Ca^{2+}-dependent phospholipid scrambling by a reconstituted TMEM16 ion channel

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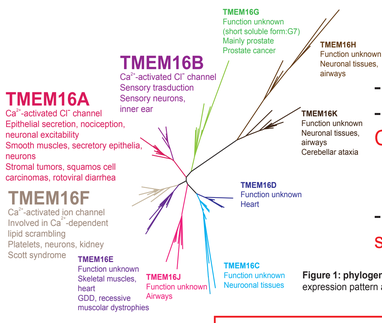
ABSTRACT

Phospholipid scramblases collapse the plasma membrane lipid asymmetry, externalizing phosphatidylserine to trigger blood coagulation and mark apoptotic cells. Despite their importance in cell physiology, the molecular identity of the scramblases has eluded researchers for decades. TMEM16F, a member of the TMEM16 family of Ca2+-gated channels, has been shown to be involved in lipid scrambling. The function of TMEM16F remains controversial, as it has been also reported to be a Ca2+-dependent cation channel and three different Cl- channels. Two other members of the TMEM16 family, TMEM16 A and B, are Ca2+-activated Cl- channels and they do not seem to be involved in lipid scrambling. Whether TMEM16F, and possibly other members of the family, are phospholipid scramblases or ion channels that regulate scrambling activity remains unclear.

To differentiate between these hypotheses we expressed, purified and reconstituted several TMEM16 family members and discovered that purified afTMEM16, from Aspergillus fumigatus, is a dual-function protein: it is a Ca2+-gated channel, with characteristics of other TMEM16 homologues, and a Ca2+-dependent scramblase, with the expected properties of mammalian phospholipid scramblases. Remarkably, we find that a single Ca2+ site, conserved among the TMEM16 homologues, regulates separate transmembrane pathways for ions and lipids. Two other purified TMEM16-channel homologues do not mediate scrambling, suggesting that the family diverged into channels and channel/scramblases.

Our results demonstrate for the first time that a member of the TMEM16 family is simultaneously a Ca2+dependent ion channel and a Ca2+ dependent lipid scramblase. We propose that the spatial separation of the ion and lipid pathways underlies the evolutionary divergence of the TMEM16 family, and that other homologues, such as TMEM16F, might also be dual-function channel/scramblases.

TMEM16 family: Ca^{2+}-gated ion channels and their involvement in phospholipid scrambling



- Most of the TMEM16s have unknown function
- All the characterized TMEM16 members are Ca^{2+}-gated channels:
  - TMEM16A and B : Ca^{2+}-activated Cl^- channels
  - TMEM16F: Ca^{2+}-activated ion channel
- TMEM16F : involved in Ca^{2+}-dependent lipid scrambling.

Figure 1: phylogenetic tree of vertebrate TMEM16 family members. Each branch contains the known or proposed functions, expression pattern and diseases associated with the protein. *GDD: GnathoDiphyseal Dysplasia* (adapted from *Duran et al.*)

PHOSPHOLIPID SCRAMBLING

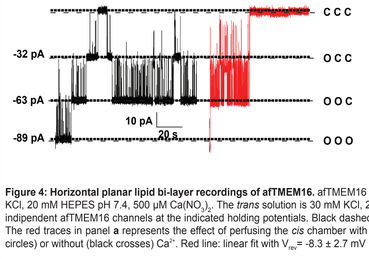


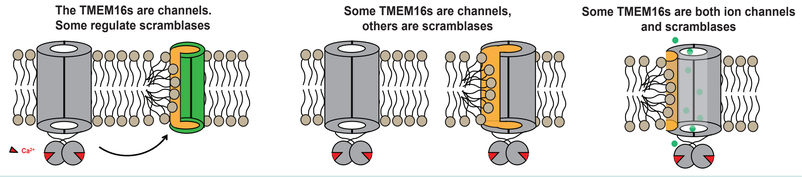
Figure 2: phospholipid scrambling. a: the lipid composition of all cell membranes (a typical erythrocyte membrane lipid composition is shown) is asymmetric and upon activation of scramblases the asymmetry collapses. **b:** classification of lipid-moving enzymes. Flippases and floppases move lipid only in one direction and they are lipid-specific while scramblases move all kinds of lipid bidirectionally

Phosphatidylserine exposure:

- Blood coagulation via recruitment of clotting factors (TMEM16F mutation causes Scott syndrome, a bleeding disorder associated with defective lipid scrambling)
- Marker of apoptosis: binding of Annexin V

QUESTION: is TMEM16F a scramblase or a key regulator of scrambling activity?

