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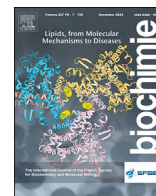
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# Established and emerging players in phospholipid scrambling: A structural perspective



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## ABSTRACT

The maintenance of a diverse and non-homogeneous lipid composition in cell membranes is crucial for a multitude of cellular processes. One important example is transbilayer lipid asymmetry, which refers to a difference in lipid composition between the two leaflets of a cellular membrane. Transbilayer asymmetry is especially pronounced at the plasma membrane, where at resting state, negatively-charged phospholipids such as phosphatidylserine (PS) are almost exclusively restricted to the cytosolic leaflet, whereas sphingolipids are mostly found in the exoplasmic leaflet. Transbilayer movement of lipids is inherently slow, and for a fast cellular response, for example during apoptosis, transmembrane proteins termed scramblases facilitate the movement of polar/charged lipid headgroups through the membrane interior. In recent years, an expanding number of proteins from diverse families have been suggested to possess a lipid scramblase activity. Members of TMEM16 and XKR proteins have been implicated in blood clotting and apoptosis, whereas the scrambling activity of ATG9 and TMEM41B/VMP1 proteins contributes to the synthesis of autophagosomal membrane during autophagy. Structural studies, *in vitro* reconstitution of lipid scrambling, and molecular dynamics simulations have significantly advanced our understanding of the molecular mechanisms of lipid scrambling and helped delineate potential lipid transport pathways through the membrane. A number of examples also suggest that lipid scrambling activity can be combined with another activity, as is the case for TMEM16 proteins, which also function as ion channels, rhodopsin in the photoreceptor membrane, and possibly other G-protein coupled receptors.

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## 1. Introduction

Biological membranes are composed mostly of phospholipids, sphingolipids, sterols and proteins, with selective permeability to the environment. Their composition results in a bilayer structure where the two leaflets are in contact via the apolar acyl chains of their constituent lipids, while the polar head groups face the hydrophilic environment. The lipid composition is adapted to specific membrane functions; for example, the high concentration of sterols and saturated lipids in the plasma membrane supports its role as a barrier. Another fundamental aspect of biological membranes is the different lipid composition of their two leaflets, known as transbilayer lipid asymmetry [1,2]. For instance, in the plasma membrane (PM) of resting eukaryotic cells, phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) are almost exclusively found in the cytosolic leaflet [3,4]. However, (patho)physiological events such as activation of the caspase proteolytic cascade or an increase in the cytosolic calcium concentration can disrupt this asymmetry and lead to PS exposure in the exoplasmic leaflet, a known signal for blood clotting and apoptosis [5].

Due to the hydrophobicity of the membrane core, transfer of polar lipid head groups between leaflets (known as flip-flop) is energetically unfavorable and therefore very slow [6,7]. Membrane asymmetry is a non-equilibrium state and as such, it has to be maintained by active transporters. For that purpose, eukaryotic organisms express ATP-dependent transporters, including flippases, which catalyze unidirectional lipid transport towards the cytosolic leaflet, and floppases, which move lipids towards the exoplasmic leaflet. Conversely, membrane asymmetry may be disrupted (for example during apoptosis) by energy-independent transporters called scramblases, which move lipids down their electrochemical potential [8]. Most flippases belong to the P4-ATPase subfamily of P-type ATPases, whereas floppases belong to the ATP-Binding-Cassette (ABC) family of transporters. *In vitro* reconstitution assays have shown that flippases and floppases move specific lipids at a rate of 10–100 lipids per second against their concentration gradient. In contrast, energy-independent scramblases facilitate the bi-directional movement of lipids at a rate of ~10,000 lipids per second [9,10]. Such a high transport rate enables fast apoptotic response, mentioned above, and also supports membrane expansion at the endoplasmic reticulum (ER), where phospholipids need to cross the membrane rapidly while being synthesized on the cytosolic leaflet of the ER membrane [11].

The first protein suggested to act as a PL scramblase is known as PLSCR. PLSCR is a single-pass transmembrane protein with the transmembrane span near the C-terminus. Upon reconstitution in proteoliposomes, Ca<sup>2+</sup>-induced PL scrambling was observed [12]. However, the disruption of PLSCR in different organisms has little effect on PS exposure making it highly unlikely that PLSCR functions as a scramblase [13,14].

Scramblases have long been elusive, and only recently have the molecular identities of some of them been revealed. Menon and co-workers demonstrated lipid scrambling activity of the G protein-

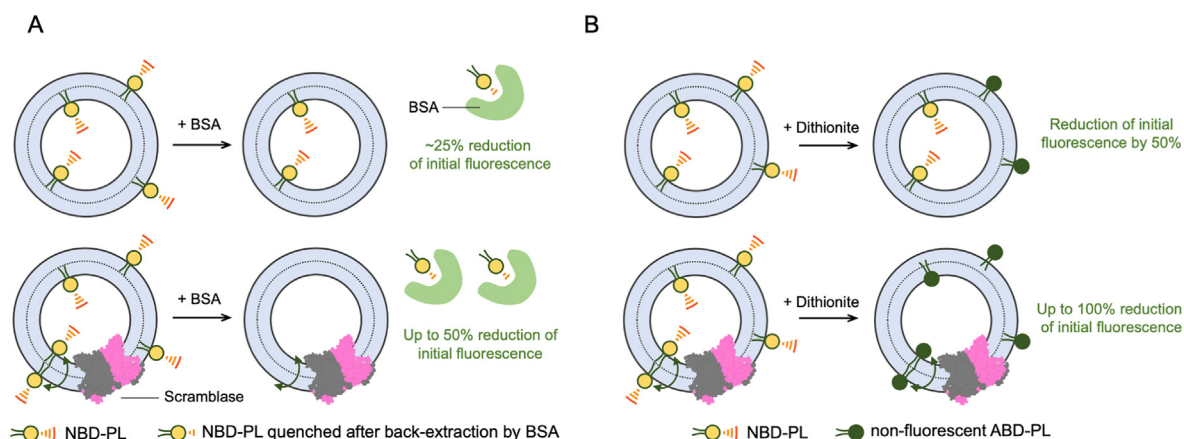
coupled receptor (GPCR) opsin after its purification and reconstitution in proteoliposomes [15]. At the same time, Suzuki and colleagues identified two candidate scramblases, Xkr8 and TMEM16F, from cell lines that strongly exposed PS on their surface [16,17]. Since then, several TMEM16 protein members have been characterized both functionally and structurally, revealing exquisite details of the lipid transport mechanism [18]. New players are also entering the game, as unrelated proteins from diverse families such as TMEM41B/VMP1, lactose permease, VDAC1/2 and protein insertases have been proposed to scramble lipids [19–22]. The diversity of protein folds involved in lipid scrambling suggests a diversity of scrambling mechanisms.

In this review, we discuss the different mechanisms at play within the established phospholipid scramblase families, with emphasis on TMEM16 and XKR proteins, and we shed light on emerging candidates for lipid scrambling activity. We highlight how molecular dynamics (MD) simulations combined with *in vitro* transport assays and high-resolution structural data have greatly improved our understanding of the lipid scrambling mechanisms.

