AnV-based scrambling assay can be integrated into a high content screening device, such as Cellomics ArrayScan VTI, to assess CaPLSase activity in the presence of the pharmacological compound libraries. Since there is no known specific antagonist for CaPLSases to serve as a positive control, we can employ genetic knockout or knockdown to eliminate the activity
of the targeted CaPLSases and serve as a control. Next, the potential hit molecules can be verified and further characterized by different configurations of PCF assay, which allows simultaneous recording of both scrambling and ion channel activities of the moonlighting TMEM16s in the presence of the testing drugs.
### Appendix A

**Table 2: Primers used for generation of mTMEM16F mutations**

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Primers</th>
</tr>
</thead>
</table>
| F518A     | Forward: 5’ GCCTCCATCATCAGGCCATCATCATGATGATC 3’  
|           | Reverse: 5’ GATCATGATGATGATGGCGCTGATGATGGAGGC 3’ |
| F518E     | Forward: 5’ CAGCCTCCATCATCAGGCCATCATCATGATGATC 3’  
|           | Reverse: 5’ GATCATGATGATGATGGCGCTGATGATGGAGGC 3’ |
| F518K     | Forward: 5’ CAGCCTCCATCATCAGGCCATCATCATGATGATC 3’  
|           | Reverse: 5’ GATCATGATGATGATGGCGCTGATGATGGAGGC 3’ |
| F518Q     | Forward: 5’ CAGCCTCCATCATCAGGCCATCATCATGATGATC 3’  
|           | Reverse: 5’ GATCATGATGATGATGGCGCTGATGATGGAGGC 3’ |
| F518L     | Forward: 5’ CAGCCTCCATCATCAGGCCATCATCATGATGATC 3’  
|           | Reverse: 5’ GATCATGATGATGATGGCGCTGATGATGGAGGC 3’ |
| Y563A     | Forward: 5’ CTGGTTCCAGTTTGTCAACCGCTTACTCCTCATGCTTCTAC 3’  
|           | Reverse: 5’ GTAGGCTCATGATCTGATCAGGCTTACTCCTCATGCTTCTAC 3’ |
| Y563E     | Forward: 5’ GTCCAGTTTGTCAACCGCTTACTCCTCATGCTTCTAC 3’  
|           | Reverse: 5’ GAAGCATGAGGACTTCTCCCTCATGCTTCTAC 3’ |
| Y563K     | Forward: 5’ CTGGTTCCAGTTTGTCAACCGCTTACTCCTCATGCTTCTAC 3’  
|           | Reverse: 5’ GTAGGCTCATGATCTGATCAGGCTTACTCCTCATGCTTCTAC 3’ |
| Y563Q     | Forward: 5’ GTCCAGTTTGTCAACCGCTTACTCCTCATGCTTCTAC 3’  
|           | Reverse: 5’ GAAGCATGAGGACTTCTCCCTCATGCTTCTAC 3’ |
| Y563W     | Forward: 5’ GTCCAGTTTGTCAACCGCTTACTCCTCATGCTTCTAC 3’  
|           | Reverse: 5’ GAAGCATGAGGACTTCTCCCTCATGCTTCTAC 3’ |
### Table 3: Primers used for generation of mTMEM16A mutations

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Primers</th>
</tr>
</thead>
</table>
| L543K     | Forward: 5' CGGCTGTATCATCAACAAAGGTGGTCATCATTCTGTGCTG 3'  
|           | Reverse: 5' CAGCAGAATGATGACCGTGCCACCTTTGATGATAACAGCGG 3' |
| S588K     | Forward: 5' CTGCTCAAGTTTGTGAACAAATACACTCCCATCTTCTATG 3'  
|           | Reverse: 5' CATAGAAGATGGGAGTGTATTTGTTCACAAACTTGAGCAG 3' |
| I637K     | Forward: 5' CCAGCTGAGCATCAAAATGCTGGGCAAGCAG 3'  
<p>|           | Reverse: 5' CTGCTTGCCCAGCATTTTGATGCTCAGCTGG 3' |</p>
<table>
<thead>
<tr>
<th>Mutations</th>
<th>Primers</th>
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</table>
| E716A     | Forward: 5’ GCTGTTTGACGGGCGCTGTGCATCCAG 3’  
Reverse: 5’ CTGGATGCACAGCGCCGTCAAACAGC 3’ |
References


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Biography

In 2012, Trieu Phuong Hai Le attended Utica College (Utica, New York), where she majored in Biochemistry. Trieu received her Bachelor Degree of Science with honors from Utica College in May 2015. In the same year, she was matriculated to PhD Program of Biochemistry Department at Duke University. Trieu became one of the first members of Dr. Huanghe Yang’s Laboratory in Spring 2016 and worked on her dissertation research under Dr. Yang’s supervision.

PUBLICATIONS:

* indicates co-first authors

Underline indicates the author


MAJOR FELLOWSHIPS/SCHOLARSHIPS:

American Heart Association Pre-doctoral fellowship (one-year; 2020)

Utica College, Annual International and Residential Grant (2012 – 2015)

MAJOR AWARDS:

ACS Division Award of Organic Chemistry Outstanding, Senior Organic Chemistry Student (2015)

Amparo M. Escarrilla Chemistry Award – Outstanding Academic Achievement in Chemistry (2015)

Utica College, Biochemistry Department, Teaching Assistant Award (2015)