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TMEM16 proteins: unknown structure and confusing functions

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Abstract

The TMEM16 family of membrane proteins, also known as anoctamins, play key roles in a variety of physiological functions that range from ion transport, to phospholipid scrambling and to regulating other ion channels. The first two family members to be functionally characterized, TMEM16A (ANO1) and TMEM16B (ANO2), form Ca^{2+}-activated Cl^- channels (CaCCs) and are important for transepithelial ion transport, olfaction, phototransduction, smooth muscle contraction, nociception, cell proliferation and control of neuronal excitability. The role(s) of other family members, such as TMEM16C (ANO3), TMEM16D (ANO4), TMEM16F (ANO6), TMEM16G (ANO7), and TMEM16J (ANO9), remain poorly understood and controversial. These homologues were reported to be phospholipid scramblases, ion channels, to have both functions or to be regulatory subunits of other channels. Mutations in TMEM16F cause Scott syndrome, a bleeding disorder caused by impaired Ca^{2+}-dependent externalization of phosphatidylserine in activated platelets, suggesting that this homologue might be a scramblase. However, overexpression of TMEM16F has also been associated with a remarkable number of different ion channel types, raising the possibility that this protein might be involved in both ion and lipid transport. The recent identification of an ancestral TMEM16 homologue with intrinsic channel and scramblase activities supports this hypothesis. Thus, the TMEM16 family might have diverged in two or three different subclasses, channels, scramblases and dual function channel/scramblases. The structural bases and functional implication of such a functional diversity within a single protein family remain to be elucidated and the links between TMEM16 functions and human physiology and pathologies need to be investigated.

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Introduction

Calcium-activated Chloride Channels (CaCCs) play important regulatory roles in a variety of physiological processes, ranging from epithelial fluid secretion to signal transduction, nociception and cell proliferation. Despite being initially characterized in the early 1980's [1, 2], their molecular identity remained unknown and controversial for nearly 30 years. In 2008 three groups independently identified two members of TMEM16 orphan family of membrane proteins (also known as anoctamins, Anion Channels with 8 TM domains), TMEM16A (ANO1) and TMEM16B (ANO2), as key constituents of CaCCs [3-5]. Several groups followed this landmark discovery by confirming these initial findings and expanded the breadth of physiological processes regulated by Ca^{2+}-activated Cl^- currents mediated by TMEM16A and B which are as diverse as nociception, epithelial secretion, neuronal signaling, smooth muscle contraction, host defense, cell proliferation, signal transduction and tumorigenesis [6-13]. While these advances greatly expanded our understanding of the roles of CaCCs in physiology, our insights into the molecular bases of Ca^{2+}-dependent Cl^- transport by TMEM16 proteins remain extremely limited as we lack key pieces of information on even their most basic structural features, such as their topological organization, the localization of the ion conduction pore [5, 14-16] and whether these channels are directly regulated by Ca^{2+} or if the association to exogenous Ca^{2+}-sensing subunits is required [14, 17-25].

One of the most surprising characteristics emerging after these initial discoveries is that not all TMEM16 homologues are ion channels, or at least that they are not only channels. In addition to mediating ion transport, the conventional role of ion channels, TMEM16 proteins have been involved in an unusually wide array of functions, such as phospholipid scrambling [26] or regulating the function of certain K^+ channels [27]. While the role of TMEM16A and B as Ca^{2+}-activated Cl^- channels has been firmly established *in vivo* and *in vitro* [3-5, 22], the function(s) of most other family members remain poorly understood and/or controversial. For example, TMEM16C (ANO3) and TMEM16F (ANO6) have been involved in Ca^{2+}-dependent externalization of phospholipids that are normally confined to the inner leaflet of the plasma membrane, such as phosphatidylserine (PS) [26, 28]. This process is called phospholipid scrambling and is mediated by proteins called scramblases [29], whose molecular identity has remained unknown for nearly 40 years. Extracellular exposure of PS is a key trigger for the initiation of blood clotting by activated platelets [30, 31] and is a required signal for the phagocytic clearance of apoptotic cells [31, 32]. It remains however unclear whether TMEM16C and F themselves are phospholipid scramblases, if they are regulators of yet unidentified scramblases or if they have multiple activities. The recent finding that an ancestral TMEM16 homologue, afTMEM16, is a dual function ion channel and phospholipid scramblase [33] supports the hypothesis that at least some TMEM16 homologues might have both roles.

In the present review we chose to focus on three key open mechanistic questions on the function of TMEM16 proteins: first, where is the ion pore? Second, how does Ca^{2+} activate these proteins? Finally, are all TMEM16s channels, or some are scramblases and/or proteins with multiple functions?

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Topology of the TMEM16 proteins

The TMEM16 family currently comprises ~1400 sequences divided in 10 different clades (Fig. 1) and its members are found only in eukaryotes. Several lines of evidence suggest that TMEM16s form homodimers [22, 33-36], but the possibility of heterodimer formation has not been investigated in depth. The topology of TMEM16 homologues is still unclear and three competing models have been proposed [14, 25, 37] (Fig. 2).The cytosolic localization of the N- and C-termini [37] implies that the TMEM16s have an even number of transmembrane (TM) segments, with most programs predicting that the TMEM16s have 8 TMs [5, 14, 37]. The precise identification of the boundaries of these regions is uncertain as different programs identify variable numbers of TM regions with different boundaries [14]. Despite this difficulty, by considering the aggregated output of multiple programs it is possible to converge to a reasonably homogeneous consensus on the location of the TM domains even for TMEM16 homologues from very divergent clades (Fig. 3). The sequence homology between the TMEM16s is low, i.e. ~25% identity between the evolutionarily distant homologues TMEM16A and afTMEM16 (Fig. 1), and is mostly localized to short stretches of sequences within the putative TM regions (Fig. 3). The degree of homology between TMEM16s from the same clade is higher (*i.e.* all TMEM16F's share >60% identity), and decreases between different clades. The evolutionarily conserved stretches are likely to underlie common mechanistic features of the family, such as the Ca^{2+} binding site. Indeed, one of the better preserved regions, between residues 700 and 760 (Fig. 3), contains amino acids important for Ca^{2+}-sensitivity [14, 21, 22, 25, 33], which appears to be one of the more conserved features among TMEM16s. In contrast, the location of the ion permeation pathway of TMEM16 channels remains poorly defined, mostly because of the poor sequence conservation and difficulty in defining the TM domains in the absence of direct structural information.