## 2. The TMEM16 family of lipid scramblases

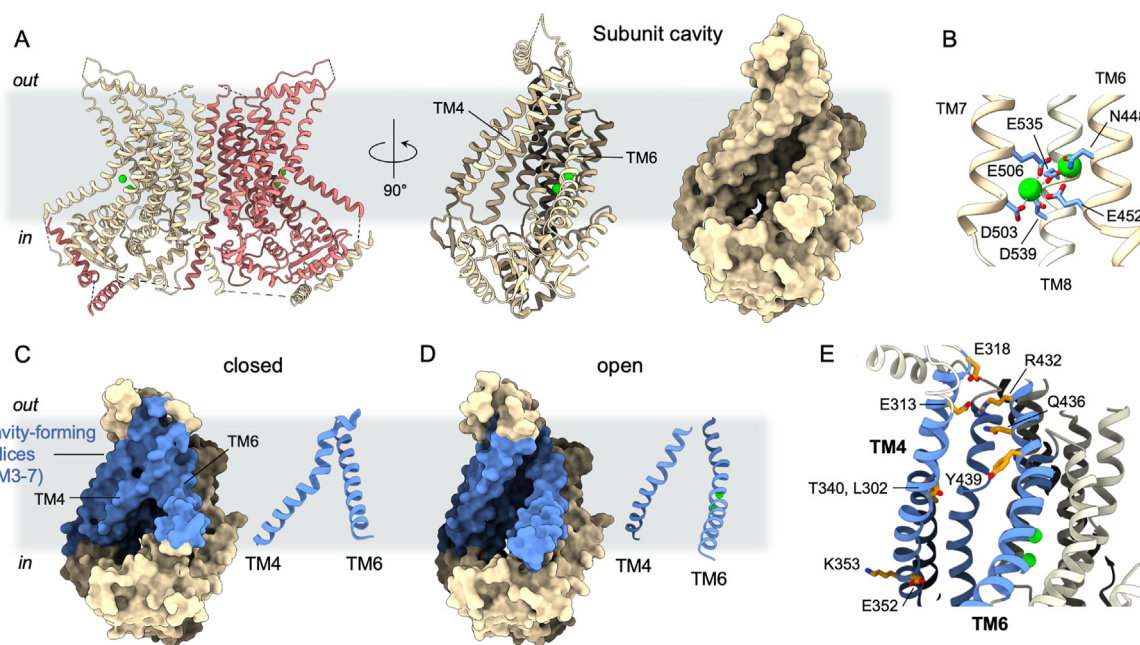
Initial characterization of the TMEM16 protein family revealed that TMEM16A is a Ca<sup>2+</sup>-activated chloride channel [23–25]. TMEM16 proteins were originally termed ‘anoctamins’, because of their anion-specific activity and an erroneous prediction of eight transmembrane spans, now revised to ten by structural studies (see below). There are ten TMEM16 family members, termed TMEM16A–K (excluding I). However, only TMEM16A and TMEM16B have been shown to be Ca<sup>2+</sup>-activated chloride channels, and most members of the family function as dual phospholipid scramblases and poorly selective cation channels, catalyzing bidirectional lipid flip-flop in an energy-independent manner (Fig. 1). TMEM16 scramblases do not distinguish specific head groups, as lipids as diverse as PS, PE, (phosphatidylcholine) PC, phosphatidylglycerol (PG) or the cationic lipid DOTAP (dioleoyl trimethylammonium propane) may be transported at rates exceeding 2.10<sup>4</sup> lipids s<sup>-1</sup> [9,26–29]. Noteworthy, TMEM16 scramblases also possess the ability to conduct ions, albeit with a low specificity. All TMEM16 proteins characterized so far are activated by Ca<sup>2+</sup> [18].

The first high-resolution X-ray structure of a TMEM16 protein was that of a fungal scramblase from the plant pathogen *Nectria haematococca* (nhTMEM16) [27]. It confirmed the previously proposed homodimeric structure of TMEM16 proteins [33]. The protein displayed a novel fold with ten membrane-spanning segments (Fig. 2A). Two Ca<sup>2+</sup> ions were bound to a conserved site contributed by amino acids located in TM6, 7 and 8 of each protomer [27] (Fig. 2A and B). Two other regions of interest were the dimer interface between the two subunits and the subunit cavities, which face the lipid bilayer at both sides of the dimer in the shape of spiral staircases. The properties of the subunit cavity, formed by TM3 to 7, supported a so-called ‘credit card’ scrambling mechanism [34], with the cavity shielding the polar head groups of phospholipids, thereby lowering the energy barrier for lipid movement across



**Fig. 1.** Principle of proteoliposome-based lipid transport assays.

For monitoring scrambling activity, the most common approach is to use fluorescent probes bound to lipids in the liposomal membrane. The most widely used fluorophore used in these assays is nitrobenzoxadiazole (NBD), which can be placed either at the polar head group or at the fatty acid chain of the lipid. Usually, NBD-lipids are used in trace amounts to avoid light-induced artifacts (usually 0.3 mol% to 1 mol%). Bovine serum albumin (BSA) back-extraction and dithionite bleaching are the most commonly used assays for testing scrambling activity [9,30,31]. (A) Fluorescence quenching by fatty acid-free BSA takes place after lipid extraction from the external membrane leaflet. The lipid probe inside BSA's globular structure experiences a different environment that decreases its quantum yield by ~50%. Therefore, following complete probe extraction from the membrane, the maximum fluorescence quenching obtained is 50% of the initial fluorescence intensity. Due to the activity of a scramblase constantly shuttling lipids bidirectionally, 50% fluorescence quenching is the theoretical quenching expected upon reconstitution of a scramblase, assuming that every liposome harbors at least one protein molecule. Mock liposomes are expected to have a maximum quenching of 25%, resulting in 75% final fluorescence [19,21]. (B) In the dithionite assay, photobleaching is due to reduction of the NBD fluorophore into an aminobenzoxadiazole (ABD) group by dithionite. Like in the BSA back-extraction assay, this assay relies on dithionite bleaching the fluorescence of the probes located in the outer leaflet of the vesicles, but not in the inner leaflet, thus providing a read-out on transbilayer lipid distribution. Liposomes should therefore be impermeant to dithionite. Liposomes harboring a scramblase should have all the fluorophore molecules bleached by dithionite, while pure lipid liposomes should have only the exterior leaflet bleached (about 50% bleaching). Note that the size of the liposome will significantly affect the extent of fluorescence bleaching by dithionite [32].



**Fig. 2.** Architecture and lipid transport pathway in TMEM16 scramblases.

(A) nhTMEM16 structure in the open conformation (PDB 6QM9), displayed as ribbon. Left: side view. Each protomer of the homodimer is represented in a different color. Middle: 90° rotation of the structure displayed on the left, in order to show the subunit cavity. Only one of the two protomers is shown for clarity. Right: same as middle structure but shown as surface. The two  $\text{Ca}^{2+}$  ions are shown as green spheres. (B) Close-up view of the calcium binding site formed by TM helices 6, 7 and 8 (PDB 6QM9). The amino acids involved in  $\text{Ca}^{2+}$  binding have been numbered. (C) nhTMEM16 structure in the closed conformation (PDB 6QM4), displayed as surface, highlighting the cavity-forming helices (TM3-7 in blue) and the position of TM4 and TM6 in the absence of calcium. (D) nTMEM16 structure in the open conformation (PDB 6QM9), displayed as surface, highlighting the opening of the subunit cavity and the lipid transport pathway. The position of TM4 and TM6 in the presence of calcium is indicated on the side. (E) Close-up view at the open subunit cavity in nhTMEM16 (PDB 6QM9). Amino acids involved in lipid transport are displayed as orange sticks. Residues that form contacts with lipid polar head groups and potentially gating at the exoplasmic leaflet are E313, E318 and R432. Other interacting residues are L302 and T340 (in TM3 and TM4, respectively) at mid-point of the membrane, Q436 and Y439 at a constriction point of the groove, and E352 and K353 in TM4, potentially gating at the cytosolic leaflet. The approximate position of the membrane is shown as a grey shaded area.



leaflets (Fig. 2A). This mechanism was also proposed by Yu et al., who showed that a subdomain of TMEM16F, comprising TM4 and TM5, conferred lipid scrambling activity to TMEM16A [35]. However, the transport mechanism remained enigmatic as no lipids could be observed in contact with the subunit cavities.