Can we explain the presence of ion channels and scramblases within the TMEM16 family?



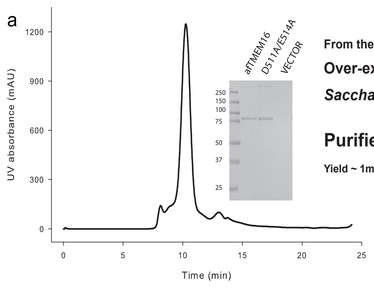
How can we solve this puzzle?

Biochemical approach :purification and functional reconstitution of TMEM16F to DIRECTLY measure scrambling activity

We could not purifiy TMEM16F, but other homologues might have scrambling activity: screening of TMEM16 homologues suitable for purification and functional reconstitution

afTMEM16

Purification of afTMEM16



From the fungus *A. fumigatus*

Over-expression system :

Saccharomyces cerevisiae

Purified in DIGITONIN

Yield ~ 1mg/L of culture

Figure 3: Purification of afTMEM16 and its homology with human TMEM16s. a: Size-exclusion cromatography profile of afTMEM16. The protein was exactred in 1% digitonin and, after cleaving the 8 His-tag via overnight TEV treatment, was run on a Sephadex S-200 size exclusion column. *Inset:* SDS-PAGE gel of afTMEM16 WT, double mutant and vector alone. The predicted MW for afTMEM16 is ~85 kDa. **b**: putative topology of TMEM16s according to Yu et al (2012) and homology of the putative transmembrane segments of afTMEM16, hTMEM16A, B, E and F

afTMEM16 IS a Ca^{2+}-ACTIVATED CHANNEL

SINGLE CHANNEL RECORDINGS IN PLANAR LIPID BI-LAYERS

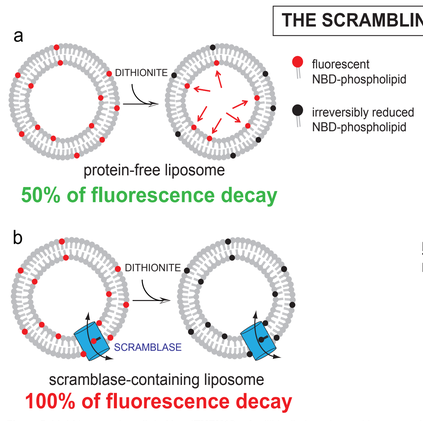
I ~ 30 pA
Conductance ~ 300 pS
P_O(-100 mV)~0.3
P_O(V>-50 mV)~1

Non selective channel:
V_{rev}(300 KCl/30 KCl)~ -9 mV
P_{K^+} / P_{Cl^-} ~ 1.5

Figure 4: Horizontal planar lipid bi-layer recordings of afTMEM16. afTMEM16 was reconstituted at 5 ug/mg of lipid (*E. coli* polar lipid:EGG PC 3:1). The *cis* (upper) solution is 300 mM KCl, 20 mM HEPES pH 7.4, 500 μM $Ca(NO_3)_2$. The *trans* solution is 30 mM KCl, 20 mM HEPES pH 7.4, 500 μM $Ca(NO_3)_2$. Sampling rate: 0.1 ms. Filter : 1 KHz. **a-b:** recordings of three indipendent afTMEM16 channels at the indicated holding potentials. Black dashed lines indicate conductance levels. The states of the channels are indicated by O (open) or C (closed). The red traces in panel **a** represents the effect of perfusing the *cis* chamber with 1 mM EDTA. **C**. Current-Voltage plot of afTMEM16 in the presence of a 10-fold KCl gradient with (red circles) or without (black crosses) Ca^{2+}. Red line: linear fit with V_{rev}= -8.3 ± 2.7 mV

afTMEM16 IS A Ca^{2+}-DEPENDENT LIPID SCRAMBLASE

THE SCRAMBLING ASSAY



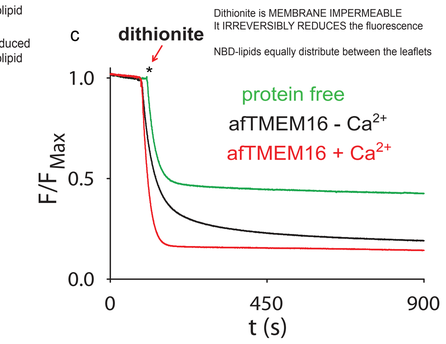
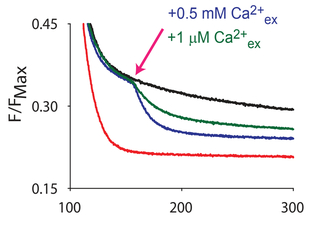