An initial model, based on epitope accessibility studies on TMEM16G (ANO7) [37], proposed that residues 610-706 form a re-entrant loop facing the extracellular milieu (Fig. 2A), similar to those found in many other channels [38]. However, further epitope accessibility studies as well as site-directed mutagenesis experiments on TMEM16A showed that residues 640-706 face the intracellular solution and that E702 and E705 are critical for the channel's sensitivity to intracellular Ca^{2+} [14]. Thus, TMEM16A must fully cross the membrane between residues 610-640, suggesting that TM6 localizes to this region. This result, however, poses a topological conundrum. Most programs identify 3 TM domains between residues 710 and 880 for a total of 9 TMs, a topology that would force the N- and C-termini of these proteins to be on opposite sides of the membrane. To obviate to this difficulty a revised model proposed that TM7a and TM7b form a re-entrant structure that crosses the membrane only once (Fig. 2B) [14]. The recent identification of two additional acidic residues in the TM7a-7b loop important for the Ca^{2+} sensitivity of TMEM16A [25] suggests that both the N- and C-termini of TM7a face the intracellular solution and participate in the Ca^{2+} binding site, most likely by forming a re-entrant hairpin [25], leading to a further refinement of the proposed topology (Fig. 2C). Interestingly, TM7a contains two highly conserved prolines, P721 and P724, which could promote the formation of such a structure. Further experiments such as epitope accessibility and site directed labelling will be needed to further refine the topology.

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TMEM16A and B: the Ca^{2+}-activated Cl^- channels

Although Ca^{2+}-activated Cl^- currents were identified in the early 1980's [1, 2], it took nearly 30 years for their molecular identity to be uncovered. In 2008 three independent groups used different approaches to show that the identified heterologous expression of TMEM16A and B leads to the appearance of CaCC currents whose properties closely resembled those seen in native cells [3-5], indicating that these proteins are key components of CaCCs. Currents mediated by TMEM16A and B in a variety of heterologous systems recapitulate the basic properties of endogenous CaCCs; they are outwardly rectifying, activated by submicro- (TMEM16A) or micro-molar (TMEM16B) intracellular Ca^{2+} and blocked by inhibitors such as niflumic acid (NFA), 5-nitro-2-(phenylpropylamino)-benzoate (NPPB) and 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid (DIDS). Furthermore, depressing the expression of TMEM16A and B led to the disappearance of CaCC currents in the appropriate tissues. Finally, definitive proof that TMEM16A is sufficient to form fully functional CaCCs came from the purification and reconstitution of the protein in liposomes [22]. Interestingly, the details of the pharmacological and biophysical properties of native CaCCs vary depending on the tissues under study. This raises the possibility that, in some cases, these channels might form complexes with unidentified regulatory subunits, or that different TMEM16 homologues or splice variants associate with form heterodimers with novel properties.

Ion selectivity of TMEM16A and B

The anion selectivity of TMEM16A and B follows a type I Eisenmann anion sequence, $I^- > Br^- > Cl^- > F^-$ [4, 5, 16] and resembles that of native CaCCs under physiological conditions. Unlike most other known anion channels, the ionic selectivity of TMEM16A and B is unique in two different respects: their anionic selectivity is not fixed, during channel activation the relative permeability of different anions varies [4, 39], and their anion-cation selectivity is imperfect [14, 40].

Early studies on native currents indicated that the CaCCs do not have a constant inter-anionic selectivity [39], an observation later recapitulated by heterologously expressed TMEM16A [4]. Recent work proposed that the dynamic selectivity of TMEM16A might be modulated by the association of the channel to calmodulin [19]. Multiple studies, however, showed that these two proteins interact weakly, if at all [22-24]. One group also failed to reproduce the calmodulin-dependent selectivity changes in TMEM16A [24] and suggested that the original observation might have been due to problems with controlling potential shifts associated with ion accumulation and/or changes in the patch series resistance. Thus, the structural basis for this dynamic selectivity remain unknown and controversial. One possible explanation is that the TMEM16 pore undergoes significant conformational rearrangements during activation so that the channel visits multiple stable or meta-stable conductive conformations while opening. If this were the case, then it is conceivable that changes in the residues lining the ion permeation pathway give rise to the diverse selectivity of these states. This possibility could account for at least some of the difficulties in reaching a consensus as to which residues line the pore of these channels, as will be discussed below.

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While most Cl^- channels display poor inter-anionic selectivity, likely due to the lack of other competing anions abundant in the environment, their selectivity against cations is nearly perfect [41]. In contrast, some groups showed that TMEM16A and B discriminate poorly between anions and cations [14, 40], with a permeability ratio $P(Cl^-)/P(X^+)$ of only ~7. This implies that these channels let cations through at a rate close to 15% that of anions. However, others reported that these channels have a more stringent anion *vs* cation selectivity [5, 22]. Indeed, the first report indicated that TMEM16A excludes cations nearly completely [5], with a $P(Cl^-)/P(X^+)$~30, and the second showed that purified TMEM16A reconstituted in proteoliposomes sustains a 300-fold KCl gradient for long periods of time [22], again indicating that the channel's permeability to K^+ is negligible. One possibly relevant difference between the electrophysiological measurements [14, 40] and the experiments with reconstituted TMEM16A [22], is that the latter were carried out in the presence of constant $[Ca^{2+}]$, a condition that is known to affect the channel's activation state so that the electrophysiological measurements and the biochemical ones might reflect the selectivity of different states. A second important point is that gating of CaCCs is strongly affected by the type and concentration of the permeant anion [42, 43] shifting the g-V curve and promoting channel deactivation. It is therefore possible that the relative weight of leak or endogenous cation currents increases when $[Cl^-]$ is lowered to measure a reversal potential in bi-ionic conditions, resulting in a spurious right-shift of the reversal potential of the currents causing an overestimation of the cation permeability through these channels. Further experiments aimed at determining the selectivity of TMEM16A and B will therefore need to carefully evaluate the contributions of endogenous currents, the ionic composition of the solutions and the Ca^{2+} concentration to minimize the contributions of leaks to the measurements of reversal potentials.