### 2.1. Lipid permeation through the hydrophilic subunit cavity

The determination of TMEM16 protein structures in multiple conformations, together with molecular dynamics simulations, has illuminated our understanding of the lipid transport mechanism. The fungal nhTMEM16 reconstituted in nanodiscs was captured in three different conformations sampling different degrees of opening of the subunit cavity [36]. In the absence of  $\text{Ca}^{2+}$ , TM4 shielded the subunit cavity from the hydrophobic membrane core (Fig. 2C). In the transition from the closed to the open state, triggered by  $\text{Ca}^{2+}$  binding, the subunit cavity gradually becomes accessible to the membrane (Fig. 2D). In the open state, TM4 is held away from TM6 along its entire length, providing a pathway ~8–11 Å wide, large enough for lipid head groups of the major classes of lipids to cross the membrane, while in the closed and intermediate states, the extracellular sides of TM4 and TM6 interact with each other, closing the pathway [36] (compare Fig. 2C and D).

MD simulations also provided support for the role of the subunit cavity in shielding the lipid head groups from the membrane core [37]. All-atom MD simulations revealed two charged residues at the nhTMEM16 extracellular entry of the subunit cavity, E313 in TM3 and R432 in TM6, responsible for stabilizing and isolating PC lipids from the surrounding membrane lipid head groups, potentially acting as gating residues. At the cytoplasmic extremity of the groove, two other charged residues, E352 and K353 in TM4, played a similar role, interacting with nearby lipid head groups (Fig. 2E). Importantly, MD simulations revealed 20–40 ns long flipping events, both from the extracellular to the cytosolic leaflet and vice versa [37]. Other atomistic MD simulations of nhTMEM16 also suggested a prominent role of the subunit cavity for lipid scrambling, notably E313, E318 and R432 that form contacts with lipid polar head groups but also residues localized at mid-point of the membrane (e.g. L302 and T340 respectively in TM3 and TM4) as well as residues intermediate to these two positions, at a constriction point of the groove (Q436 and Y439 in TM6) (Fig. 2E) [26,38]. Further mutational analysis of TMEM16 proteins, combined with cell-based or proteoliposome-based lipid transport assays, pinpointed the pivotal role of subunit cavity residues in the transport process. Tryptophan scanning mutagenesis in TM3, TM4, TM5, and TM6 confirmed that E313 and R432 in nhTMEM16 are involved in extracellular lipid selection and act as gating residues, and that mutation of Y439 strongly impairs lipid permeation (Fig. 2E) [26]. As further evidence of the essential nature of the subunit cavity in lipid scrambling, mutating several bulky residues lining the conduction pore in TMEM16F to alanine proved to be strongly activating [39]. Other bulky residues of the subunit cavity, e.g. F518 in TM4 of the mouse TMEM16F (mTMEM16F), have been proposed to form an inner activation gate, blocking the access to the hydrophilic groove in the absence of  $\text{Ca}^{2+}$ , while  $\text{Ca}^{2+}$ -induced movements distanced TM4 from TM6, thus opening the hydrophilic groove [40]. Overall, replacing the residues that form the gate with smaller, more polar or charged groups, removed the steric hindrance and destabilized the gate, allowing constitutive scrambling of phospholipids, i.e. in a  $\text{Ca}^{2+}$ -independent manner [40,41]. Interestingly, replacing L543 in TMEM16A, equivalent to F518 in TMEM16F, with a lysine converted the  $\text{Ca}^{2+}$ -activated chloride channel into a lipid scramblase [40].

### 2.2. TMEM16-induced membrane deformations and out-of-the-groove transport

Bethel and Grabe used continuum-atomistic modeling to study the effects of nhTMEM16 on membrane deformations [37]. They found that nhTMEM16 induces a distortion of the membrane, with a more pronounced bending at the subunit cavity. Due to this distortion, the path from one leaflet to the other would decrease from 28.5 Å for a flat POPC membrane to 18.3 Å at the subunit cavity, thereby lowering the energetic cost for lipid movement through the membrane core. This ability of nhTMEM16 to modify the curvature and thickness of the surrounding lipid environment was also shown by experimental work, using nhTMEM16 either embedded in detergent micelles or in nanodiscs, and was also suggested by studies of other TMEM16 proteins including mTMEM16F [36,42–44]. Recent cryo-EM structures of nanodisc-reconstituted afTMEM16 from *Aspergillus fumigatus* revealed membrane thinning by ~50 % near the hydrophilic cavity [43], suggesting that membrane thinning acts in concert with opening of the cavity to facilitate lipid movement. Consistent with this hypothesis, increasing the thickness of proteoliposomes by using C22:1 instead of C18:1 acyl chains strongly reduced the scrambling activity of afTMEM16 in a proteoliposome-based lipid transport assay even in the presence of  $\text{Ca}^{2+}$  [42]. Since the cryo-EM structure of afTMEM16 in C22:1 nanodiscs in the presence of  $\text{Ca}^{2+}$  indicates that the groove is open, this shows that an open groove is not sufficient for efficient scrambling. Conversely,  $\text{Ca}^{2+}$ -independent scrambling did not require groove opening as scrambling was possible with a closed groove (only particle classes with a closed groove could be detected by cryo-EM). This argues that lipid scrambling in the absence of  $\text{Ca}^{2+}$  does not occur while the groove is transiently open but rather in an 'out-of-the-groove' manner [9]. An alternative out-of-the-groove pathway may help explain the origin of the basal,  $\text{Ca}^{2+}$ -independent scrambling activity of all TMEM16 proteins characterized so far, as well as the unexpected ability of afTMEM16 to facilitate bidirectional movement of phospholipids with polyethylene glycol (PEG) head groups, i.e. much larger than the width of the subunit cavity [9]. However, it remains to be established whether  $\text{Ca}^{2+}$ -independent, out-of-the-groove transport is physiologically relevant. The aforementioned exquisite sensitivity of afTMEM16 activity to the lipid composition of proteoliposomes makes it difficult to predict the level of  $\text{Ca}^{2+}$ -independent activity in cell membranes. Furthermore, such a  $\text{Ca}^{2+}$ -independent transport is difficult to reconcile with the strong phospholipid asymmetry generally observed in the PM at resting state.

## 3. The XKR family of lipid scramblases

While TMEM16F and several other TMEM16 family members are responsible for exposing PS in activated platelets, a scaffold for blood clotting enzymes, XKR family members including XKR4, 8 and 9 are associated with PS exposure in apoptotic cells, which serves as an eat-me signal for macrophages [45]. XKR8 interacts with Basigin (BSG), a type I membrane protein required for correct targeting of the complex to the PM [46]. While a wealth of structural information already exists for TMEM16 proteins, recent breakthroughs in the scramblase field are the elucidation of three-dimensional structures of an XKR8-BSG complex [47] and XKR9 [48]. Both XKR8 and XKR9 are stimulated by caspases via cleavage of a short C-terminal fragment of the protein, and XKR8 may be independently activated by phosphorylation on its C-terminus [49]. Another member of this family, XKR1, which is not activated by caspases, has recently been demonstrated to promote lipid scrambling in an *in vitro* reconstitution assay [50].

