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Review

Exposure of phosphatidylserine on the cell surface

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Phosphatidylserine (PtdSer) is a phospholipid that is abundant in eukaryotic plasma membranes. An ATP-dependent enzyme called flippase normally keeps PtdSer inside the cell, but PtdSer is exposed by the action of scramblase on the cell's surface in biological processes such as apoptosis and platelet activation. Once exposed to the cell surface, PtdSer acts as an 'eat me' signal on dead cells, and creates a scaffold for blood-clotting factors on activated platelets. The molecular identities of the flippase and scramblase that work at plasma membranes have long eluded researchers. Indeed, their identity as well as the mechanism of the PtdSer exposure to the cell surface has only recently been revealed. Here, we describe how PtdSer is exposed in apoptotic cells and in activated platelets, and discuss PtdSer exposure in other biological processes.
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Facts

- ATP11A and ATP11C, members of the P4-ATPase family, act as flippases at the plasma membrane and are cleaved by caspase during apoptosis.
- Of the TMEM16-family proteins, which carry 10 transmembrane segments, TMEM16F and four other family members function as Ca^{2+}-dependent scramblases at the plasma membrane.
- TMEM16F exposes phosphatidylserine (PtdSer) and release microparticles on activated platelets for blood clotting. It is also involved in releasing hydroxyapatite in osteoblasts for bone mineralization.
- XK-related protein 8 (Xkr8) and two other Xkr-family proteins are cleaved during apoptosis and promote apoptotic PtdSer exposure.

Open Questions

- What are the physiological roles of the plasma membrane flippases that are present only in the brain and testis?
- What are the physiological roles of TMEM16-family members expressed only in the brain and intestine? Do their Ca^{2+}-dependent scramblase activity at the plasma membrane plays a specific role there?
- What are the physiological roles of Xkr-family members expressed only in the brain and intestine? Do their caspase-dependent scramblase activities play a specific role?
- Is PtdSer exposure in activated lymphocytes, pyrenocytes, aged reticulocytes, capacitated sperm, tumor-associated endothelial cells and enveloped viruses regulated by the P4-type ATPase, TMEM16 and Xkr families?
- How the flippases and scramblases translocate phospholipids between inner and outer leaflets of plasma membranes?

PtdSer is Distributed Asymmetrically in the Plasma Membrane

In eukaryotic cells, phospholipids in the plasma membrane are distributed asymmetrically.[1,2] The amine-containing phospholipids PtdSer and phosphatidylethanolamine (PtdEtn) are confined to the cytoplasmic leaflet of the plasma membrane, while phosphatidylcholine (PtdCho) and sphingomyelin (SM) are more concentrated in the exoplasmic leaflet. Phospholipid distribution in the plasma membrane is regulated by three types of phospholipid translocases.[3] Flippase (or aminophospholipid translocase) specifically translocates PtdSer and PtdEtn from the outer to the inner leaflet of the lipid bilayer, in an ATP-dependent manner. Floppase is ATP-dependent, and is thought to translocate phospholipids, especially PtdCho, from the inner to outer leaflet. Scramblase non-specifically translocates or scrambles phospholipids between the lipid bilayers in both directions, without consuming ATP.

Plasma Membrane Flippases and their Down-regulation by Ca^{2+} and Caspase

Red blood cells can incorporate aminophospholipids in an ATP-dependent manner,[4] and this ability was termed 'flippase' activity. Subsequently, flippase was abundantly detected in the chromaffin granules of bovine adrenal glands[5] and was partially purified. Since the purified granule flippase had an ATPase activity that was biochemically similar to ATPase II, a major ATPase in chromaffin granules,[6] ATPase II was

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Abbreviations: PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; SM, sphingomyelin; P4-ATPase, type IV P-type ATPase; TMEM, transmembrane protein; Xkr, XK-related; MEF, mouse embryonic-fibroblast; TNF, tumor necrosis factor
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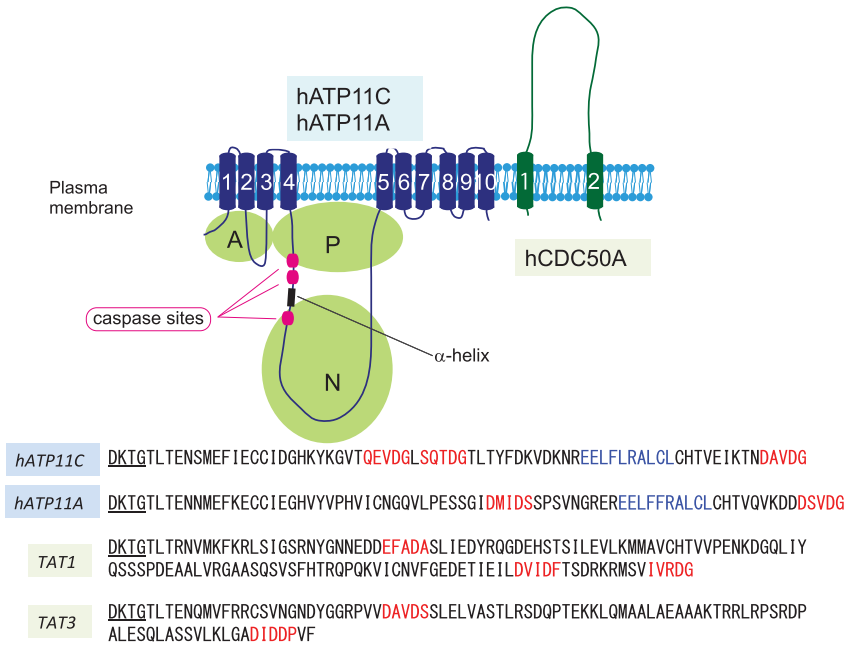

Figure 1 Structure of flippases and its cleavage by caspase. The structure of ATP11A/ATP11C and CDC50A is schematically shown. ATP11A and ATP11C carry 10 transmembrane segments. The ATPase domain in the cytoplasm is divided into A, actuator; N, nucleotide-binding; P, phosphorylation domains. CDC50A carrying two transmembrane regions functions as a chaperone for proper localization of ATP11A and ATP11C at plasma membranes. CDC50A forms a complex with ATP11A or ATP11C in the plasma membrane, and may be necessary for the flippase activity. ATP11A and ATP11C contain 2 and 3 caspase-recognition sites (Red) that flank a α-helix (Blue), and are cleaved during apoptosis for the PtdSer exposure. TAT-1 and TAT-3, P4-ATPases in *C. elegans* also carry putative caspase-recognition sites in the corresponding positions. DTKT, the conserved phosphorylation site, is underlined

proposed to be the flippase. The molecular cloning of bovine chromaffin flippase/ATPase (ATPase II) cDNA indicated that ATPase II is a type IV P-type ATPase (P4-ATPase),[7] and ATPase II was thus designated ATP8A1.[8,9] The P4-ATPases, which exist only in eukaryotic cells, comprise a large family, with 5, 6, 15 and 14 members in yeast, *Caenorhabditis elegans*, mice and humans, respectively.[10–12] P4-ATPases have 10 transmembrane segments and 2 large cytoplasmic loops that contain nucleotide-binding-site and ATPase domains. P4-ATPases are chaperoned to their proper subcellular location by CDC50, which carries two transmembrane segments with cytoplasmic N- and C-termini.[13] CDC50A seems to be necessary for the flippase and lipid-transport activity of these ATPases.[14]

ATP8A1 and its yeast ortholog Drs2p localize mainly to intracellular vesicles such as granules and trans-Golgi networks,[5,15,16] and a Drs2p deficiency in yeast has no effect on the PtdSer-flippase activity at plasma membranes.[17] Thus, Drs2p, and probably mammalian ATP8A1, appear to be flippases that function in intracellular vesicles. Dnf1p and Dnf2p, two yeast P4-ATPases present in the plasma membrane, have been proposed to be plasma-membrane flippases,[18] but their function to flip PtdSer is controversial.[19] TAT-1, one of five P4-type ATPases found in *C. elegans*, was reported to function as a plasma-membrane flippase;[20] however, it is also intracellularly localized[21] suggesting that it may act as a flippase at intracellular membranes, too. Thus, the molecular identity of the plasma-membrane PtdSer flippase remained uncertain.