Identification of the ion pore of TMEM16A

Initial experiments on TMEM16A, based on the original topology (Fig. 2A), identified three positively charged residues in the putative loop region (R621, K645 and K668) important for the anion *vs* cation selectivity of the channel [5]. Most notably the R621E mutation decreased $P(Cl^-)/P(Na^+)$ by ~30-fold, so that the channel became nearly non-selective [5]. However, others could not reproduce this result, but rather found that the selectivity of this mutant was indistinguishable from that of the WT channel [14]. Furthermore, mutations in the 610-700 region were reported to cause a decrease in the number of active channels at the plasma membrane, suggesting that this region influences gating and/or trafficking rather than permeation [15]. Thus, the altered selectivity of the mutants might reflect an increase in the contribution of endogenous currents rather than effects of the mutations on TMEM16A's intrinsic permeation properties. A more systematic analysis of the accessibility of the TM6 region suggested that some residues in this helix line ion permeation pathway [14]. These experiments identified 6 residues that are important for ion conduction through TMEM16A as their mutation to cysteine affected ion selectivity, and their subsequent reaction with MTS compounds drastically reduced the currents. Since the TMEM16's are dimers it is unlikely that the pore is formed by a single TM helix from each subunit. Rather, it is plausible that portions of multiple helices contribute to pore formation. Further systematic mutagenesis and systematic accessibility studies of the helices are required to identify which regions of the channel directly line the ion permeation pathway.

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Mechanism of Ca^{2+} sensing by TMEM16 proteins

One of the most surprising features of the TMEM16 family members is that while they all sense Ca^{2+} their sequences lack canonical Ca^{2+} binding sites such as EF-hands or RCK domains [5]. Furthermore, the TMEM16s display a wide range of Ca^{2+}-sensitivities, spanning nearly 3 orders of magnitude from ~200 nM to ~20 μM [3-5, 40, 44]. Initial investigations focused on a Ca^{2+}-bowl like stretch of negatively charged residues in the first intracellular loop (TM1-2, Fig. 3). However, these residues were shown to affect the coupling between the Ca^{2+}- and voltage-sensitivity of these channels rather than altering Ca^{2+} affinity [45, 46], indicating that these residues are not directly involved in binding. The difficulties in identifying the Ca^{2+} binding site, coupled to the observation that TMEM16A and B have several putative calmodulin sites [17, 20] led to the proposal that the Ca^{2+} sensitivity of these channels is extrinsic and mediated by calmodulin [17-20]. These groups reported that TMEM16A and B can associate with and form stable complexes with calmodulin [17-20] and two groups reported that inhibition of calmodulin leads to loss of function of TMEM16A channels [17, 20]. However, these groups come to different conclusions on what the role of the TMEM16/calmodulin interaction is. One group found that it is required for channel activation [17], while others reported that it modulates the kinetics of channel activation and inactivation [20] or controls the channel's selectivity [19]. This confusing picture is further complicated by the findings of other laboratories showing that the association between TMEM16A and calmodulin is weak (if at all present) and not necessary for the channel's Ca^{2+} sensitivity [23, 24]. Finally, the observation that purified TMEM16A reconstituted in proteoliposomes recapitulates the functional properties of TMEM16A including its sub-micromolar affinity for Ca^{2+} [22] provides direct evidence that the Ca^{2+} sensitivity of TMEM16A is intrinsic.

Despite extensive mutagenesis the location of the Ca^{2+} binding site on the TMEM16's as well as the origin for the different Ca^{2+} sensitivities of the various homologues remain unclear. Work from several laboratories showed that a pair of conserved amino acids in the third intracellular loop (TM6-7a) plays a key role in the Ca^{2+} sensitivity of several TMEM16 homologues [14, 22, 33, 40]. Support for this conclusion came from a studies showing that three non-charged amino acids in the TM7a-7b loop were shown to be responsible for the ~10-fold different Ca^{2+}-sensitivity of TMEM16A and B [21] and from the identification of a second pair of conserved acidic residues in the TM7a-7b loop that is critical for Ca^{2+} sensitivity [25].

These results collectively argue that the TMEM16s bind Ca^{2+} directly and that association with calmodulin is not required. It is reasonable to hypothesize that the four conserved acidic residues near TM7a participate in the formation of the Ca^{2+} binding site, but direct binding measurements will be required to test this hypothesis and rule out the possibility that these loops control gating rather than binding. It remains to be seen whether these residues are sufficient to form the binding site or if other regions of the channels are also involved. Finally, although association with calmodulin is not required for function, it is possible that in some conditions this interaction becomes stable enough to be physiologically significant.

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TMEM16F: channel, scramblase or both?