### 3.1. Architecture

The XKR8/BSG complex and XKR9 have been purified and the detergent-solubilized samples subjected to structural analysis by both X-ray crystallography and/or single particle cryo-EM. As expected, given the high sequence similarity between XKR8 and XKR9 (53 % similarity), the two proteins exhibit very similar structures. They display a novel fold, with a cuboid-like membrane domain made of 8 TM helices ( $\alpha$ 1-2,  $\alpha$ 4-7, and  $\alpha$ 9-10), 2 helices penetrating the membrane halfway ( $\alpha$ 3 and  $\alpha$ 8) and one cytoplasmic interfacial helix ( $\alpha$ 11) [47]. The C-terminal  $\alpha$ 11 helix, which contains the caspase cleavage site, sits in a groove formed by the cytoplasmic ends of TM2, 4, 5 and 6, thereby maintaining the protein in an inactive state [48,51]. Interaction between XKR8 and BSG is mediated by  $\alpha$ 9 from XKR8 and the TM span of BSG (Fig. 3A).

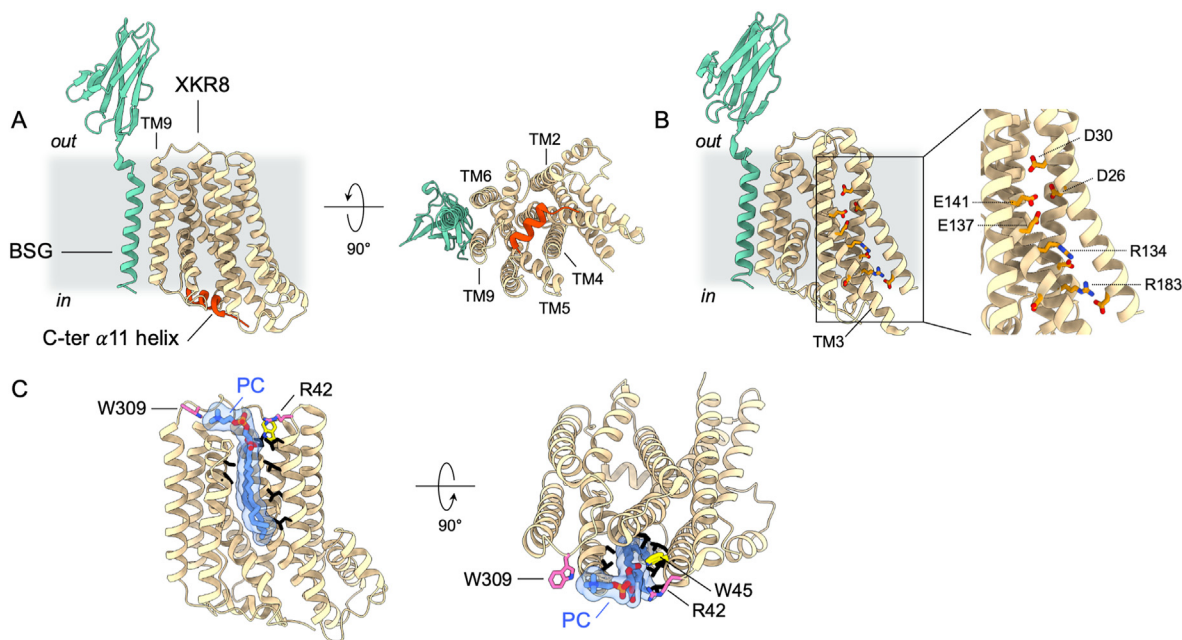
### 3.2. What do the structures tell us about the lipid transport pathway?

A rather unusual feature of XKR8 and XKR9 is the presence of a number of charged residues within their transmembrane domains, specifically TM3. For XKR8, the impact of mutating several of these residues was examined using a cell-based assay, revealing their prominent role in PS, PE and sphingomyelin (SM) scrambling at the PM [47]. These six residues are aligned in such a way that they line a possible path for phospholipid scrambling (Fig. 3B). The suggested involvement of acidic residues in scrambling activity implies that repulsive forces between these residues and the negatively charged phospholipid head groups or other acidic residues may widen the passage, facilitating phospholipid transit.

The structure of XKR8 also revealed the presence of a hydrophobic cleft contributed by residues located in  $\alpha$ 2,  $\alpha$ 4,  $\alpha$ 6,  $\alpha$ 7, where a density attributed to PC could be observed [47] (Fig. 3C). A lipid

bound at the same location was modelled in the density map of XKR9 as well, although with a different orientation of the head group [48]. Although the mutation of some of the residues of the hydrophobic cleft seemed to compromise the stability of the complex, some other mutations (e.g. W309A and R42A, see Fig. 3C) dramatically impaired PS exposure without seemingly altering the expression level and localization of the complex, raising the question as to whether the PC molecule plays a structural, stabilizing effect, or rather a role in the scrambling mechanism [47]. Among those residues interacting with the PC head group, mutating W45 to alanine had the striking effect of enhancing the scrambling of all lipids tested (PS, PE and SM). Interestingly, the W45A mutation makes XKR8 constitutively active, bypassing the requirement for phosphorylation of XKR8 C-terminal tail, indicating that this residue plays a critical role in the regulation of lipid scrambling [47].

In contrast to TMEM16 proteins, examination of the structure of XKR proteins does not reveal any obvious lipid transport pathway. For instance, whether the PC molecule identified in the structure represents a lipid on its way to the other side of the membrane or whether it is merely a stabilizing lipid is not clear. A further divergence between XKR and TMEM16 proteins stems from the fact that no membrane distortion was observed in the vicinity of the protein, either in detergent micelles or in nanodiscs [47,48,51], precluding an 'out-of-the-groove' transport mechanism. Also, the strong conservation within all XKR family members of some of the hydrophilic/charged residues in TM3 does not entirely support the idea that these residues play a specific role in phospholipid scrambling, which is only found in some family members. Along those lines, the structures of the full-length and caspase-cleaved XKR9 are essentially identical (RMSD 0.72 Å), suggesting that caspase cleavage is not sufficient to trigger conformational changes and that additional components are required for activation. In the case of XKR8, such component could be BSG but that does not seem



**Fig. 3. Structure and potential lipid transport pathways in XKR8.**

Ribbon structure of the XKR8-Basigin (BSG) complex (PDB 8XEJ). (A) Overall structure of XKR8-BSG highlighting the interaction of Basigin with TM9 of XKR8, and the  $\alpha$ 11 helix containing the caspase cleavage site at the C-terminus. The structure is displayed from the side (left) and from the cytosolic leaflet (right). BSG is in green while the  $\alpha$ 11 helix is in red. (B) Close-up view of the charged pathway in the transmembrane domain of XKR8. Residues D26, D30, K134, E137, E141 and R183 constituting the charged pathway and with a prominent role in lipid scrambling are displayed as orange sticks. Reentrant helix  $\alpha$ 3 is omitted for clarity. (C) Ribbon structure highlighting the hydrophobic cleft where a PC molecule binds. Hydrophobic residues forming the cavity are indicated in black. PC, in blue, is displayed as sticks and transparent surface. Residues W309, R42, and W45, which interact with the PC head group and are important for lipid transport are shown as pink (W309 and R42) or yellow (W45) sticks.

to apply to XKR9 as no interaction with BSG could be detected [48]. Noteworthy, XKR4 has recently been shown to require both cleavage of its C-terminus by caspases and binding of a caspase-derived fragment of the nuclear protein XRCC4 for its full activation [52]. It should also be stressed that neither XKR8 nor XKR9 exhibited phospholipid scrambling activity after purification and reconstitution in proteoliposomes, further substantiating a possible requirement for an as yet unidentified component for full activation, and raising the question as to whether the available structures for XKR proteins describe a state competent for lipid transport.