To identify plasma-membrane PtdSer flippases in mammalian cells, we performed a forward genetic screen using KBM7,[22] a human myeloid-cell line with a near-haploid karyotype.[23] KBM7 cells were randomly mutagenized with gene-trap retroviruses,[24] and cells that could not efficiently incorporate fluorescently labeled PtdSer were collected by repeated cell sorting. We sequenced virus-insertion sites in the sorted population using a next-generation sequencer, and identified ATP11C and CDC50A as flippase candidates (Figure 1). ATP11C, a P4-ATPase, localized to the plasma membrane in a CDC50A-dependent manner, and an *ATP11C* deficiency severely reduced the PtdSer-flippase activity (by ~80%) at the plasma membranes. On the other hand, *CDC50A*-deficient cells completely lost the ability to flip PtdSer at the plasma membrane, and constitutively exposed PtdSer on the cell surface.[22] These results identified ATP11C as the major plasma-membrane PtdSer flippase, but also indicated that other CDC50A-dependent P4-ATPases may contribute to the flipping of PtdSer at the plasma membrane. In fact, by establishing stable transformants expressing each member of human P4-ATPase family in ATP11C-null cells, not only ATP11C but also ATP11A and ATP8A2 were found to be localized at plasma membranes, and to flip PtdSer and PtdEtn[25] (Table 1). ATP11A and ATP11C were ubiquitously expressed in various cells, while ATP8A2 is expressed only in the brain and testis.

Increasing the intracellular Ca^{2+} in human erythrocytes inhibits their ability to incorporate aminophospholipids,[26] suggesting that Ca^{2+} can regulate the flippase activity at plasma membranes. In fact, ATP11A- or ATP11C-mediated PtdSer flipping at the plasma membrane was inhibited by increasing the intracellular Ca^{2+} concentration.[25] Their PtdSer-dependent ATPase activity was also inhibited by a

Table 1 Human P4-ATPase family

Class	P4-ATPase	Subunit	Substrate for PM flippase	Localization	Caspase-recognition site	Expression
1a	ATP8A1	CDC50A		Golgi, recycling endosome, (PM)		Ubiquitous
	ATP8A2	CDC50A	PtdSer (PtdEtn)	PM, (Golgi)	No	Brain, testis
1b	ATP8B1	CDC50A		PM		Liver/intestine
	ATP8B2	CDC50A		PM		
	ATP8B3	CDC50C		Acrosome		Testis
	ATP8B4	CDC50A		PM		
2	ATP9A	No		Golgi, recycling endosome		
	ATP9B	No		Golgi		
5	ATP10A	CDC50A		PM		
	ATP10B	CDC50A		Late endosome, lysosome		
	ATP10D	CDC50A		PM		
6	ATP11A	CDC50A	PtdSer, PtdEtn	PM	Yes (2 sites)	Ubiquitous
	ATP11B	CDC50A		Recycling endosome		
	ATP11C	CDC50A	PtdSer, PtdEtn	PM	Yes (3 sites)	Ubiquitous

Abbreviation: PM, plasma membrane
The substrates of P4-ATPases are from the study by Segawa *et al.*[25] except ATP8B3 and ATP10A. The cellular localization and tissue expression are from Segawa et al.,[25] Tanaka et al.,[11] and Panatala et al.[12] The specific expression ATP8B3 at testis was shown by Gong *et al.*[122] The localization of ATP10A at plasma membranes and its ability to translocate PtdCon were shown by Naito *et al.*[123] ATP8B1, 8B2 and 8B3 were claimed to work as flippases at plasma membrane against PtdSer, PtdCho, or Cardiolipin,[124–126] but we could not confirm[25]

high concentration of Ca^{2+}, suggesting that Ca^{2+} directly binds to ATP11A and 11C to inhibit their activity.

Apoptosis is mediated by caspases, which cleave >400 cellular substrates to induce cell death.[27] Apoptosis is almost universally accompanied by PtdSer exposure, which also requires caspase activation.[28] Flippase is inactivated during apoptosis,[29,30] suggesting that flippase might be a caspase target. In fact, ATP11A and ATP11C carry two or three evolutionarily well-conserved caspase 3 recognition sites in its large cytoplasmic domains[22] (Figure 1). Point mutations in these caspase-recognition sites generate a caspase-resistant flippase. Cells expressing this caspase-resistant flippase fail to expose PtdSer on the cell surface during apoptosis and are not engulfed by macrophages, indicating that the caspase-mediated cleavage of the flippase is essential to expose PtdSer during apoptosis. In this regard, it is noteworthy that the cell transformants expressing ATP8A2 that does not carry a caspase-recognition site fail to expose PtdSer during apoptosis, suggesting that ATP8A2 may have a specific function in the brain and testis.

Like many other processes in the cell-death pathway, apoptotic PtdSer exposure is phylogenetically well-conserved.[31] Dying cells in *C. elegans*[32] and *Drosophila*[33] expose PtdSer downstream of caspase. Among the five P4-ATPases found in *C. elegans,* TAT-1 and TAT-3 have putative caspase-recognition sites in positions corresponding to those of human ATP11A and 11C (Figure 1).

Calcium-Dependent Scramblase

When platelets are activated, PtdSer is exposed on the cell surface and forms a scaffold for coagulation factors.[34] This process is regulated by intracellular calcium, suggesting the involvement of one or more calcium-activated phospholipid scramblases.[35] Although PLSCR1 (phospholipid scramblase 1) was once reported to be a scramblase,[36,37] its molecular properties and the phenotypes of *PLSCR*-deficient mice and *Drosophila* ruled PLSCR1 out as a phospholipid scramblase.[38,39]

To identify phospholipid scramblases, we characterized calcium-dependent PtdSer exposure using the mouse cell line Ba/F3.[40] Cells stimulated with the Ca^{2+} ionophore A23187 in the presence of 0.5 mM Ca^{2+} exposed PtdSer to the cell surface within 15 min and underwent necrotic cell death. However, cells treated with A23187 without extracellular Ca^{2+} transiently exposed PtdSer; the PtdSer was internalized again within 12 h. We used this property of transient, Ca^{2+}-dependent PtdSer exposure to establish a Ba/F3 subline that strongly exposes PtdSer. Ba/F3 cells were stimulated with A23187 in the absence of Ca^{2+}, and a population (1–5%) that strongly exposed PtdSer was collected by flow cytometry and expanded. This process was repeated 19 times, with a decreasing ionophore concentration each time (from 1 μM to 63 nM). This procedure produced Ba/F3-PS19, a Ba/F3 subline that strongly exposes PtdSer at an A23187 concentration (63 nM) too low to induce PtdSer exposure in the parental Ba/F3 cells.

To isolate scramblases, we constructed a cDNA library in a retrovirus-based mammalian expression vector using 2.5-6.0-kb cDNA prepared from the Ba/F3-PS19 cells. The original Ba/F3 cells were infected with the retroviral cDNA library, stimulated with A23187 and sorted by PtdSer exposure. After undergoing this process three times, a significant population of Ba/F3 cells was found to constitutively expose PtdSer. These cells carried transmembrane protein (TMEM) 16 F with a point mutation (Asp to Gly at amino-acid position 409) that appears to have been spontaneously introduced during the sorting procedure of the Ba/F3-P19 cells. Ba/F3 and other mouse cell lines transformed with the mutant TMEM16F constitutively exposed both PtdSer and PtdEtn, and internalized PtdCho and SM. Treating the transformants with an intracellular Ca^{2+} chelator, BAPTA-AM, blocked the PtdSer exposure. These results indicated that TMEM16F supports Ca^{2+}-dependent phospholipid scrambling.[40] A topological analysis of TMEM16F based on its amino-acid sequence suggested that it carries eight transmembrane regions. However, a recent structural analysis revealed that a TMEM16F ortholog from the fungus *Nectria haematococca* carries 10 transmembrane regions[41]