After the initial identification of TMEM16A and B as constitutive components of CaCCs it was generally assumed that other family homologues would encode for similar types of ion channels. However, work by several groups has painted a much more confusing picture, bringing into question whether all TMEM16's are channels. TMEM16F in particular has been the subject of intense scrutiny by multiple groups who reached different conclusions [26, 31, 40, 47-52]. Mutations in TMEM16F cause Scott syndrome [26, 47, 53, 54], an inherited bleeding diathesis caused by the impaired Ca^{2+}-dependent exposure of the lipid phosphatidylserine (PS) in red blood cells, which is a key trigger for blood coagulation. Mice lacking TMEM16F recapitulate this bleeding disorder [40], displaying increased bleeding and impaired Ca^{2+}-dependent PS exposure. Knockout mice also have additional defects, such as impaired bone mineralization [48], that are absent in Scott syndrome patients. Collectively these results indicate that TMEM16F is an important piece of the signaling machinery necessary for PS translocation from the inner to outer leaflet in red blood cells and thus for blood coagulation. What remains highly controversial is its role in this process.

Initial reports suggested that the heterologous expression of TMEM16F is associated with three different types of currents: volume-activated Cl^- channels [55], hyperpolarization-activated Cl^- channels [56] and Ca^{2+}-activated Cl^- channels [18]. In addition to having different activation triggers, the reported currents displayed different current-voltage relationships and kinetics of activation and inactivation, suggesting that TMEM16F cannot directly mediate all three types of currents. The recent discovery that the LRRC8/SWELL family encodes for the volume regulated Cl^- channels [57, 58], is consistent with the idea that some of these initial reports did not measure TMEM16F-mediated currents.

A new and unexpected role for TMEM16F emerged when strong genetic and functional data linked this homologue to lipid scrambling [26, 47, 48]. The first reports showed that heterologous expression of TMEM16F is associated with Ca^{2+}-dependent lipid scrambling in a variety of cell lines [26, 28, 32]. A mutation in the first intracellular loop, D409G, leads to the constitutive activation of the scrambling activity [26], an effect that is further potentiated by a 21 amino acid insertion in the N-terminus of the protein [32], suggesting that TMEM16F directly mediates lipid scrambling. Further support for this hypothesis comes from the identification of 3 mutations in hTMEM16F associated with Scott syndrome [26, 47]. Importantly, one report showed that no ionic currents could be detected when TMEM16F was overexpressed in HEK293T cells [28], suggesting that this protein does not have ion channel activity. This surprising conclusion was supported by the finding that TMEM16A and B are the only TMEM16 family homologues mediating ionic currents; overexpression of all other 8 family members did not give rise to novel currents and that TMEM16C, D, F, G and J are involved in Ca^{2+}-dependent lipid scrambling [28]. These findings are in partial contrast with previous data showing that TMEM16D, F, G and J mediate Ca^{2+}-dependent currents [18]. Despite these contradictions, these observations led to the proposal that the TMEM16 family is comprised of Ca^{2+}-gated Cl^- channels and Ca^{2+}-dependent phospholipid scramblases, and that TMEM16F belongs to this second subgroup [26, 28].

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This conclusion however was challenged by multiple reports which, while concurring that TMEM16F is a central player in Ca^{2+}-dependent phospholipid scrambling, show that this protein is (also) an ion channel [31, 40, 47-52]. These results however, rather than clarifying the confusing picture provided additional contradictory and confusing evidence: no two groups reported TMEM16F-mediated currents with similar properties.

A very thorough investigation of the electrical properties of TMEM16F in mice and in heterologous systems concluded that TMEM16F is a small conductance Ca^{2+}-activated cation channel (SCAM) that is also permeable to divalents, such as Ca^{2+} [40]. While deletion of TMEM16F impaired phospholipid scrambling in red blood cells, its heterologous expression did not lead to Ca^{2+}-dependent phospholipid scrambling in multiple cell lines. This led to the proposal that TMEM16F is not a scramblase, but rather that it is a cation channel that regulates a yet unknown phospholipid scramblase. Several lines of evidence support this conclusion. First, megakaryocytes, which are precursor cells to platelets, from TMEM16F knockout mice lack a non-selective cation current with properties closely resembling those induced by overexpression of TMEM16F in heterologous systems. Second, mutations of a conserved di-acidic motif in the third intracellular loop shift the Ca^{2+}-dependence of these currents by nearly two orders of magnitude. Third, mutating a charged residue in the putative pore of the channel weaken its cationic selectivity and the converse charge mutation in TMEM16A reduces its anionic selectivity. Fourth, the D409G mutation causes a pronounced rightward shift in the voltage-dependent activation of the channel, inducing a drastic reduction in the total amount of current at the cell surface. These results strongly argue that TMEM16F is a Ca^{2+}-activated cationic channel that indirectly regulates an unknown scramblase, possibly through a Ca^{2+}-dependent feed-forward circuit [40].

In contrast to these results multiple groups showed that overexpression of TMEM16F is associated with Ca^{2+}-dependent Cl^{-} currents, albeit with differing properties in their ionic selectivity, time-course of activation, Ca^{2+}- and voltage-dependencies [49-52]. Interestingly, two reports showed that TMEM16F is also associated with Ca^{2+}-dependent phospholipid scrambling [50, 52], suggesting that this protein might have both functions. However, while one group found that the ionic and lipid transport functions of TMEM16F were pharmacologically separable [50], the other showed that compounds blocking the ionic currents also drastically reduce the scrambling activity [52].

Taken together, these results concur in assigning to TMEM16F a central role in Ca^{2+}-dependent phospholipid scrambling and possibly in Ca^{2+}-regulated ionic transport. However, activated platelets and B-lymphoblasts from Scott syndrome patients do not display the impaired Ca^{2+} entry [59] that would be expected if TMEM16F were a plasma-membrane ion channel permeable to Ca^{2+}. It will be interesting to see whether the currents recorded from platelets obtained from Scott syndrome patients are altered, as would be expected if TMEM16F is a channel, if the defect is limited to impaired PS exposure, suggesting that TMEM16F is a scramblase, or if both processes are affected.

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What is TMEM16F?