#### 4. G protein-coupled receptors (GPCRs) as moonlighting phospholipid scramblases

##### 4.1. Phospholipid scrambling by opsin

Rod photoreceptor cells are highly specialized ciliated cells with a specific and unique membrane lipid composition. The cilium consists of rod outer segments composed of disc membranes surrounded by a PM. The disc membranes contain long-/very long-chain polyunsaturated fatty acids and higher levels of PE than the PM [53,54]. In addition, the disc membranes are extremely densely packed with rhodopsin, the major protein component that converts visible light into a signaling cascade [53]. The ability of lipids to rapidly diffuse from one leaflet of photoreceptor disc membranes to the other has been noticed by Wu and Hubbel [55] and Hessel et al. [56], suggesting disc membranes are equipped with proteins facilitating the transbilayer movement of lipids. Given its abundance in photoreceptor disc membranes, opsin, a class A GPCR, emerged as a candidate for such activity. Whether purified from disc membranes or after expression in HEK293 cells and reconstitution in proteoliposomes, opsin exhibited ATP-independent phospholipid scramblase activity with poor head group selectivity: NBD-PC, -PS, -PE, -SM, as well as a bulky glycosylphosphatidylinositol (M4-H8) and PEG-conjugated lipids, could all be transported by opsin, at scrambling rates of  $\sim 10^4$ – $10^5$  lipids  $s^{-1}$ , surprisingly high considering that lipid scrambling is not the primary function of GPCRs [9,15,57]. Whether the inactive apo-protein opsin or different active/inactive versions of retinal-bound rhodopsin were used in *in vitro* scramblase assays, phospholipids could be transported in all cases, and at similar rates [57]. Therefore, lipid scrambling does not seem to depend on the protein's conformational state, although both opsin activation and lipid scrambling (see below) involve rather large motions of the intracellular end of TM6 [58–60]. This suggests that in contrast to TMEM16 proteins, opsin is a constitutively active scramblase. Furthermore, opsin reconstituted as oligomers in proteoliposomes and the use of opsin variants that lack the ability to self-associate revealed that both monomeric and oligomeric opsin are competent for transport of lipids *in vitro* [61,62].

##### 4.2. Contribution of MD simulations

There are currently no high-resolution structures of opsin with a lipid engaged in the translocation pathway, possibly due to the transient nature of phospholipid interaction with opsin, which acts more like a channel than a transporter. Iterative large-scale ensemble atomistic MD simulations enabled cumulative simulation times of  $\sim 50$   $\mu s$ , compatible with the detection of rare flipping events [63]. Analysis of MD trajectories suggests the presence of a pathway located between TM6 and TM7. The opening of this pathway is dynamic and would provide sufficient space for the lipid head group to slide from one side of the membrane to the other. While these *in silico* approaches provide valuable information on the transport mechanism, MD simulations need to be

experimentally validated to unambiguously identify the lipid transport pathway. Notably, the ability to scramble phospholipids is not restricted to opsin, as other class A GPCRs, namely the  $\beta_1$ - and  $\beta_2$ -adrenergic receptors, as well as the A2A receptor, have also been shown to transport lipids across the two leaflets of proteoliposomes [15,57]. Whether the ability to transport lipids is common to all class A GPCRs or can be extended to other GPCR classes remains to be determined.

##### 4.3. Modulation of GPCR lipid scrambling by cholesterol

A constitutive activity of opsin can be tentatively reconciled with the fact that it is required to counteract unidirectional lipid transport by ABCA4 and ATP8A2 towards the cytosolic leaflet of the highly specialized photoreceptor disc membranes, and thus promote membrane stability [64]. However, the proposed constitutive phospholipid scrambling activity of other class A GPCRs is puzzling. Most GPCRs reside at the PM, where a strong transbilayer asymmetry of PS has to be maintained, and the observation that GPCRs act as scramblases raises the question of the physiological relevance of such activity.

To help resolve this conundrum, the Menon and Khelashvili groups performed MD simulations of opsin in the presence and absence of cholesterol, which suggest that cholesterol binds at the interface between TM6 and TM7, thereby stabilizing a closed conformation of the groove and preventing lipid scrambling [65]. In this study, it was further revealed that the conformational space explored by opsin is restricted in the presence of cholesterol, and that the unexplored regions correspond to structures with an open translocation pathway.

Another recent study from the Menon group provides some experimental support for the effect of cholesterol on GPCR-mediated phospholipid scrambling [66]. Upon loading of opsin- or  $\beta_1$ -adrenergic receptor-containing proteoliposomes with cholesterol, a significant reduction of scrambling-competent proteoliposomes was observed. Interestingly, the observed inhibition by cholesterol appeared to happen beyond a given threshold (corresponding to about 25 mol% for opsin), in a general agreement with the concentration of cholesterol found in the PM ( $\sim 40$  mol%) and in the ER ( $\sim 5$  mol%). This observation would provide a basis for silencing of GPCR scramblase activity at the PM (and therefore maintenance of phospholipid asymmetry), whereas the low cholesterol concentration in the ER would not prevent GPCRs to scramble lipids to support membrane expansion or, in the specific case of photoreceptor disc membranes, to counteract the flippase activity of ABCA4 and ATP8A2. It also highlights the importance of the lipid composition when studying scramblase activity in proteoliposome-based assays.

#### 5. New scramblases identified in non-vesicular lipid transport pathways

In recent years, a number of new players have emerged with the unexpected ability to promote lipid flip-flop between bilayer leaflets. These include scramblases belonging to two evolutionary conserved families: ATG9 [19,67–71] and TMEM41B/VMP1 [19,72–74]. ATG9 is present in most eukaryotic species [75]. TMEM41B and VMP1 are eukaryotic integral ER membrane proteins that, individually or as a complex, function as lipid scramblases [19]. They both share a conserved domain belonging to the DedA superfamily, which is also present in archaea and in bacteria [76] and contains the only candidates for lipid scramblases in prokaryotes [77]. For example, a recent study in *Bacillus subtilis* used a fluorescent analog of duramycin as a probe to monitor surface-exposed PE, showing that the DedA protein PetA alters the



transbilayer distribution of PE [78].