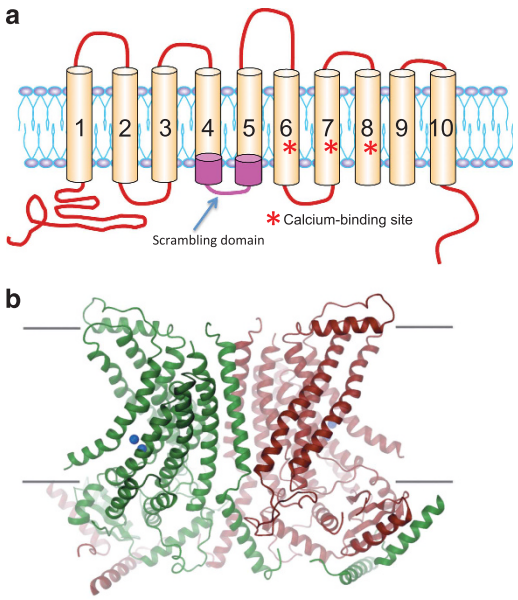

Figure 2 Structure of TMEM16F. (**a**) A schematic representation of TMEM16F. The scrambling domain (SCRD) between transmembrane regions IV and V, and the Ca^{2+}-binding site comprised of Aspartate and Glutamate in transmembrane regions VI–VIII are shown by red asterisks. (**b**) A tertiary structure of TMEM16 from *N. haematococca*. From the study by Brunner *et al.*[41]

(Figure 2). Structural and chemical cross-linking analyses indicated that TMEM16F exists as a homodimer.[41,42]

TMEM16F is one of the TMEM16-family proteins, also known as anoctamins; this family has 10 members (TMEM16A-H, J and K; or Ano1-10).[43] In 2008, three groups independently reported that TMEM16A, which is expressed ubiquitously, functions as a Ca^{2+}-activated Cl^- channel.[44–46] Since TMEM16B, which is specifically expressed in the retina, also acts as a Ca^{2+}-dependent Cl^- channel, all of the TMEM16-family members were thought to be Cl^- channels.[43,47,48] Accordingly, several groups reported that some TMEM16-family members, including TMEM16F, act as ion channels.[49–52] The expression of the wild-type TMEM16F makes the cells sensitive to Ca^{2+}-ionophore-induced scrambling of phospholipids.[40] Mouse fetal-thymocyte and embryonic-fibroblast (MEF) cells lacking *TMEM16F* lose phospholipid-scrambling ability,[53] clearly indicating that TMEM16F is indispensable for Ca^{2+}-dependent phospholipid scrambling. On the other hand, while TMEM16A and 16B are robust Cl^- channels under physiological conditions, we were not able to detect Cl^--channel activity for TMEM16F.[53] Two groups recently confirmed that the ability of TMEM16F to support phospholipid scrambling, and concluded that TMEM16F's ion-channel activity is a consequence of phospholipid translocation[54] or a secondary activity triggered under extreme, not necessarily physiological, conditions.[55]

Meanwhile, we expressed the 10 TMEM16 members in a *TMEM16F*-deficient cell line and showed that not only TMEM16F, but also TMEM16C, 16D, 16G and 16J can scramble phospholipids at the plasma membrane[53] (Table 2). TMEM16E, 16H and 16 K localize to intracellular membranes, where they might also function as phospholipid scramblases. The TMEM16 family is represented in all eukaryotes.[56,57] *Saccharomyces cerevisiae* and *Aspergillus fumigatus* carry a single TMEM16 ortholog, whose biochemical and physiological functions are unknown. Nematodes carry two genes encoding TMEM16 homologs, *ANOH1* and *ANOH2*; ANOH1 was recently found to be involved in Ca^{2+}-dependent PtdSer exposure,[58] as we will discuss later.

A mutation analysis with TMEM16A indicated that six Glutamate and Aspartate residues located in transmembrane segments of VI–VIII are involved in direct binding of Ca^{2+}.[59] These amino-acid residues are evolutionally well-conserved, and the structure of TMEM16 from *N. haematococca* indicates that these Ca^{2+}-binding sites are positioned close to the cavity between two subunits.[41] Regarding the scrambling of phospholipids, Yu *et al.*[54] compared the amino-acid sequences of TMEM16F paralogues from 24 animals using a program for Type II-divergence,[60] and showed that a domain of 35 amino acids located between the transmembrane IV and V is sufficient to confer the lipid scrambling activity to TMEM16A. This domain was named as a scrambling domain (SCRD). It would be interesting to study whether any other members of the TMEM16 family have the SCRD or not.

PtdSer Exposure in Activated Platelets

Normal, growing cells have a high intracellular ATP concentration, while the Ca^{2+} concentration is kept low by Ca^{2+}-ATPase, a Ca^{2+} pump that removes Ca^{2+} from the cells.[61] Under these conditions, ATP11A and ATP11C actively and specifically translocates PtdSer and PtdEtn from the outer to the inner leaflet of the lipid bilayer, creating their asymmetrical distribution in the plasma membrane. When platelets are activated with thrombin and collagen, the intracellular Ca^{2+} concentration increases locally and transiently,[62] likely reaching several hundred micromolar near or beneath the plasma membrane.[63] This local Ca^{2+} increase would transiently activate TMEM16F's scramblase function and inactivate ATP11A's and 11C's flippase (Figure 3), thereby swiftly exposing PtdSer on limited regions of the cell's surface. Intracellular Ca^{2+} levels decrease when the cell returns to a resting state, causing TMEM16F to stop scrambling, and ATP11A's and 11C's flippase activity to resume, thus re-establishing the asymmetrical distribution of PtdSer in the plasma membrane.

Caspase-Dependent Scramblases

In contrast to the Ca^{2+}-induced PtdSer exposure, *TMEM16F*-deficient cells have normal apoptotic PtdSer exposure,[53] confirming previous suggestions that two independent scrambling systems exist in human lymphocytes or mouse platelets.[64,65] To identify the scramblases responsible for apoptotic PtdSer exposure, we again performed expression cloning using the Ba/F3-PS19 cDNA library. Assuming that scramblases, like channels, should be large membrane proteins, we first screened a library of long cDNAs (>2.5 kb) and obtained the TMEM16F cDNA, encoding 911 amino acids. However, this library did not appear to contain the cDNA for a scramblase for apoptotic PtdSer exposure, so we switched to a library of smaller cDNAs (1–2.5 kb). The library

Table 2 Human TMEM16 Family

	Cl^- channel	Phospholipid scrambling	Celluar localization	Tissue distribution	Disease
TMEM16A	+	–	PM	Most tissues	
TMEM16B	+	–	PM	Eye	
TMEM16C	–	+	PM	Brain	Craniocervical dystonia
TMEM16D	–	+	PM	Brain, eye, ovary, uterus	
TMEM16E	–	–	Intracellular	Muscle, bone, testis	Muscle dystrophy, gnathodiaphyseal dysplasia (bone fracture)
TMEM16F	–	+	PM	Ubiquitous	Scott syndrome (hemophilia)
TMEM16G	–	+	PM	Intestine	
TMEM16H	–	–	Intracellular	Ubiquitous	
TMEM16J	–	+	PM	Intestine, skin	
TMEM16K	–	–	Intracellular	Ubiquitous	Cerebellar ataxia

Abbreviation: PM, plasma membrane
The Cl^--channel activity for TMEM16A and 16B is from Yang *et al.*,[44] Schroeder *et al.*,[45] Caputo *et al.*[46] and Suzuki *et al.*[40,53] The scramblase activity and tissue distribution are from Suzuki *et al.*[53] The diseases associated with TMEM16 are from Munchau *et al*[127] and Charlesworth *et al.*[128] for TMEM16C, from Tsutsumi *et al.*[129] and Savarese *et al.*[130] for TMEM16E, from Suzuki *et al.*[40] and Castoldi *et al.*[93] for TMEM16F, and Vermeer *et al.*[131] and Balreira *et al.*[132] for TMEM16K