Figure 5: Lipid transport mediated by afTMEM16. a-b: dithionite-based scramblase assay. NBD- lipids (red) are irreversibly reduced (black) by dithionite. In protein-free liposomes only outer-leaflet fluorophores are reduced (a) while in afTMEM16 proteoliposomes all fluorophores are inactivated (b). **c:** fluorescence loss of NBD-posphatidylethanolamine (PE): green, protein-free liposomes with 0.5 mM Ca^{2+}; afTMEM16 proteoliposomes with 0.5 mM Ca^{2+} (red) or in 0 Ca^{2+} (black). * denotes addition of dithionite.

Ca^{2+}-dependence of phospholipid scrambling



afTMEM16 +Ca^{2+} : τ_{Fast} ~ 20s
afTMEM16 -Ca^{2+} : τ_{Fast} ~ 20s ; τ_{Slow}~ 400s

scrambling rate $\gamma = N_{Lipid}/(N_{Protein}*\tau)$

$\gamma(+Ca^{2+})$ > 10^4 lipid/s
$\gamma(-Ca^{2+})$ ~ 400 lipid/s

Ca^{2+} increases scrambling rate by > 20 fold

Figure 6: Ca^{2+}-dependence of phospholipid scrambling. Scrambling in symmetrical 0.5 mM Ca^{2+} (red) or 0 Ca^{2+} (black). The blue and green traces correspond to experiments started in symmetrical 0 Ca2+ and at t~ 160 s 0.5 mM (blue) or 1 μM (green) Ca2+ex is added to the extraliposomal solution. Dithionite was added at τ=100 s in all cases and is not shown. For clarity, only the F/F_{max} interval between 0.15 and 0.45 is shown. To determine the scrambling rate (γ) the number of lipids in one liposome (N_{Lipid}) is assumed to be ~10^6 and the number of proteins in one liposomes ($N_{Protein}$) is 6

Fluorescence decay IS NOT due to dithionite permeation through the channel

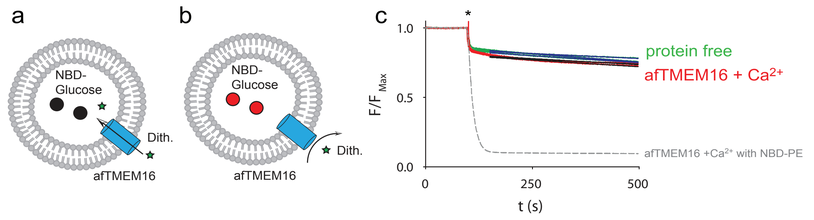
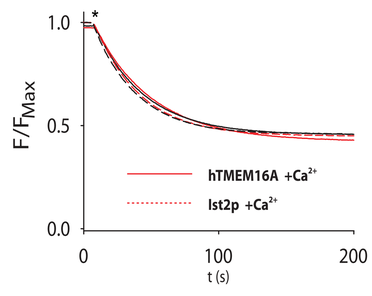


Figure 7: Dithionite does not permeate through afTMEM16. Schematic representation of the NBD-Glucose accessibility experiments. If dithionite permeates through afTMEM16 then it will enter the liposomes and reduce the NBD-Glucose leading to areduction in fluorescence (**a**). If dithionite does not permeate through afTMEM16 (**b**) then the fluorescence should remain constant. **c)** Replicate time courses of the NBD-Glucose fluorescence decay upon addition of dithionite (*) in protein free liposomes (green) and proteoliposomes containing 5 μg/mg afTMEM16 (red). All experiments were performed in the presence of 0.5 mM Ca^{2+} to fully activate afTMEM16. The fits are shown as blue (protein-free liposomes) and black (afTMEM16) lines. The dashed gray line is a trace of phospholipid scrambling by afTMEM16 in the standard condition conditions and is shown to illustrate the kinetic difference between the two processes.