The results discussed above are confusing, contradictory and cannot all be simultaneously correct. TMEM16F cannot mediate five different types of Cl^- currents, be a cation selective channel, a scramblase rather than a channel, and a protein with both channel and scramblase functions. These inconsistencies cannot be simply ascribed to the use of different expression systems or experimental approaches; in many cases nominally identical cell lines, clones and techniques are utilized. One possibility is that the overexpression of TMEM16F has pleotropic effects on the expression and/or trafficking of many different channels and scramblases in cells, and that slight differences in the experimental procedures (i.e. transfection techniques, assay details, solution composition, $[Ca^{2+}]$ used) might underlie the plethora of divergent results. While further experiments will be required to resolve this controversy, we can envision three scenarios that could give rise to this confusion. Although none of these scenarios can account for all the data, they can serve as starting points to design further experiments. The first possibility is that TMEM16F is a Ca^{2+}-dependent scramblase with no channel activity. In this case, it is possible that its overexpression alters the lipid composition of trafficking vesicles, resulting in impaired signaling and mis-targeting of multiple channels to the plasma membrane. This would account for the multiple reports associating the expression of TMEM16F to lipid scrambling and to the contrasting results on its ion channel activity. A second scenario is that TMEM16F is a Ca^{2+}-dependent ion channel, either cation- or anion-selective. In this case TMEM16F might regulate the activity of yet unidentified scramblases, either directly via a protein-protein interaction or indirectly by controlling the electrical state of the cell and/or by allowing entry of extracellular Ca^{2+} into the cytosol. This would be consistent with the reports indicating that TMEM16F is a channel and account for its critical role in phospholipid scrambling. A third possibility is that TMEM16F possesses intrinsic ion channel and scramblase functions. While this scenario accounts for most of the fundamental observations, it cannot explain the contrasts in channel properties or the lack of scrambling associated with its expression. It will take a careful and concerted effort by the numerous groups involved to resolve this controversy. Ideally, this conundrum will be resolved by purifying and reconstituting TMEM16F, so that its intrinsic function(s) can be directly determined. However, while waiting for this breakthrough we will have to settle for more traditional approaches, such as site-directed mutagenesis.

afTMEM16: a TMEM16 homologue with intrinsic ion channel and phospholipid scramblase functions

While purification of TMEM16F itself has not yet been successful, the recent purification and functional reconstitution of afTMEM16, an ancestral TMEM16 homologue from the pathogenic fungus *Aspergillus fumigatus*, provided the first insights into the intrinsic properties of TMEM16 proteins as this homologue was shown to be a dual function ion channel and scramblase, with both functions being Ca^{2+}-dependent [33].

The ion channel formed by afTMEM16 is poorly selective, $P(Cl^-)/P(K^+)$~0.66, an intermediate value between the cationic selectivity of TMEM16F, $P(Cl^-)/P(X^+)$~0.15 [40], and the anion preference of TMEM16A and B, $P(Cl^-)/P(X^+)$~7 [14, 40]. As expected, charge-neutralization of the di-acidic motif in the TM6-7a loop nearly abolishes the Ca^{2+}-

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dependence of activation of the channel further supporting the role of this region in divalent sensitivity. It is important to note that the characterization of the channel properties of afTMEM16 was only preliminary and was limited by its poor activity in reconstituted membranes. Additional experiments will be needed to fully characterize this channel.

The availability of a biochemically pure preparation of afTMEM16 allowed the hypothesis that TMEM16 proteins directly mediate lipid scrambling to be tested. In addition to its channel activity afTMEM16 is also a scramblase. This multiplicity of functions is not a general feature of the family since neither TMEM16A nor Ist2p, a yeast homologue [60], mediate lipid scrambling. The broad lipid specificity of afTMEM16 resembles the poor selectivity of mammalian scramblases: it mediates the transmembrane movement of lipids with headgroups with different charges, sizes and chemical composition as well as lipids with different chains. Addition of Ca^{2+} greatly increases the rate at which afTMEM16 scrambles phospholipids, and the conserved di-acidic motif in the third intracellular loop plays a key role in Ca^{2+} sensing. It is worth noting, however, that even in the absence of Ca^{2+} afTMEM16 scrambles lipids at a finite rate. The physiological significance of this is unclear. It is possible that the liposomal bilayer is only an imperfect mimic of a cellular membrane, in that it might be more flexible as it lacks the high percentage of sterols characteristic of the plasma membrane as well as the cytoskeletal network. Alternatively, it is possible that in higher eukaryotes lipid scrambling is more tightly regulated than in lower organisms since lipid externalization is a pro-apoptotic and a pro-coagulant signal. Further work will be needed to clarify this point.

Mechanistic speculations

The finding that a TMEM16 homologue has intrinsic ion channel and scramblase activities provides direct evidence that the TMEM16 structural scaffold can accommodate both functions and that the two are not mutually exclusive [33]. This raises the possibility that other homologues, such as TMEM16F, might also be dual-function channel/scramblases. The finding that TMEM16A is only a channel but not a scramblase [33] suggests that the two activities are not functionally linked and that, in at least this case, evolution has successfully separated them. It remains to be seen whether the TMEM16 family has diverged in just two separate subtypes, channel-only and dual function channel/scramblases, or if a third subtype, scramblase-only, exists. The reports indicating that TMEM16C is not a channel [18, 27, 28] and that its overexpression leads to Ca^{2+}-dependent scrambling [28] is consistent with this latter notion. The modulatory function that TMEM16C exerts on the SlacK K^+ channel could either be a consequence of the direct association between the two proteins or be an indirect, lipid-mediated, effect [27].