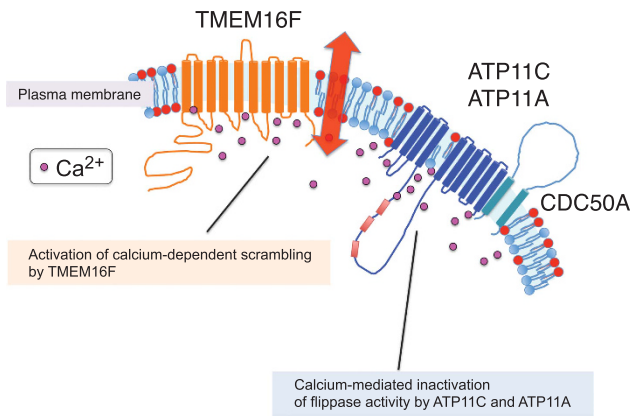

Figure 3 The molecular mechanism for PtdSer exposure in cells with high Ca^{2+}-concentration. The flippase comprised of P4-ATPase (ATP11A or ATP11C) and CDC50A, and a Ca^{2+}-dependent scramblase (TMEM16F) are schematically shown. In activated platelets, the intracellular Ca^{2+} concentration increases and activates TMEM16F to scramble phospholipids, while it inactivates P4-ATPases and reduces their flipping activity. When the Ca^{2+} concentration returns to normal level, TMEM16F stops scrambling phospholipids, while P4-ATPases resume flipping PtdSer and PtdEtn. Thus, PtdSer is only transiently exposed to the cell surface in this process, and likely depends on the intracellular concentration of ATP and Ca^{2+}. The constant flipping of PtdSer prevents the PtdSer-exposing cells to be engulfed by macrophages[78]

was introduced into Ba/F3 cells, and their transformants were screened for cells that strongly exposed PtdSer when treated with A23187. By repeated sorting, we found a cell population that constitutively exposes PtdSer. These cells carried the cDNA for Xkr8, which consists of 401 amino acids and has 6–10 transmembrane regions (depending on the topography-prediction program) with cytoplasmic N- and C-termini[66] (Figure 4). Except for Ba/F3 cells, transformation with Xkr8 did not cause constitutive PtdSer exposure in the cell lines we tested (mouse WR19L T-cell leukemia, and human Raji lymphoma and PLB985 leukemia), but strongly enhanced the apoptotic PtdSer exposure. MEF and mouse fetal-thymocyte cell lines lacking *Xkr8* lost the ability to scramble phospholipids (to expose PtdSer and PtdEtn and internalize PtdCho and SM) on apoptosis, while the Ca^{2+}-dependent phospholipid scrambling was not affected. It was also found that human Raji and PLB985 cells that are defective apoptotic PtdSer exposure[67,68] do not express Xkr8 due to hypermethylation in the *Xkr8* promoter region. Xkr8 carries a well-conserved caspase 3 recognition site in its C-terminal tail region, and its cleavage by caspases 3/7 is essential for its scramblase activity[66] (Figure 4).

Xkr8 belongs to the Xkr family, which has nine and eight members in humans and mice, respectively.[69] To examine whether any other Xkr-family proteins function as apoptotic scramblases, we expressed all of the Xkr-family members in human PLB985 or *Xkr8*$^{-/-}$ mouse fetal-thymocyte cells. Our results showed that not only Xkr8, but also Xkr4 and Xkr9 support apoptotic PtdSer exposure[70] (Table 3). Like Xkr8, Xkr4 and Xkr9 carry a caspase-recognition site in their C-terminal region, and this site is cleaved during apoptosis to activate the scramblase and expose PtdSer. Xkr8 is ubiquitously expressed in various tissues, and is expressed strongly in the testes. Xkr4 is ubiquitously expressed at low levels, but is strongly expressed in the brain and eyes. Xkr9 is strongly expressed in the intestines. Whether Xkr4 and Xkr9 are redundant to Xkr8 or play specific roles in the brain/eyes and intestines remains to be studied. PtdSer is exposed in apoptotic cells not only in mammals, but also in *Drosophila* and *C. elegans*.[31] Accordingly, flies and nematodes carry an Xkr8 orthlog (CG32579 in *D. melanogaster*, and CED8 in *C. elegans*)[66] (Figure 4). CED8 has a caspase (CED3)-recognition site in its N terminus[71] and is indispensable for CED3-dependent PtdSer exposure.[66] On the other hand, a *Drosophila* ortholog (CG32579) has no apparent caspase-recognition site, and it is not clear how this molecule is activated or whether it is involved in apoptotic PtdSer exposure.

PtdSer Exposure on Apoptotic and Other Dying Cells

Although apoptosis has been considered to be responsible for all programmed cell death,[72] recent studies indicate that cell death can also occur by programmed necrosis[73] by

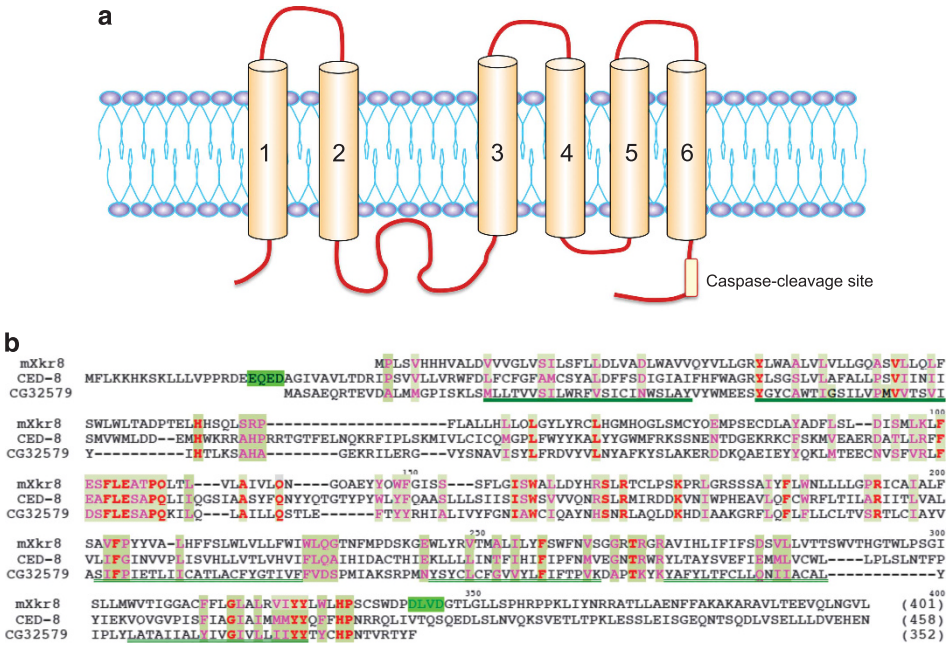

Figure 4 Structure of Xkr8, and its caspase-cleavage site. (**a**) The structure of mouse Xkr8, a caspase-dependent phospholipid scramblase is schematically shown. The caspase-recognition site at C-terminal region is indicated. In addition to Xkr8, Xkr4 and Xkr9 also carry the caspase-recognition site at the C-terminal tail region, and work as a caspase-dependent scramblase. (**b**) The amino-acid sequences of mouse Xkr8 (mXkr8), CED8 of *C. elegans* and CG32579 of *Drosophila* are aligned to obtain the maximal homology. The caspase-recognition sites at the C-terminal region of mXkr8[66] and at the N-terminal region of CED8[71] are highlighted in green. Transmembrane regions are underlined. reproduced from Suzuki *et al.*[66] with permission

Table 3 Human Xkr family

	Phospholipid scrambling	Caspase cleavage	Celluar localization	Tissue distribution	Disease
Xkr1	–	–	PM	Brain, pancreas, skeletal muscle	McLeod syndrome (neuropathy)
Xkr2	–	–	Intracellular? PM?	Placenta, adrenal gland	
Xkr3	ND	ND	ND	Testis	
Xkr4	+	+	PM	Brain, eye, skin	
Xkr5	–	–	PM	ND	
Xkr6	–	–	PM	ND	
Xkr7	–	+	PM	ND	
Xkr8	+	+	PM	Ubiquitous	
Xkr9	+	+	PM	Intestine, stomach	

Abbreviation: PM, plasma membrane
The scrambling activity of the Xkr family, and tissue distribution of Xkr4, Xkr8 and Xkr9 are from the study by Suzuki *et al.*[70] Xkr1, Xkr2, and Xkr3 are also called XK, XPLAC and XTES, respectively, and their tissue distribution was determined by Calenda *et al.*[69] The fusion protein with GFP shows that most of Xkr2 is intracellularly localized,[70] but some may be present on plasma membrane.[69] The disease associated in XK is from the study by Danek *et al.*[133]

necroptosis or pyroptosis, which are induced by tumor necrosis factor (TNF) and bacterial infection, respectively. Cells that undergo apoptosis, necroptosis or pyroptosis expose PtdSer on their surface and are engulfed by phagocytes in a PtdSer-dependent manner.[74–77]