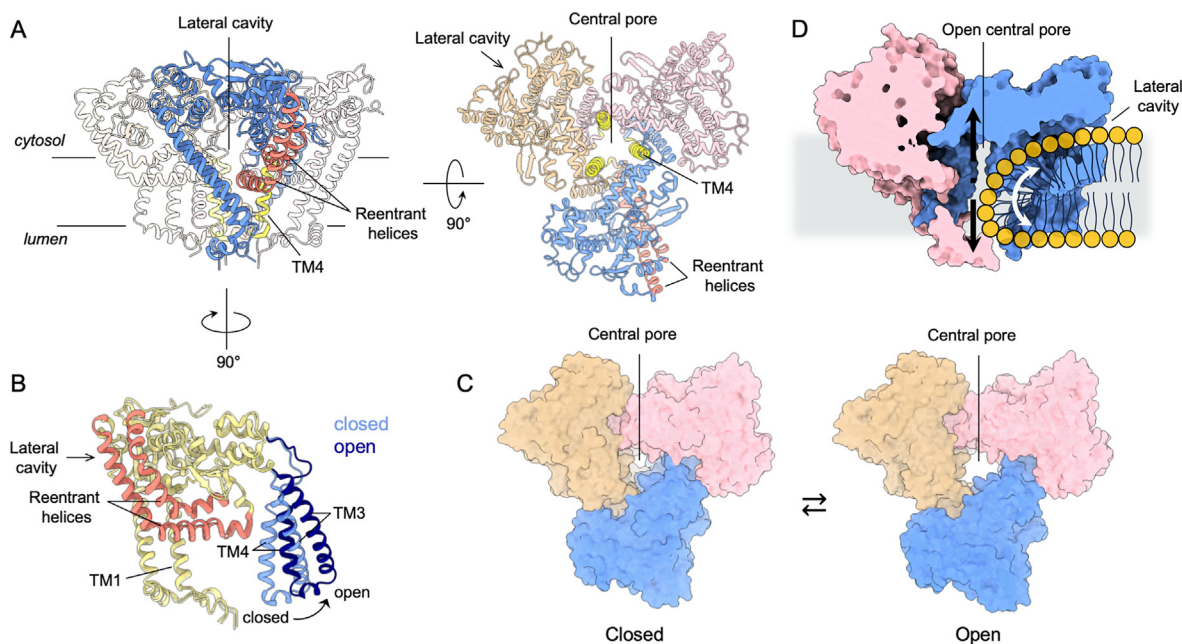
Interestingly, scramblases from both of these families are involved in the process of autophagy, which allows the degradation and recycling of molecular aggregates, organelles or cellular pathogens [79]. This process requires *de novo* formation of a bilayer membrane organelle to form the autophagosome. The new membrane can be derived from the ER or contributed by the Golgi-derived ATG9 vesicles. In addition, recent evidence from several different groups shows the requirement for lipid transfer by lipid transfer proteins (LTPs) between the cytosolic leaflet of the donor membrane and the newly formed organelle, and partnership between LTPs and lipid scramblases to equilibrate phospholipid distribution between both membrane bilayers [19,80,81]. One mechanism has been illustrated between the ER and the autophagosome where phospholipids are scrambled by the ER scramblases TMEM41B/VMP1, then transferred by the ATG2 LTP to the autophagosome, and finally equilibrated by the autophagosome scramblase ATG9 [19,69,70,81]. However, the ATG9-ATG2 complex appears to not be restricted to autophagy. A recent genetic study demonstrated that *ATG9* knock-out cells have impaired lipid distribution in lipid droplets (LD) and in mitochondria. Endogenous ATG9 can colocalize with the ER, the LD and the mitochondria, where it may regulate lipid homeostasis [82]. Whether these are direct or indirect effects remains to be further explored.

### 5.1. Structure and mechanism of ATG9/Atg9 scramblases

ATG9 is a eukaryotic protein with variable and disordered N- and C-terminal extremities, and a highly conserved central region composed of six transmembrane helices (TM), two of which are reentrant helices, anchored only into the cytoplasmic leaflet, and around ten cytoplasmic helices (Fig. 4A) [69,70]. ATG9 forms a

trimer within the bilayer membrane resulting in a very large central pore (~60 Å long) at the interface between the three protomers, in addition to three (40 Å long) lateral cavities, one per protomer, capable of accommodating amphipathic molecules (Fig. 4A) [69,70]. Each protomeric lateral cavity connects the trimer laterally to the central pore and residues responsible for this trimeric architecture are conserved among the ATG9 homologues. Furthermore, the central pore, connecting the cytosol and the lumen, is predominantly hydrophilic and exhibits an overall positive charge, which is consistent with a general model whereby scramblases possess a hydrophilic pathway at the surface of their TM regions [83]. Additionally, the structure of human ATG9A revealed two distinct conformations: open when obtained in detergent micelles and closed when obtained in nanodiscs (Fig. 4B and C). This distinction is based on hinge movements of the TM3-TM4 hairpin, which push TM3-TM4 away from TM1, resulting in an open central pore (Fig. 4B and C) [69]. Atomistic MD simulations led the authors to propose the following scrambling process: multiple lipids enter simultaneously into the lateral cavity, where the hydrophilic heads can diffuse while the hydrophobic tails stay inlaid in the membrane. Subsequently, they can cross the leaflet in a non-directional manner through a funnel-shaped solvent pool formed by the central pore connected to the three lateral pores (Fig. 4D) [69].

Dithionite scrambling assay indicates that Atg9 from *Schizosaccharomyces pombe* transports PE, PS, and PC from the inner to the outer leaflet of proteoliposomes, while quick-freezing and freeze-fracture replica labeling (QF-FRL) shows that Atg9 can also transport phosphoinositide-3-phosphate from outer to the inner autophagosome leaflet [70]. Phospholipid scrambling was also confirmed by other groups for the human homolog ATG9A [19,69] and its paralog ATG9B [67], the *Saccharomyces cerevisiae* Atg9 [71] and Atg9 from the plant fungus *Magnaporthe oryzae* [68]. Of note,



**Figure 4.** Structure and possible scrambling mechanism of ATG9A.

(A) Structures obtained from trimeric ATG9A reconstituted in nanodiscs, in a closed conformation (PDB:7JLP), shown as ribbon, and either viewed from the side (left) or from the cytosol (right). Membranes boundaries are indicated by solid lines. Re-entrant helices are shown in salmon color, while the pore-forming TM4 helix is displayed in yellow. In the side view, the lateral cavity is highlighted while in the view from the cytosol, the central pore is highlighted. (B) Overlay of open (detergent) and closed (nanodisc) ATG9A structures shown in ribbon representation, emphasizing movements of TM helices 3 and 4 associated with pore opening. For clarity, a single ATG9A protomer is shown. (C) View from the cytosolic side of the ATG9A trimer, highlighting opening of the central pore associated with TM3-TM4 movement. (D) Cutaway view of a hypothetical lipid transport pathway in ATG9A. Only two of the three protomers are shown for clarity. The white arrow depicts the connection between the lateral cavity and the central pore, whereas the black arrow shows the putative lipid flux within the central pore. (A, C and D) Each protomer of the ATG9A trimer is represented in a different color.



permeability assays as well as BSA back-extraction lipid scramblase assay ensured that dithionite quenching is not a consequence of dithionite permeation into proteoliposomes, as could be anticipated based on the large central pore observed in ATG9 [69,70].

Combinations of mutations of polar residues lining the central pore, changed to hydrophobic residues, have been shown to impair lipid transport, suggesting that scramblase activity of ATG9 relies on the hydrophilic pore properties [70]. Mutations in ATG9 that lead to autophagy deficiency often, but not always, correlate with impaired scramblase activity. In *S. cerevisiae*, such mutations in the Atg9 central pore impair both scrambling and the autophagy pathway [70], as do mutations in the equivalent human ATG9A [69]. Conversely, mutating the highly conserved residue F627, located in TM3 and not lining the central pore, leads to autophagy defect *in vivo* but does not block scramblase activity *in vitro* [84].