All TMEM16 homologues have at least 8 putative transmembrane domains whose boundaries are reasonably well preserved between different subtypes (Fig. 2, 3). This suggests that the overall architecture of the different TMEM16 types is conserved and that the family's functional divergence does not arise from drastic structural changes, such as the addition or subtraction of functional domains, but rather that the accumulation of single side chain differences might have selectively affected one function or the other. In this scenario it is easy to envision how the two or three different functional subtypes might have emerged

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randomly during evolution. A corollary of this hypothesis is that the two activities should be functionally separable in the channel/scramblase subtypes. Indeed, inhibition of the ion transport activity of afTMEM16 is not associated with the loss of scrambling activity [33], and at least one report on TMEM16F comes to a similar conclusion [50].

The simplest structural models that account for these observations are either that the TMEM16 protein scaffold forms two distinct pathways, one for lipids and one for ions, or that both substrates permeate through a single permeation pathway. Within the framework of the first model it is easy to envision how random mutations might impair substrate permeation through either pathway, giving rise to the ion channel- or scramblase-only subtypes. Similarly, this model also predicts and is consistent with the observation that inhibition of one pathway should not necessarily result in block of other [33, 50]. However, it is not easy to envision how a TMEM16 dimer could form these two pathways, one of which has to be sufficiently large to accommodate lipid translocation while simultaneously preventing movement of ions. Furthermore, if the ion pore is formed at the symmetry axis of the TMEM16 dimer, like in most channels, then what kind of symmetry could the lipid pathway have? Do the TMEM16's form two lipid pathways, one per monomer? If so, could the monomer function independently?

A second alternative is that the TMEM16s form deep lesions at the membrane-protein interface leading to the appearance of pores that are only partly delimited by protein; rather these pores would also be directly lined by the hydrophobic tails of the phospholipids forming the membrane. The hybrid nature of these pores would allow transport of ions and/or lipids, with preferences which depend on the nature of the side chains lining them. While unusual, hybrid protein/lipid pores are not without precedent in the ion channel literature as the diphtheria toxin channel was proposed to be formed by a single helical hairpin and phospholipids [61]. A consequence of this hybrid structure would be, for example, that the charge and composition of the lipids surrounding the protein should affect the ionic selectivity of the TMEM16 channels, possibly accounting for the disparate selectivities reported for the TMEM16F channels [40, 49-51]. Similarly, the nature, size and charge of the ions present in solution should affect the rate and selectivity of scrambling and both transport activities should be affected in parallel by small molecule inhibitors. At least one report suggests this might be the case for TMEM16F [52].

Both models are consistent with subsets of the available data but cannot fully account for all experimental observations. Further work will be required first to sort out the contrasting pieces of evidence present in the literature and then to test the predictions of these simplistic models with the goal of generating more sophisticated ones with better predictive power. It is otherwise possible that only the structure of a TMEM16 homologue will provide answers to these questions.

Conclusions

Since its identification in 2008, the TMEM16 family has been at the center of a whirlwind of exciting discoveries and raging controversies. Six years of extensive studies by numerous groups have yielded only partial glimpses of the complex puzzle that is the structure and

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function of the TMEM16 proteins, few of these pieces fit well together and many are missing. On few observations there is broad agreement while many are irreproducible and in direct contrast with each other. TMEM16A and B are Ca^{2+} - activated Cl^{-} channels, but the location of their pore and mechanism of Ca^{2+} sensitivity remain unclear and controversial. TMEM16F is involved in lipid scrambling, but it is unclear whether it is a channel, a scramblase or both. The other 7 family members are less controversial simply because they are understudied!

While extensive structural, functional and physiological work is required to solve these contrasts, several questions are beginning to emerge. Structurally, it will be fascinating to see how the TMEM16 scaffold can allow for the Ca^{2+}-dependent transport of substrates as diverse as ions and lipids, as well as seeing what changes underlie the functional divergence seen within the family, with up to three different subtypes in channels, scramblases and dual function channel/scramblases. The TMEM16's are central players in numerous physiological processes, but their role(s) are often unknown and controversial. Finally, we cannot but speculate on what the role of the dual function channel/scramblase TMEM16's is. Why is it important to directly link the electrical state of a cell to its membrane composition? While it is possible that the co-existence of both activities on a single protein characterizes the some ancestral homologues, the reported involvement of TMEM16F in both ion and lipid transport suggests that this feature might be conserved. Could the ability of TMEM16 proteins to mediate ion and/or lipid transport be important in allowing them to control cell proliferation and programmed death? Indeed, TMEM16's have been involved in controlling cell migration, uncontrolled cell proliferation and cell death [62-65], TMEM16A (known as DOG1 in the cancer literature) [66] and G [67] are considered biomarkers for cancers and inhibition of the ion channel function of TMEM16A stops tumorigenesis [11, 68]. While many ion channels have long been known to regulate cell proliferation, cancer and apoptosis, the link between the electrical state of the cells and these processes remains poorly understood. However, the distribution of charged lipids regulates the signaling activity of proteins crucial for cell proliferation [69-71]. It is therefore tempting to speculate that the TMEM16's might directly link between these processes: the scramblases by directly affecting the transbilayer distribution of lipids and the channels by affecting the membrane potential.

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Highlights

- Members of the TMEM16 family display an unusually high degree of functional diversity.
- TMEM16 proteins can be ion channels, phospholipid scramblases or simultaneously have both functionalities.
- An ancestral homologue, afTMEM16, is a dual function channel/scramblase.
- TMEM16F is involved in lipid scrambling and might also be a channel.
- TMEM16A and B form Ca^{2+}-activated Cl^{-} channels.
- The role of the other family members is unclear.
- The location of the ion pore and lipid translocation pathway are unknown.
- Ca^{2+} directly binds to the TMEM16's and is required for ion and lipid transport.