In the apoptotic caspase cascade, caspases 3 and 7 in the downstream of the cascade cleave the flippases of ATP11A and ATP11C and inactivate them. At the same time, caspases 3/7 cleave the Xkr8 scramblase, and activate it (Figure 5). Cleavage by caspases, which is irreversible, thus leading to irreversible PtdSer exposure. Living cells that expose PtdSer by the constitutive active forms of TMEM16 F are not engulfed by macrophages,[78] but cells exposing PtdSer due to the lack of flippase activity are recognized and engulfed by macrophages.[22] PtdSer-expressing cells by the constitutive-active scramblase, carry active flippase. They seem to have a low affinity to the PtdSer receptor on macrophages,[78] probably because the PtdSer is constantly being flipped. In the absence of flippase, the PtdSer remains exposed and does not return to the inner leaflet, increasing the cell's affinity for the PtdSer receptor. The difference between complete, irreversible flippase inactivation and partial, transient inactivation may explain why PtdSer-exposing apoptotic cells are engulfed by macrophages, but activated platelets are not.[79] Once established, it takes days to disrupt the asymmetrical distribution of phospholipids because spontaneous translocation across the bilayer is extremely low.[80,81] In this regard, a scramblase(s) is essential for quick PtdSer exposure during apoptosis.

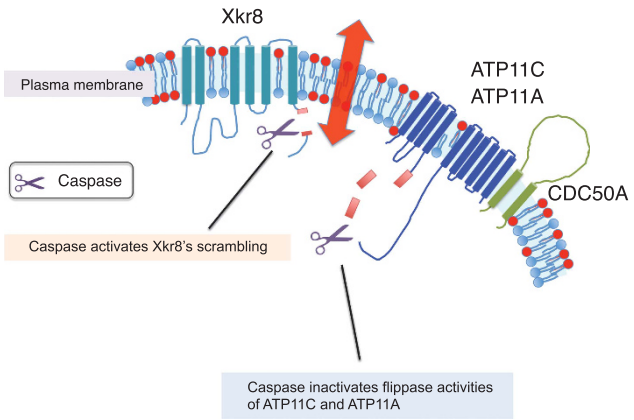

Figure 5 The PtdSer exposure in apoptotic cells. A caspase-dependent phospholipid scramblase of Xkr8 and flippase (ATP11A/ATP11C associated with CDC50A) are schematically shown. When cells undergo apoptosis, caspase 3 or caspase 7 in the downstream of the caspase cascade cleaves Xkr8 to activate its scramblase activity, while the same caspases cleave and inactivate ATP11A and ATP11C. This is the irreversible process, and the PtdSer exposed on the cell surface is recognized by macrophages for engulfment

Recently, a scramblase is reported to function in PtdSer exposure in necrotic cell death in nematodes. Dominant mutations in *mec-4* (the core subunit of a mechanically gated sodium channel) in touch neurons in *C. elegans* cause hyper channel activity, which results in excess Ca^{2+} influx and necrotic cell death.[82,83] Li *et al.*[58] recently showed that necrotic cells in *mec-4* nematodes expose PtdSer by the action of ANOH1 (a TMEM16 homolog) and CED7 (an ABCA1-transporter homolog). TNF-induced necroptosis and bacteria-induced pyroptosis are mediated by calcium influx.[84,85] It will be interesting to investigate whether TMEM16F or other TMEM16 proteins play a role in exposing PtdSer in necroptosis or pyroptosis.

Defects in Flippases and Scramblases

Mouse WR19L cells that lack *CDC50A* or that express constitutively active TMEM16F expose PtdSer but grow normally,[22,78] suggesting that cell growth may not require an asymmetrical distribution of phospholipids at the plasma membrane. On the other hand, and *ATP11C*-null mice suffer from B-cell lymphopenia, cholestasis, anemia, dystocia and hepatocellular carcinoma.[86–89] In *ATP11C*-null B cells and erythrocytes, the flippase activity is reduced and the population of erythrocytes that expose PtdSer is slightly increased.[88,89] How this small effect on flippase activity in *ATP11C*$^{-/-}$ cells leads to the strong phenotypes seen in *ATP11C*-null mice is unclear. Lymphocytes or erythrocytes might express only the ATP11C flippase at a specific developmental stage; an *ATP11C*-null mutation may cause the PtdSer exposure in cells at this stage, leading macrophages to recognize and engulf these cells or causing the destabilization and abnormal assembly of plasma-membrane proteins.[90,91]

Scott syndrome, a mild autosomal bleeding disorder, is caused by the failure of Ca^{2+}-dependent PtdSer exposure in activated platelets.[92] Patients in two families carrying Scott syndrome had a homozygous null mutation or compound heterozygous mutations in the *TMEM16F* gene.[40,93] Canine Scott syndrome, a naturally occurring bleeding disorder, is found in German shepherd dogs.[94] As with human patients, the affected dogs lack procoagulant activity in activated platelets and carry loss-of-function mutations in the *TMEM16F* gene.[95]

Two mouse lines lacking *TMEM16F* in platelets have been established, by Yang *et al.*[51] and by us.[96] Yang *et al.* established conventional *TMEM16F*-knockout mice in a 129/B6-mixed background; the phenotype of these mice differs significantly from that of human or canine Scott syndrome. Although the PtdSer exposure in activated platelets depends significantly on TMEM16F, these mice produce tissue-factor-induced thrombin without TMEM16F; however, the tail-bleeding time is greatly increased in these mice. Yang *et al.* concluded that this phenotype was due to a defect in TMEM16F's cation-channel activity. On the other hand, we were unable to detect ion-channel activity in TMEM16F, and our platelet-specific deletion of the *TMEM16F* gene in a C57/B6 background produced mice with phenotypes matching those in human and canine Scott syndrome. That is, the thrombin/collagen-induced PtdSer exposure in platelets is fully impaired in these mice, as is the tissue-factor-induced thrombin activation. However, similar to human and canine Scott syndrome, the tail-bleeding time is not affected in our platelet-specific *TMEM16F*-null mouse. The reason for the differences in these two *TMEM16F*-null mouse lines[51,96] is not clear.

A defect in Xkr8 causes the loss of PtdSer exposure in apoptotic cells, and the dead cells are not engulfed.[66] Similarly, mutations in *CED8*, an Xkr8 ortholog in nematodes, causes the failure of PtdSer exposure in dying cells, and the inefficient or delayed engulfment of dead cells.[71] Masking PtdSer or deleting molecules that recognize PtdSer, such as MFG-E8, Tim4 and TAM receptors, blocks the engulfment of apoptotic cells, and induces the development of systemic lupus erythematosus-type autoimmune diseases.[97–101] Thus, it is likely that *Xkr8*-deficient mice develop similar autoimmune diseases, but this remains to be confirmed.

Perspectives

We have here described one flippase and two scramblase families (Ca^{2+}-dependent and caspase-dependent), and have discussed how PtdSer is exposed on activated platelets and apoptotic cells. PtdSer and PtdEtn are exposed not only on activated platelets, but also on galectin-treated neutrophils,[102] IgE-stimulated mast cells,[103] activated CD4 T cells,[104] capacitated sperm,[105,106] aged red blood cells,[107] and cells activated through the $P2X_7$ ATP receptor.[104] PtdSer exposure on galectin-treated neutrophils,[108] activated mast cells,[109] activated lymphocytes and cells activated via the $P2X_7$ receptor[110] is mediated by (or at least accompanied by) increased intracellular calcium. It is tempting to speculate that PtdSer exposure in these processes involves TMEM16F or other TMEM16-family members (TMEM16C, 16D, 16F, 16G or 16J) with Ca^{2+}-dependent scramblase activity.

Pyrenocytes (nucleus with a thin rim of cytoplasm surrounded by a plasma membrane) extruded from erythroblasts,[111] milk-fat globules from mammary glands,[112] exsosomes,[113] enveloped viruses[114] and microparticles

released from activated platelets[96] expose PtdSer. Pyrenocytes do not carry mitochondria, and quickly lose their ATP once separated from erythroblasts, causing a rapid Ca^{2+} influx.[111] A similar process may occur for milk-fat globules and enveloped viruses. Whether this loss of ATP and increased intracellular Ca^{2+} is responsible for activating or inactivating specific flippases or scramblases to expose PtdSer in these particles remains to be studied.