Not all TMEM16s are phospholipid scramblases



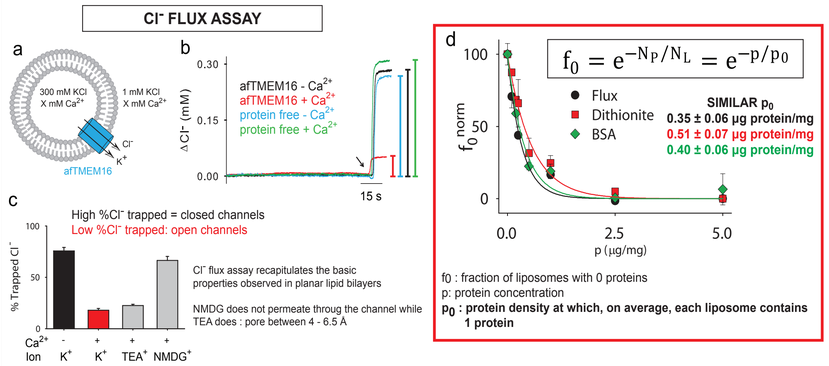
hTMEM16A (a known CaCC) and **Ist2p** (from Saccharomyces cerevisiae) are not scramblases

The TMEM16 family is split into Ca^{2+}-gated channels and dual function proteins/scramblases

Figure 8: functional divergence within the TMEM16 family. Fluorescence loss in liposomes reconstituted with hTMEM16A (solid lines) or Ist2p (dashed lines) in the presence (red) or absence (black) of Ca^{2+}. * denotes addition of dithionite.

afTMEM16 IS A DUAL FUNCTION PROTEIN

Cl^- FLUX ASSAY



Cl- flux assay recapitulates the basic properties observed in planar lipid bilayers

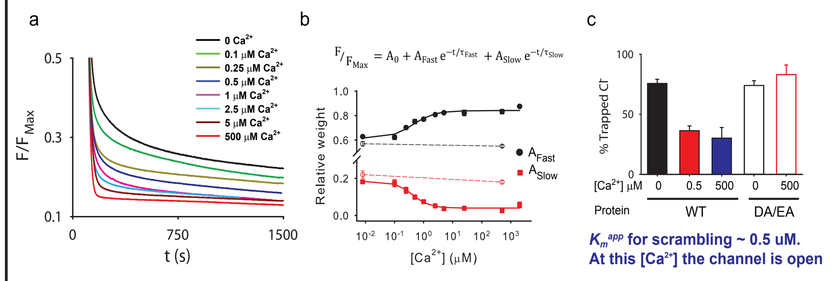
NMDG does not permeate throug the channel while TEA does : pore between 4 - 6.5 Å

f0 : fraction of liposomes with 0 proteins
p: protein concentration
p_0 : protein density at which, on average, each liposome contains 1 protein

Figure 9: afTMEM16 is a dual function protein. a: Cl^- efflux assay scheme. **b:** Measure of Cl^- content in protein-free liposomes in 0.5 (green) or 0 (blue) mM Ca^{2+} and in afTMEM16 proteoliposomes with 0.5 (red) or 0 (black) mM Ca^{2+}. Arrow denotes the addition of detergent to solubilize liposomes. Vertical bars represent the value of ΔCl^-. **c:** Fraction of trapped Cl^- in proteoliposomes reconstituted with or without 0.5 mM Ca^{2+} and with K^+, TEA+ or $NMDG^+$. **d:** Protein dependence of f_{0norm} measured by flux (black) and lipid scrambling (dithionite (red) or BSA (green) assays). Solid lines are fits to $f_{0norm} = e^{-p/p0}$. Average of 3-6 independent experiments, error bars are s.e.m.

A COMMON Ca^{2+}-BINDING SITE CONTROLS BOTH CHANNEL and SCRAMBLING ACTIVITIES

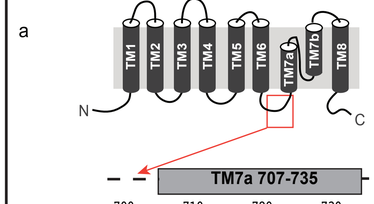
Ca^{2+} MODULATION of CHANNEL and SCRAMBLING ACTIVITIES



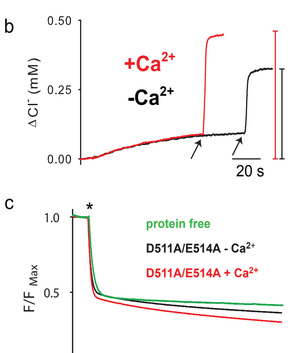
K_m^{app} **for scrambling ~ 0.5 uM. At this [Ca^{2+}] the channel is open**