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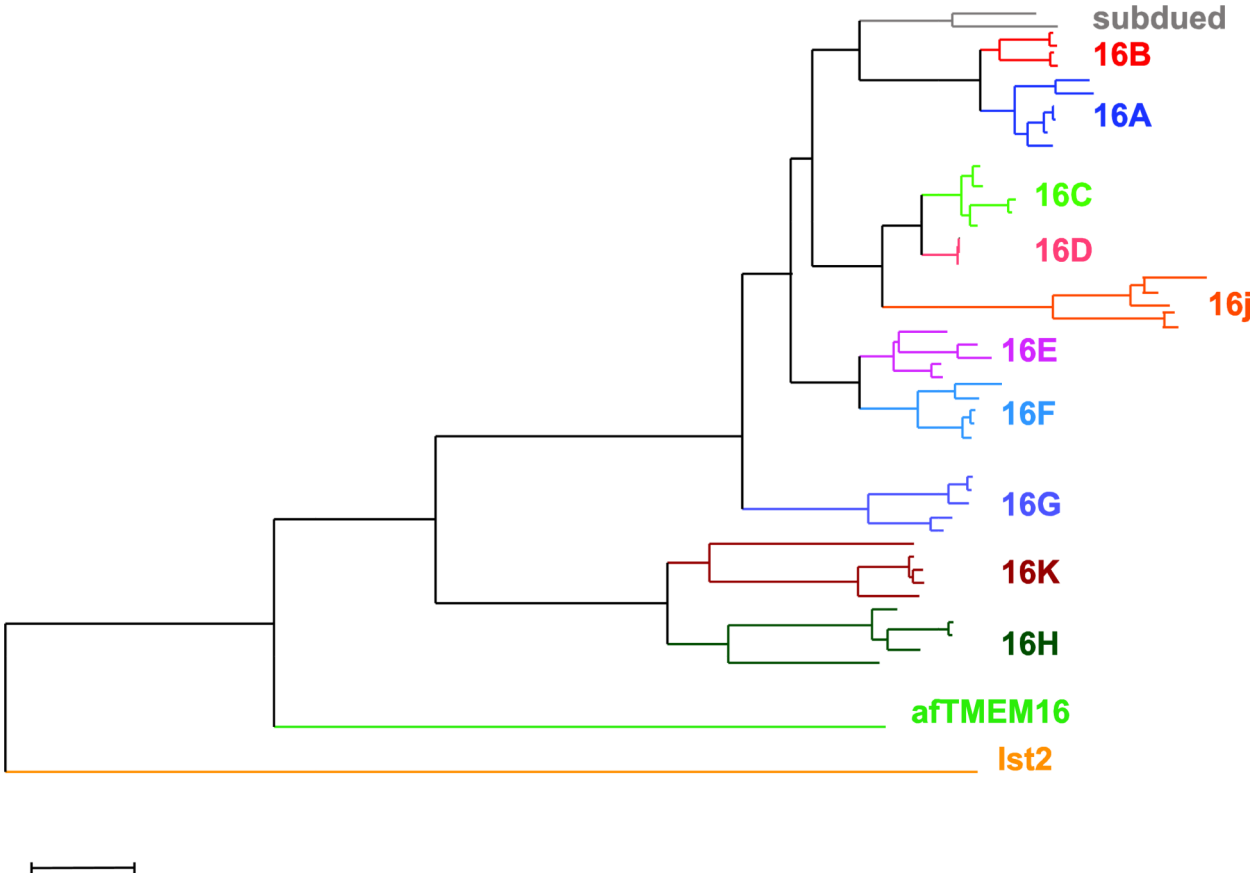

Figure 1. Phylogeny of the TMEM16 family

60 TMEM16 protein sequences were obtained from the PFAM database from Mammalia (30), Actinopterygii (13), Arthopoda (10), Fungi (3), no kingdom (4). Multiple sequence alignment was performed using CLUSTALW. From this initial alignment the 5 most divergent sequences were removed and a new alignment built. Phylogenetic analysis was conducted using PhyMOL 3.0 [72] and the phylogenetic tree was built using Seaview [73]. Scale bar indicates 1 nucleotide substitution per site.

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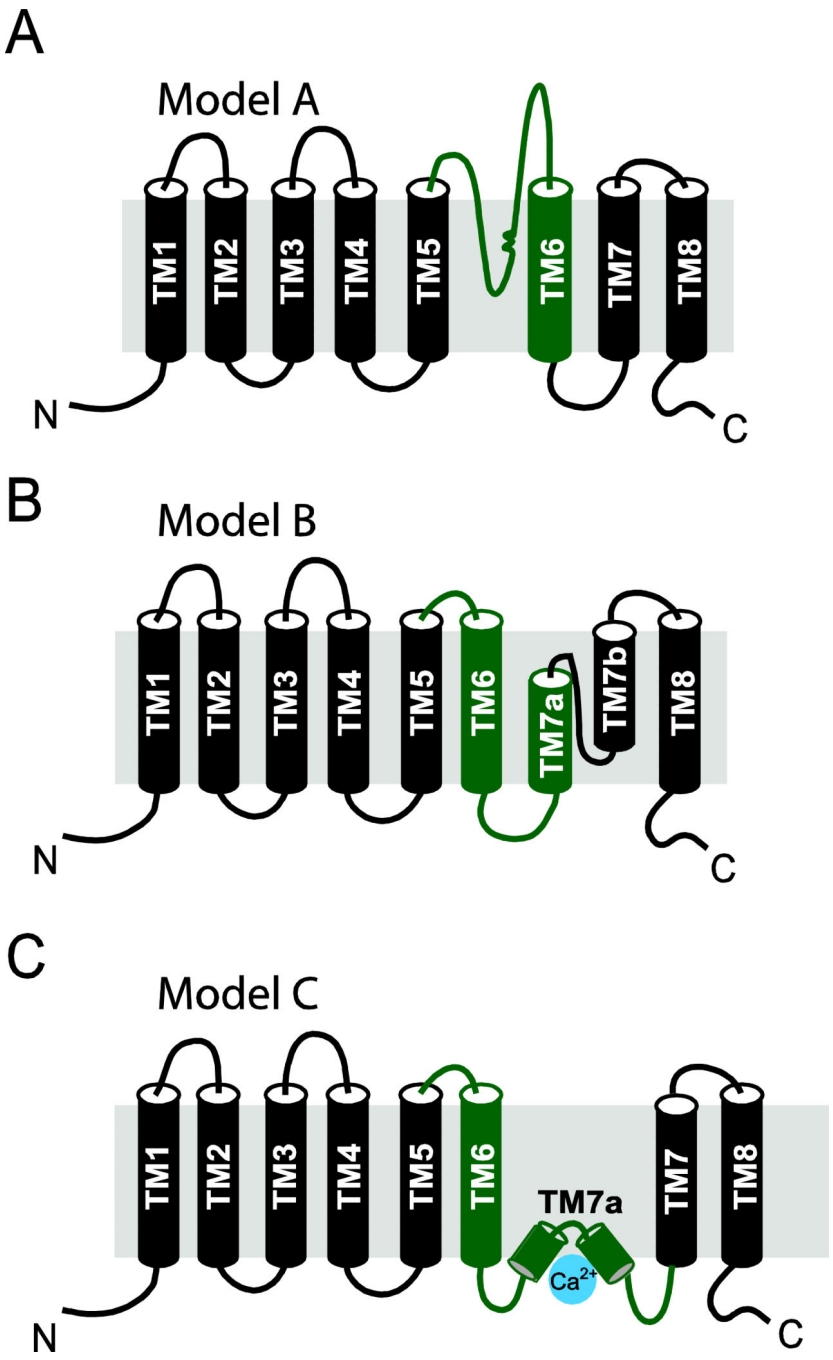