Tumor-associated endothelial cells expose PtdSer.[115,116] A monoclonal antibody against PtdSer was proposed as a treatment for cancer, to kill tumor-associated endothelial cells via antibody- or complement-mediated cytotoxicity.[117] This treatment appears promising.[118–120] Recent reports suggest that the PtdSer in the tumor-associated endothelial cells and the PtdSer exposed on tumor cells after treatment with anti-cancer drugs create an immune-suppressive tumor environment; the antibody against PtdSer reverses this effect, creating anti-tumor immunity.[121] It will be interesting to study whether the flippase and scramblases discussed in this review are involved in the PtdSer exposure on tumor-associated endothelial cells.

Conflict of Interest

The authors declare no conflict of interest.

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1. van Meer G, Voelker D, Feigenson G. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* 2008; **9**: 112–124.
2. Leventis PA, Grinstein S. The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys* 2010; **39**: 407–427.
3. Balasubramanian K, Schroit AJ. Aminophospholipid asymmetry: a matter of life and death. *Annu Rev Physiol* 2003; **65**: 701–734.
4. Seigneuret M, Devaux P. ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc Natl Acad Sci USA* 1984; **81**: 3751–3755.
5. Zachowski A, Henry JP, Devaux PF. Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. *Nature* 1989; **340**: 75–76.
6. Moriyama Y, Nelson N. Purification and properties of a vanadate- and N-ethylmaleimide-sensitive ATPase from chromaffin granule membranes. *J Biol Chem* 1988; **263**: 8521–8527.
7. Tang X, Halleck MS, Schlegel RA, Williamson P. A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* 1996; **272**: 1495–1497.
8. Paulusma C, Oude Elferink R. The type 4 subfamily of P-type ATPases, putative aminophospholipid translocases with a role in human disease. *Biochim Biophys Acta* 2005; **1741**: 11–24.
9. Folmer D, Elferink R, Paulusma C. P4 ATPases—lipid flippases and their role in disease. *Biochim Biophys Acta* 2009; **1791**: 628–635.
10. Halleck MS, Pradhan D, Blackman C, Berkes C, Williamson P, Schlegel RA. Multiple members of a third subfamily of P-type ATPases identified by genomic sequences and ESTs. *Genome Res* 1998; **8**: 354–361.
11. Tanaka K, Fujimura-Kamada K, Yamamoto T. Functions of phospholipid flippases. *J Biochem* 2011; **149**: 131–143.
12. Panatala R, Hennrich H, Holthuis JC. Inner workings and biological impact of phospholipid flippases. *J Cell Sci* 2015; **128**: 2021–2032.
13. Saito K, Fujimura-Kamada K, Furuta N, Kato U, Umeda M, Tanaka K. Cdc50p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in Saccharomyces cerevisiae. *Mol Biol Cell* 2004; **15**: 3418–3432.
14. Lenoir G, Williamson P, Puts CF, Holthuis JCM. Cdc50p plays a vital role in the ATPase reaction cycle of the putative aminophospholipid transporter Drs2p. *J Biol Chem* 2009; **284**: 17956–17967.
15. Hua Z, Fatheddin P, Graham TR. An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Mol Biol Cell* 2002; **13**: 3162–3177.
16. Lee S, Uchida Y, Wang J, Matsudaira T, Nakagawa T, Kishimoto T *et al.* Transport through recycling endosomes requires EHD1 recruitment by a phosphatidylserine translocase. *EMBO J* 2015; **34**: 669–688.
17. Siegmund A, Grant A, Angeletti C, Malone L, Nichols JW, Rudolph HK. Loss of Drs2p does not abolish transfer of fluorescence-labeled phospholipids across the plasma membrane of Saccharomyces cerevisiae. *J Biol Chem* 1998; **273**: 34399–34405.
18. Pomorski T, Lombardi R, Riezman H, Devaux PF, Van Meer G, Holthuis JCM. Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. *Mol Biol Cell* 2003; **14**: 1240–1254.
19. Stevens HC, Malone L, Nichols JW. The putative aminophospholipid translocases, DNF1 and DNF2, are not required for 7-nitrobenz-2-oxa-1,3-diazol-4-yl-phosphatidylserine flip across the plasma membrane of Saccharomyces cerevisiae. *J Biol Chem* 2008; **283**: 35060–35069.
20. Darland-Ransom M, Wang X, Sun CL, Mapes J, Gengyo-Ando K, Mitani S *et al.* Role of C. elegans TAT-1 protein in maintaining plasma membrane phosphatidylserine asymmetry. *Science* 2008; **320**: 528–531.
21. Chen B, Jiang Y, Zeng S, Yan J, Li X, Zhang Y *et al.* Endocytic sorting and recycling require membrane phosphatidylserine asymmetry maintained by TAT-1/CHAT-1. *PLoS Genet* 2010; **6**: e1001235.
22. Segawa K, Kurata S, Yanagihashi Y, Brummelkamp T, Matsuda F, Nagata S. Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science* 2014; **344**: 1164–1168.
23. Kotecki M, Reddy PS, Cochran BH. Isolation and characterization of a near-haploid human cell line. *Exp Cell Res* 1999; **252**: 273–280.
24. Carette JE, Guimaraes CP, Wuethrich I, Blomen VA, Varadarajan M, Sun C *et al.* Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. *Nat Biotechnol* 2011; **29**: 542–546.
25. Segawa K, Kurata S, Nagata S. Human type IV P-type ATPases that work as plasma membrane phospholipid flippases, and their regulation by caspase and calcium. *J Biol Chem* 2016; **291**: 762–772.
26. Bitbol M, Fellmann P, Zachowski A, Devaux PF. Ion regulation of phosphatidylserine and phosphatidylethanolamine outside-inside translocation in human erythrocytes. *Biochim Biophys Acta* 1987; **904**: 268–282.
27. Luthi AU, Martin SJ. The CASBAH: a searchable database of caspase substrates. *Cell Death Differ* 2007; **14**: 641–650.
28. Martin SJ, Finucane DM, Amarante-Mendes GP, O'Brien GA, Green DR. Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. *J Biol Chem* 1996; **271**: 28753–28756.
29. Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J Biol Chem* 1997; **272**: 26159–26165.
30. Verhoven B, Schlegel RA, Williamson P. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J Exp Med* 1995; **182**: 1597–1601.
31. van den Eijnde SM, Boshart L, Baehrecke EH, De Zeeuw CI, Reutelingsperger CP, Vermeij-Keers C. Cell surface exposure of phosphatidylserine during apoptosis is phylogenetically conserved. *Apoptosis* 1998; **3**: 9–16.
32. Venegas V, Zhou Z. Two alternative mechanisms that regulate the presentation of apoptotic cell engulfment signal in Caenorhabditis elegans. *Mol Biol Cell* 2007; **18**: 3180–3192.
33. Tung TT, Nagaosa K, Fujita Y, Kita A, Mori H, Okada R *et al.* Phosphatidylserine recognition and induction of apoptotic cell clearance by Drosophila engulfment receptor Draper. *J Biochem* 2013; **153**: 483–491.
34. Bevers EM, Comfurius P, Zwaal RF. Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta* 1983; **736**: 57–66.
35. Bevers EM, Comfurius P, Zwaal RF. Platelet procoagulant activity: physiological significance and mechanisms of exposure. *Blood Rev* 1991; **5**: 146–154.
36. Basse F, Stout JG, Sims PJ, Wiedmer T. Isolation of an erythrocyte membrane protein that mediates Ca2+-dependent transbilayer movement of phospholipid. *J Biol Chem* 1996; **271**: 17205–17210.
37. Zhou Q, Zhao J, Stout J, Luhm R, Wiedmer T, Sims P. Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. *J Biol Chem* 1997; **272**: 18240–18244.
38. Bevers EM, Williamson PL. Phospholipid scramblase: an update. *FEBS Lett* 2010; **584**: 2724–2730.
39. Sahu S, Gummadi S, Manoj N, Aradhyam G. Phospholipid scramblases: an overview. *Arch Biochem Biophys* 2007; **462**: 103–114.
40. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010; **468**: 834–838.
41. Brunner JD, Lim NK, Schenck S, Duerst A, Dutzler R. X-ray structure of a calcium-activated TMEM16 lipid scramblase. *Nature* 2014; **516**: 207–212.