## 5.2. Interplay between scramblases and inter-organelle lipid transport

Scramblases also associate with other proteins involved in lipid homeostasis, such as LTPs. In a well-studied example, the ATG9A scramblase interacts with the channel-like cytosolic LTP ATG2A, which supplies lipids to the outer leaflet of an acceptor membrane organelle, thereby creating asymmetry with respect to the inner leaflet. To allow bilayer expansion, lipids are rapidly transported to the inner leaflet as they arrive in the outer leaflet [19]. The interaction with ATG9A is mediated by the C-terminal region of ATG2A, located close to the opening of its lipid transfer cavity. Although ATG2A can interact with ATG9A independently of membrane binding, the binding interface between ATG2A and ATG9A is required for ATG2A membrane binding and tethering, suggesting the necessity of this interaction for efficient autophagy. For ATG9A, the N-terminal part of the core domain and the disordered C-terminus are both interacting with ATG2A. However, the C-terminal domain appears to be less critical for the interaction and may play a regulatory role in the binding of ATG2A. Conversely, the N-terminal part of the core domain possesses a major ATG2A binding site, which is not required for scramblase activity [85]. Additionally, the residues responsible for efficient binding are also required for efficient autophagy. Finally, ATG2A can also interact with TMEM41B/VMP1 by its N-terminal region, linking the ER to the autophagosome through membrane contact sites [19].

Mainly studied during the autophagy process, ATG9A and ATG2A are not only involved in the interaction with TMEM41B/VMP1 at the ER, but also in the transfer of fatty acids from LDs to mitochondria. ATG2A can bind to the mitochondrial translocase TOMM40, establishing contact sites between mitochondria and ATG9A vesicles, close to mitochondria-associated ER membranes [86]. Genetic approaches have shown that ATG9A or ATG2A knockout cells exhibit impaired FA transfer, leading to an increase in the number and size of LDs. This phenotype is attributed to non-autophagic lipid transport from LDs rather than lipophagy defects (LD degradation), since the LD number and size in cells lacking ATG7 (essential for lipophagy) remains unaltered. Correlative light and electron microscopy (CLEM) experiments have further supported the hypothesis of ATG9A-ATG2A involvement in the control of lipid homeostasis between LDs and mitochondria by demonstrating close proximity between ATG9A vesicles and the ER, lipid droplets, or mitochondria [82]. The tethering property of ATG2A could mediate the interaction between ATG9A and TMEM41B or TOMM40 at contact sites between organelles. Despite the possibility of passive diffusion of fatty acids between bilayers, it is increasingly established that fatty acids can cross inter-membrane space through a protein-mediated mechanism that allows regulated intracellular translocation and uptake [87].

Interestingly, some TMEM16 scramblases have also been implicated in membrane tethering, which is important for lipid transport and also for  $\text{Ca}^{2+}$  signaling [88]. TMEM16H and TMEM16K both reside in the ER and have been shown to interact with the PM and the endosomes, respectively [89,90]. The molecular mechanisms of TMEM16-mediated membrane tethering remain to be explored. Conversely, in yeast, the TMEM16 homolog Ist2 contains an elongated cytosolic tail that tethers the ER to the PM and also interacts with the LTPs Osh6 and Osh7 to promote PS transport to the PM [91–94]. However, a lipid scrambling activity for Ist2 has so far not been demonstrated [29]. Finally, a recent study has shown that the LTP Vps13 or VPS13A, belonging to the same family as ATG2, which tethers ER and mitochondria, interacts with Mcp1 (outer mitochondrial membrane scramblase) or XKR1 (see chapter 3), in yeast and human, respectively [50].

## 5.3. Insights into TMEM41B/VMP1 scramblase structure and function

As previously mentioned, TMEM41B/VMP1 belong to the DedA superfamily. To date, no DedA domain protein has been structurally assessed. However, the DedA domain is predicted to consist of two canonical TM helices and two reentrant loops (helix-loop-helix) facing each other in the membrane. *In silico* and biochemical analyses predict that TMEM41B possesses an extramembrane helix at the N-terminus of the DedA domain [95], similar to chloride channel proteins, and two additional TM helices located upstream and downstream of the DedA domain [76]. It has been proposed that the TM helices form a positively-charged hydrophilic cavity allowing interaction with negatively charged molecules [72]. Regarding VMP1, the DedA domain is connected to four TM helices, three in the N-terminus and one in the C-terminus [77].

Experimental validation of TMEM41B/VMP1 scramblase activity has been only recently achieved using a dithionite scrambling assay [19,72–74,77] and in cells using metabolic labelling of PC with a clickable alkyl-choline combined with permeabilization of cell membranes with different detergents [72]. They both can scramble phospholipids *in vitro* in a non-specific ATP- and  $\text{Ca}^{2+}$ -independent manner [19,72,73]. In cells, deletion of TMEM41B or VMP1 also affected the distribution of cholesterol between different organelles, as assessed by the use of fluorescent probes, suggesting that they may regulate the physical properties of membranes [73].

Besides a role in autophagy, TMEM41B and VMP1 are involved in homeostasis of neutral lipids and lipoproteins [96,97]. It also has been suggested that they may indirectly contribute to protein transport through vesicle formation [98], since vesicle formation at the ER requires PL availability and therefore lipid scrambling [76].

## 5.4. Lipid scrambling at the outer mitochondrial membrane

Other newly identified scramblases include the  $\beta$ -barrel membrane proteins VDAC1 and VDAC2, which facilitate transport of metabolites through the outer mitochondrial membrane (OMM). Mitochondria rely in part on lipids supplied by the ER. For example, PS must be transported from the ER to the OMM before it is flipped towards the intermembrane space and decarboxylated to form PE. A rapid transverse movement of phospholipids, e.g. PC and PI, across the OMM was already observed years ago [99,100], but the identity of the OMM scramblase remained unknown. A recent report showed that human VDAC2 catalyzes energy-independent transport of PC and PI after reconstitution in proteoliposomes, with a scrambling rate of  $>10^3$  lipids  $\text{s}^{-1}$ . VDAC1 was also shown to transport NBD-PC, although less efficiently than VDAC2 [21]. This suggests that, as GPCRs, VDAC1 and VDAC2 moonlight as phospholipid scramblases. Owing to the fact that enforcing dimerization of