Figure 2. Competing TMEM16 topological models

Models were drawn according to Das et al., 2008 [37] (A), Yu et al 2012 [14] (B) and Tien et al 2014 [25] (C). Highlighted in green are residues 610-706 whose topological organization is debated.

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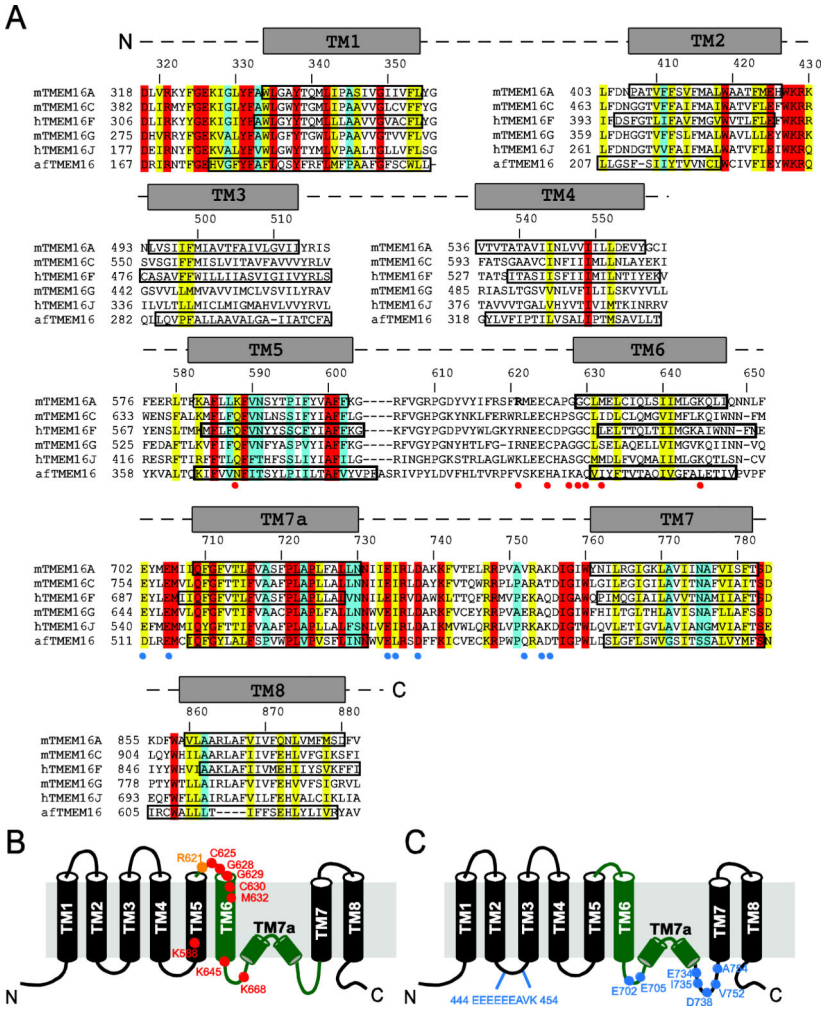

Figure 3. Sequence alignment of the TMEM16 family

A) Alignment of the putative transmembrane segments of mTMEM16A(ac), mTMEM16C, hTMEM16F, mTMEM16G, hTMEM16J and afTMEM16. Boxes on mTMEM16A, hTMEM16F and afTMEM16 indicate the boundaries of the TM regions calculated by averaging the predictions from the following programs: DAS, HMMTOP, SPLIT, TOPPRED, TMHM, PREDTMR, SOSUI and MMETSAT. Circles indicate the position of residues important for ion selectivity (red) and for Ca^{2+}-sensitivity (blue). Red highlight: complete conservation; yellow: high homology and cyan: low homology. B) The position of residues important for ion selectivity is shown as red circles: K645, K668 [5]; K588 [40]; C625, G628, G629, C630, M632 [14]; the controversial residue R621 is shown as an orange circle [5, 14]. C) Residues important for Ca^{2+}-sensitivity are shown as blue circles: 444EEEEEAVK454 [45, 46]; E702, E705 [14, 22, 33, 40]; E734, D738 [25]; I735, V752, A754 [21]. In both cases the most recent TMEM16 topological model is utilized.