42. Suzuki T, Suzuki J, Nagata S. Functional swapping between transmembrane proteins TMEM16A and TMEM16F. *J Biol Chem* 2014; **289**: 7438–7447.
43. Galietta L. The TMEM16 protein family: a new class of chloride channels? *Biophys J* 2009; **97**: 3047–3053.
44. Yang Y, Cho H, Koo J, Tak M, Cho Y, Shim W *et al.* TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature* 2008; **455**: 1210–1215.
45. Schroeder B, Cheng T, Jan Y, Jan L. Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 2008; **134**: 1019–1029.
46. Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E *et al.* TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 2008; **322**: 590–594.
47. Hartzell HC, Yu K, Xiao Q, Chien LT, Qu Z. Anoctamin/TMEM16 family members are Ca^{2+}-activated Cl^- channels. *J Physiol* 2009; **587**: 2127–2139.
48. Flores CA, Cid LP, Sepúlveda FV, Niemeyer MI. TMEM16 proteins: the long awaited calcium-activated chloride channels? *Braz J Med Biol Res* 2009; **42**: 993–1001.
49. Schreiber R, Uliyakina I, Kongsuphol P, Warth R, Mirza M, Martins J *et al.* Expression and function of epithelial anoctamins. *J Biol Chem* 2010; **285**: 7838–7845.
50. Shimizu T, Iehara T, Sato K, Fujii T, Sakai H, Okada Y. TMEM16F is a component of a Ca^{2+}-activated Cl^- channel but not a volume-sensitive outwardly rectifying Cl^- channel. *Am J Physiol Cell Physiol* 2013; **304**: C748–C759.
51. Yang H, Kim A, David T, Palmer D, Jin T, Tien J *et al.* TMEM16F forms a Ca^{2+}-activated cation channel required for lipid scrambling in platelets during blood coagulation. *Cell* 2012; **151**: 111–122.
52. Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingsawat J, Sirianant L *et al.* Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? *Pflug Arch Eur J Phy* 2014; **466**: 407–414.
53. Suzuki J, Fujii T, Imao T, Ishihara K, Kuba H, Nagata S. Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J Biol Chem* 2013; **288**: 13305–13316.
54. Yu K, Whitlock JM, Lee K, Ortlund EA, Yuan Cui Y, Hartzell HC. Identification of a lipid scrambling domain in ANO6/TMEM16F. *eLife* 2015; **4**: e06901.
55. Scudieri P, Caci E, Venturini A, Sondo E, Pianigiani G, Marchetti C *et al.* Ion channel and lipid scramblase activity associated with expression of tmem16F/ANO6 isoforms. *J Physiol* 2015; **593**: 3829–3848.
56. Wang Y, Alam T, Hill-Harfe K, Lopez AJ, Leung CK, Iribarne D *et al.* Phylogenetic, expression, and functional analyses of anoctamin homologs in *Caenorhabditis elegans*. *Am J Physiol Regul Integr Comp Physiol* 2013; **305**: R1376–R1389.
57. Milenkovic VM, Brockmann M, Stöhr H, Weber BH, Strauss O. Evolution and functional divergence of the anoctamin family of membrane proteins. *BMC Evol Biol* 2010; **10**: 319.
58. Li Z, Venegas V, Nagaoka Y, Morino E, Raghavan P, Audhya A *et al.* Necrotic cells actively attract phagocytes through the collaborative action of two distinct PS-exposure mechanisms. *PLoS Genet* 2015; **11**: e1005285.
59. Yu K, Duran C, Qu Z, Cui Y-y, Hartzell HC. Explaining calcium-dependent gating of anoctamin-1 chloride channels requires a revised topology. *Circ Res* 2012; **110**: 990–999.
60. Gu X. A simple statistical method for estimating type-II (cluster-specific) functional divergence of protein sequences. *Mol Biol Evol* 2006; **23**: 1937–1945.
61. Carafoli E. Calcium pump of the plasma membrane. *Physiol Rev* 1991; **71**: 129–153.
62. Hussain JF, Mahaut-Smith MP. Reversible and irreversible intracellular Ca2+ spiking in single isolated human platelets. *J Physiol* 1999; **514**: 713–718.
63. Marsault R, Murgia M, Pozzan T, Rizzuto R. Domains of high Ca2+ beneath the plasma membrane of living A7r5 cells. *EMBO J* 1997; **16**: 1575–1581.
64. Williamson P, Christie A, Kohlin T, Schlegel RA, Comfurius P, Harmsma M *et al.* Phospholipid scramblase activation pathways in lymphocytes. *Biochemistry* 2001; **40**: 8065–8072.
65. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD *et al.* Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009; **114**: 663–666.
66. Suzuki J, Denning DP, Imanishi E, Horvitz HR, Nagata S. Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science* 2013; **341**: 403–406.
67. Fadeel B, Gleiss B, Högstrand K, Chandra J, Wiedmer T, Sims PJ *et al.* Phosphatidylserine exposure during apoptosis is a cell-type-specific event and does not correlate with plasma membrane phospholipid scramblase expression. *Biochem Biophys Res Commun* 1999; **266**: 504–511.
68. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem* 2001; **276**: 1071–1077.
69. Calenda G, Peng J, Redman CM, Sha Q, Wu X, Lee S. Identification of two new members, XPLAC and XTES, of the XK family. *Gene* 2006; **370**: 6–16.
70. Suzuki J, Imanishi E, Nagata S. Exposure of phosphatidylserine by Xk-related protein family members during apoptosis. *J Biol Chem* 2014; **289**: 30257–30267.
71. Chen Y-Z, Mapes J, Lee E-S, Skeen-Gaar RR, Xue D. Caspase-mediated activation of Caenorhabditis elegans CED-8 promotes apoptosis and phosphatidylserine externalization. *Nat Commun* 2013; **4**: 1–9.
72. Rich T, Watson CJ, Wyllie A. Apoptosis: the germs of death. *Nat Cell Biol* 1999; **1**: E69–E71.
73. Berghe TV, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol* 2014; **15**: 135–147.
74. Brouckaert G, Kalai M, Krysko DV, Saelens X, Vercammen D, Ndlovu MN *et al.* Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. *Mol Biol Cell* 2004; **15**: 1089–1100.
75. Krysko DV, Denecker G, Festjens N, Gabriels S, Parthoens E, D'Herde K *et al.* Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. *Cell Death Differ* 2006; **13**: 2011–2022.
76. Wang Q, Imamura R, Motani K, Kushiyama H, Nagata S, Suda T. Pyroptotic cells externalize eat-me and release find-me signals and are efficiently engulfed by macrophages. *Int Immunol* 2013; **25**: 363–372.
77. Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. *Cell* 2010; **140**: 619–630.
78. Segawa K, Suzuki J, Nagata S. Constitutive exposure of phosphatidylserine on viable cells. *Proc Natl Acad Sci USA* 2011; **108**: 19246–19251.
79. Segawa K, Nagata S. An apoptotic 'eat me' signal: phosphatidylserine exposure. *Trends Cell Biol* 2015; **25**: 649–650.
80. Kornberg RD, McConnell HM. Inside-outside transitions of phospholipids in vesicle membranes. *Biochemistry* 1971; **10**: 1111–1120.
81. Nakano M, Fukuda M, Kudo T, Matsuzaki N, Azuma T, Sekine K *et al.* Flip-Flop of phospholipids in vesicles: kinetic analysis with time-resolved small-angle neutron scattering. *J Physical Chem B* 2009; **113**: 6745–6748.
82. Driscoll M, Chalfie M. The mec-4 gene is a member of a family of Caenorhabditis elegans genes that can mutate to induce neuronal degeneration. *Nature* 1991; **349**: 588–593.
83. Bianchi L, Gerstbrein B, Frøkjær-Jensen C, Royal DC, Mukherjee G, Royal MA *et al.* The neurotoxic MEC-4(d) DEG/ENaC sodium channel conducts calcium: implications for necrosis initiation. *Nat Neurosci* 2004; **7**: 1337–1344.
84. Cai Z, Jitkaew S, Zhao J, Chiang H-C, Choksi S, Liu J *et al.* Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat Cell Biol* 2014; **16**: 55–65.
85. Bergsbaken T, Fink SL, den Hartigh AB, Loomis WP, Cookson BT. Coordinated host responses during pyroptosis: caspase-1-dependent lysosome exocytosis and inflammatory cytokine Maturation. *J Immunol* 2011; **187**: 2748–2754.
86. Siggs OM, Schnabl B, Webb B, Beutler B. X-linked cholestasis in mouse due to mutations of the P4-ATPase ATP11C. *Proc Nat Acad Sci USA* 2011; **108**: 7890–7895.
87. Siggs OM, Arnold CN, Huber C, Pirie E, Xia Y, Lin P *et al.* The P4-type ATPase ATP11C is essential for B lymphopoiesis in adult bone marrow. *Nat Immunol* 2011; **12**: 434–440.
88. Yabas M, Teh CE, Frankenreiter S, Lal D, Roots CM, Whittle B *et al.* ATP11C is critical for the internalization of phosphatidylserine and differentiation of B lymphocytes. *Nat Immunol* 2011; **12**: 441–449.
89. Yabas M, Coupland LA, Cromer D, Winterberg M, Teoh NC, D'Rozario J *et al.* Mice deficient in the putative phospholipid flippase ATP11C exhibit altered erythrocyte shape, anemia, and reduced erythrocyte life span. *J Biol Chem* 2014; **289**: 19531–19537.
90. Manno S, Takakuwa Y, Mohandas N. Identification of a functional role for lipid asymmetry in biological membranes: phosphatidylserine-skeletal protein interactions modulate membrane stability. *Proc Nat Acad Sci USA* 2002; **99**: 1943–1948.
91. Matsuzaka Y, Hayashi H, Kusuhara H. Impaired hepatic uptake by organic anion-transporting polypeptides is associated with hyperbilirubinemia and hypercholanemia in Atp11c mutant mice. *Mol Pharmacol* 2015; **88**: 1085–1092.
92. Wielders S, Broers J, ten Cate H, Collins P, Bevers E, Lindhout T. Absence of platelet-dependent fibrin formation in a patient with Scott syndrome. *Thromb Haemost* 2009; **102**: 76–82.
93. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011; **117**: 4399–4400.
94. Brooks M, Catalfamo J, Brown H, Ivanova P, Lovaglio J. A hereditary bleeding disorder of dogs caused by a lack of platelet procoagulant activity. *Blood* 2002; **99**: 2434–2441.
95. Brooks MB, Catalfamo JL, MacNguyen R, Tim D, Fancher S, McCardle JA. A TMEM16F point mutation causes an absence of canine platelet TMEM16F and ineffective activation and death-induced phospholipid scrambling. *J Thromb Haemost* 2015; **13**: 2240–2252.
96. Fujii T, Sakata A, Nishimura S, Koji Eto, Nagata S. TMEM16F is required for phosphatidylserine exposure and microvesicle release in activated mouse platelets. *Proc Nat Acad Sci USA* 2015; **112**: 12800–12805.
97. Miyanishi M, Segawa K, Nagata S. Synergistic effect of Tim4 and MFG-E8 null mutations on the development of autoimmunity. *Int Immunol* 2012; **24**: 551–559.
98. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y *et al.* Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 2004; **304**: 1147–1150.
99. Asano K, Miwa M, Miwa K, Hanayama R, Nagase H, Nagata S *et al.* Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. *J Exp Med* 2004; **200**: 459–467.
100. Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL *et al.* Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature* 2001; **411**: 207–211.
101. Behrens EM, Gadue P, Gong SY, Garrett S, Stein PL, Cohen PL. The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. *Eur J Immunol* 2003; **33**: 2160–2167.