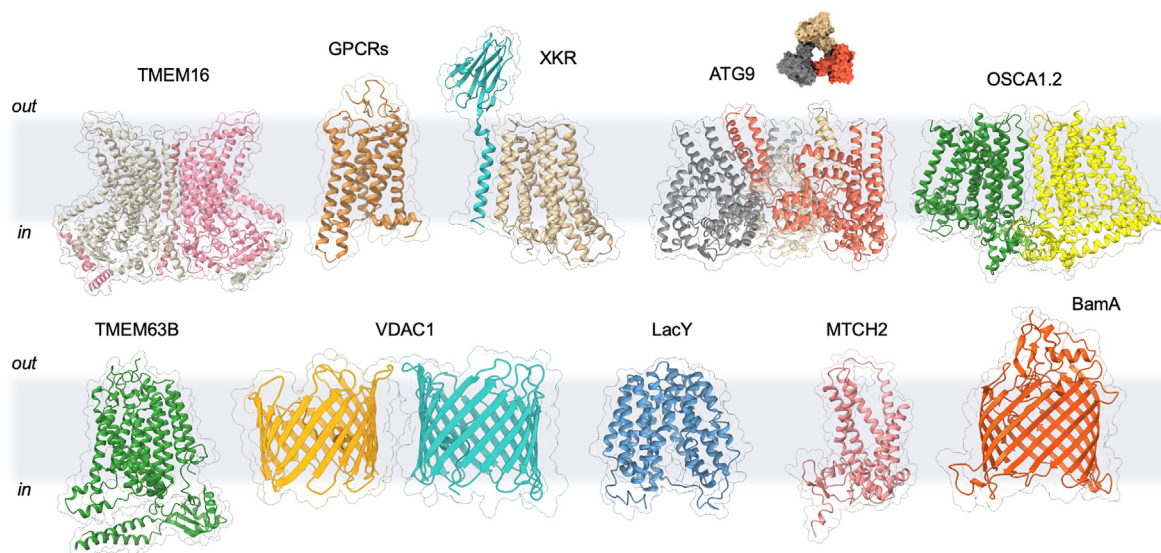
VDAC1 with cross-linkers revealed its lipid scrambling activity, an oligomeric structure seems required for efficient scrambling by VDAC proteins. Whether the same applies to VDAC2 remains to be studied. Such implication of VDAC multimers in lipid scrambling would suggest that the pore of VDAC does not constitute a pathway for lipid transport, in agreement with MD simulations, which predict that lipid flip-flop occurs at the interface between each protomer of the dimeric VDAC1. This also correlates with a pronounced thinning of the membrane in the vicinity of the dimer interface [21]. Specifically, the presence of a negative charge on the side chain carboxylate group of E73, a residue that is facing the dimer interface in VDAC1, leads to a marked thinning of the membrane around this residue, and this was shown to be accompanied by frequent lipid flip-flop events at this site [101]. However, the E84V mutation in VDAC2 (equivalent to E73V in VDAC1) had no detectable deleterious effect on lipid transport, suggesting that membrane distortion is not essential to promote rapid lipid flip-flop [21]. Future experiments are needed to identify and experimentally validate the lipid transport pathway in VDAC. Also, the chemical cross-linking approach could suffer from the possible influence of these chemicals on the protein structure, which could in turn be manifested by local distortion/thinning of the membrane. The role of VDAC-mediated lipid transport in a cellular context remains to be studied.

### Concluding remarks

The list of potential candidates for lipid scrambling activity is rapidly expanding. Besides the aforementioned new players in this field, very recent reports suggest that other unrelated protein families may be able to facilitate lipid scrambling in cell membranes. This is for example the case of the OSCA proteins from *A. thaliana* and their close TMEM63 homologues in higher eukaryotes, which had so far been described as ion channels that convert mechanical cues into electric signals [102]. OSCA/TMEM63 mechano-sensitive channels are close homologues of TMEM16 proteins, and the overall structure of OSCA1.2 from *A. thaliana* resembles that of TMEM16 proteins [103,104] (Fig. 5). Both form heterodimers, and ten transmembrane helices from OSCA1.2 (out of

11) follow closely the topology and organization of nhTMEM16 (Fig. 5). Reminiscent of the situation observed upon mutation of F518K in TMEM16F, which is proposed to form an inner activation gate (see above), introduction of single lysine mutation in the equivalent position in OSCA1.2 (L438K) resulted in PS exposure in cells overexpressing the OSCA1.2 mutant [105]. Strikingly, lipid scrambling has also been proposed to be a general feature of proteins called insertases, which catalyze the insertion of transmembrane proteins with  $\alpha$ -helical regions in membranes of the ER and of organelles that are disconnected from vesicular trafficking, such as mitochondria [22]. Combining biochemical reconstitution of candidate scramblases and *in vitro* lipid transport assays with coarse-grain (CG) MD simulations, Vanni and Reinisch groups suggested that several if not all members of different insertase families, including the ER GET complex, the recently identified mitochondrial insertase MTCH2 and the *E. coli*  $\beta$ -barrel membrane protein BamA (Fig. 5), support lipid scrambling in model membranes [22]. Using CG-MD simulations, it has been further proposed that the pathway taken by the translocated lipid is common to the pathway that promotes membrane protein insertion, i.e. through a hydrophilic transbilayer groove [22,106]. Finally, the well-characterized  $H^+$ /lactose symporter LacY has also been shown to rapidly equilibrate NBD-PC, -PE, and -PI across the membrane of proteoliposomes, a process that apparently occurs in a manner independent of  $H^+$ /lactose transport, thereby increasing the number of proteins with a moonlighting scramblase function [20].

This ever-growing number of proteins with *in vitro* scramblase activity poses the question of physiological significance. It should be stressed that for some putative scramblases described in this review, such as most of the XKR proteins, evidence that these proteins are able to transport lipids *in vitro* is still lacking. On the other hand, *in vitro* assays are especially problematic for constitutively active scramblases where transport cannot be triggered by a specific stimulus. In such cases, fluorescence bleaching above 50 % could be interpreted either as genuine scrambling activity or as an asymmetric distribution of NBD-lipids induced by the process of protein reconstitution into the liposomes. Iodide collisional quenching assay specifically addresses this issue [107]. The iodide ions exclusively interact with lipids in the outer leaflet of the



**Fig. 5.** The different folds associated with lipid scrambling.

Side view structures of several membrane protein families with a reported role as scramblases. The proteins displayed are as follows: nhTMEM16 (PDB 6QM9), rhodopsin (PDB 4J4Q), Xkr8-BSG complex (PDB 8XEJ), ATG9 (along with its view from the top) (PDB 7DOI), OSCA1.2 (PDB 6MGV), TMEM63B (PDB 8EHX), VDAC1 (PDB 5XDO), LacY (PDB 2CFQ), MTCH2 (AlphaFold model AF-Q9Y6C9), and BamA (PDB 6QGW). The approximate position of the membrane is shown as a grey shaded area.

liposome, but unlike in the dithionite or BSA back-extraction methods, they quench the fluorophores without oxidizing them or affecting their quantum yield, leaving the lipid probes free and intact. In protein-free liposomes, a 50 % decrease of fluorescence upon quenching of all outer leaflet probes with iodide ions is expected. For proteoliposomes, a similar 50 % fluorescence reduction is expected, as scramblases shuffle lipids back and forth constantly, leaving a 50/50 distribution of NBD probes at any time [107]. A fluorescence reduction exceeding 50 % would indicate that the NBD fluorophore exhibits a non-uniform distribution within the proteoliposomes. Consequently, a large fluorescence quenching observed using the dithionite assay does not necessarily imply lipid scrambling. Along the same lines, the extent of fluorescence quenching by dithionite (which is associated with the ratio of lipids in the outer leaflet of the proteoliposome over those in the inner leaflet) is dependent on the size of proteoliposomes.

We anticipate that such assays are fundamental to assess the involvement of a given protein in lipid scrambling. Other caveats may also arise from the available *in vitro* transport assays, owing to the sensitivity of TMEM16 and GPCR transport activity (and membrane proteins in general) to the lipid composition of proteoliposomes. In this context, further research is required to ascertain the physiological significance of  $\text{Ca}^{2+}$ -independent lipid scrambling activity exhibited by TMEM16 proteins, since this activity is largely influenced by the bilayer thickness, and proteoliposomes may not faithfully reflect the cell membrane properties.

Many more membrane proteins are likely to be identified as putative lipid scramblases, as suggested most recently by the work on protein insertases [22]. The diversity in the tertiary and quaternary structures adopted by the scramblases identified so far suggests a possible diversity in scrambling mechanisms. Combining structural analyses by cryo-EM with the increasing computational power of MD simulations, as well as cell-based and *in vitro* biochemical assays, is likely to contribute to the elucidation of lipid transport mechanisms by scramblases.

## CRediT authorship contribution statement

**Heitor Gobbi Sebinelli:** Writing – review & editing, Writing – original draft, Validation, Investigation, Conceptualization. **Camille Syska:** Writing – review & editing, Writing – original draft, Validation, Investigation. **Alenka Čopić:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition. **Guil-laume Lenoir:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

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