Figure 10: Ca^{2+}-modulation of afTMEM16: a: phospholipid scrambling by afTMEM16 at the indicated Ca^{2+} concentrations. **b:** Ca^{2+}-dependence of A_{Fast} (black circles) and A_{Slow} (red squares). Solid lines represent fits to a Hill equation with $K_{m^{App}}$(Fast)=0.44 ± 0.13 μM, n(Fast)=1.2 ± 0.4 and $K_{m^{App}}$(Slow)= 0.44 ± 0.07 μM, n(Slow)=1.5 ± 0.3. WT afTMEM16: solid symbols and lines; D511A/E514A mutant: empty symbols and dashed lines. **c:** Ca^{2+}-dependence of the Cl^- trapped in liposomes with wild-type afTMEM16 or the D511A/E514A mutant.

D511A/E514A mutant shows altered Ca^{2+}-sensitivity in channel and scrambling assays



Neutralization of this di-acidic motif severly alters Ca^{2+}-sensitivity in TMEM16 A and F



liposomes retain Cl^- gradient regardless of the presence of Ca^{2+}

Scrambling severly reduced (slow, Ca^{2+}-indipendent component)

Channel and scrambling activities have similar selectivity for **divalent ions**

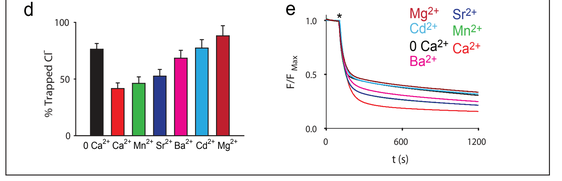
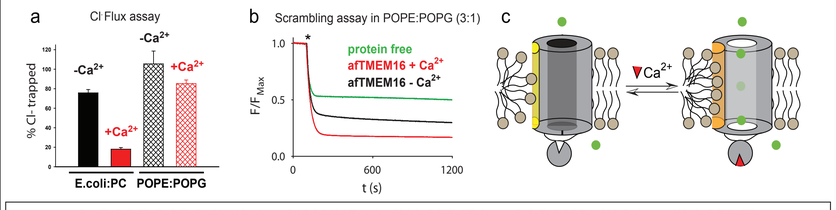


Figure 11: A common Ca^{2+} binding site regulates channel gating and phospholipid scrambling by afTMEM16. a: positions of the di-acidic pair (*) important for Ca^{2+}-sensing in TMEM16 proteins. **b-c:** Cl^- efflux (b) and phospholipid scrambling (c) mediated by the D511A/E514A mutant with 0.5 (red) or 0 (black) mM Ca^{2+}. **d-e:** Cl^- efflux (b) and phospholipid scrambling (c) mediated by afTMEM16 WT with different divalent ions. * denotes addition of dithionite.

ION and LIPID PATHWAYS are SEPARATE



In POPE:POPG (3:1) the CHANNEL is INACTIVE but SCRAMBLING is still ACTIVE

Blocking one pathway does not block both functions (as predicted with one common pathways for ions and lipids

Figure 12: Separation of the ion and lipid pathways of afTMEM16: a-b: Cl^- efflux (a) or phospholipid scrambling (b) by afTMEM16 in PE:PG with 0.5 (red) or 0 (black) mM Ca^{2+}. In a the WT values of Cl^- trapped are shown as a comparison. **c**: Cartoon representation of the structural bases of the dual function of afTMEM16. With no Ca^{2+} the channel is closed (black) and the lipid pathway (yellow) is partly occluded. Ca^{2+} (red) binding to a single site opens the channel (white) and lipid pathway (mustard).

CONCLUSIONS

- afTMEM16 IS A DUAL FUNCTION PROTEIN: a Ca^{2+}-gated channel and a Ca^{2+}-dependent phospholipid scramblase.
- Ca^{2+} increases the scrambling rate by > 20 fold compared to scrambling in 0 Ca^{2+}.
- A common Ca^{2+} binding site regulates both functions.
- Not all TMEM16s are scramblases: the family is divided in ion channels and dual function proteins/scramblases
- Ion and lipid pathways are separate.
- Our data raise the possibility that other TMEM16s, specifically TMEM16F, might be dual function proteins. Dual function TMEM16 channels/scramblases directly couple electrical, lipid and Ca^{2+} signaling through one single protein.

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