102. Stowell SR, Karmakar S, Stowell CJ, Dias-Baruffi M, McEver RP, Cummings RD. Human galectin-1, -2, and -4 induce surface exposure of phosphatidylserine in activated human neutrophils but not in activated T cells. *Blood* 2007; **109**: 219–227.
103. Martin S, Pombo I, Poncet P, David B, Arock M, Blank U. Immunologic stimulation of mast cells leads to the reversible exposure of phosphatidylserine in the absence of apoptosis. *Int Arch Aller Imm* 2000; **123**: 249–258.
104. Elliott JI, Surprenant A, Marelli-Berg FM, Cooper JC, Cassady-Cain RL, Wooding C *et al.* Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes. *Nat Cell Biol* 2005; **7**: 808–816.
105. de Vries KJ, Wiedmer T, Sims PJ, Gadella BM. Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. *Biol Reprod* 2003; **68**: 2122–2134.
106. Gadella BM, Harrison RA. Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. *Biol Reprod* 2002; **67**: 340–350.
107. Lutz HU, Bogdanova A. Mechanisms tagging senescent red blood cells for clearance in healthy humans. *Front Physiol* 2013; **4**: 387.
108. Karmakar S, Cummings RD, McEver RP. Contributions of Ca2+ to galectin-1-induced exposure of phosphatidylserine on activated neutrophils. *J Biol Chem* 2005; **280**: 28623–28631.
109. Smrz D, Draberova L, Draber P. Non-apoptotic phosphatidylserine externalization induced by engagement of glycosylphosphatidylinositol-anchored proteins. *J Biol Chem* 2007; **282**: 10487–10497.
110. Taylor SRJ, Gonzalez-Begne M, Dewhurst S, Chimini G, Higgins CF, Melvin JE *et al.* Sequential shrinkage and swelling underlie P2X7-stimulated lymphocyte phosphatidylserine exposure and death. *J Immunol* 2008; **180**: 300–308.
111. Yoshida H, Kawane K, Koike M, Mori Y, Uchiyama Y, Nagata S. Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells. *Nature* 2005; **437**: 754–758.
112. Hanayama R, Nagata S. Impaired involution of mammary glands in the absence of milk fat globule EGF factor 8. *Proc Natl Acad Sci USA* 2005; **102**: 16886–16891.
113. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009; **9**: 581–593.
114. Amara A, Mercer J. Viral apoptotic mimicry. *Nat Rev Microbiol* 2015; **13**: 461–469.
115. Stafford JH, Thorpe PE. Increased exposure of phosphatidylethanolamine on the surface of tumor vascular endothelium. *Neoplasia* 2011; **13**: 299–308.
116. Ran S, Downes A, Thorpe PE. Increased exposure of anionic phospholipids on the surface of tumor blood vessels. *Cancer Res* 2002; **62**: 6132–6140.
117. Huang X, Bennett M, Thorpe PE. A monoclonal antibody that binds anionic phospholipids on tumor blood vessels enhances the antitumor effect of docetaxel on human breast tumors in mice. *Cancer Res* 2005; **65**: 4408–4416.
118. Zörnig M, Grzeschiczek A, Kowalski M-B, Hartmann KU, Möröy T. Loss of Fas/Apo-1 receptor accelerates lymphogenesis in Em L-*MYC* transgenic mice but not in animals infected with MoMuLV. *Oncogene* 1995; **10**: 2397–2401.
119. Digumarti R, Bapsy PP, Suresh AV, Bhattacharyya GS, Dasappa L, Shan JS *et al.* Bavituximab plus paclitaxel and carboplatin for the treatment of advanced non-small-cell lung cancer. *Lung Cancer* 2014; **86**: 231–236.
120. Chalasani P, Marron M, Roe D, Clarke K, Iannone M, Livingston RB *et al.* A phase I clinical trial of bavituximab and paclitaxel in patients with HER2 negative metastatic breast cancer. *Cancer Med* 2015; **4**: 1051–1059.
121. Yin Y, Huang X, Lynn KD, Thorpe PE. Phosphatidylserine-targeting antibody induces M1 macrophage polarization and promotes myeloid-derived suppressor cell differentiation. *Cancer Immunol Res* 2013; **1**: 256–268.
122. Gong EY, Park E, Lee HJ, Lee K. Expression of Atp8b3 in murine testis and its characterization as a testis specific P-type ATPase. *Reproduction* 2009; **137**: 345–351.
123. Naito T, Takatsu H, Miyano R, Takada N, Nakayama K, Shin HW. Phospholipid flippase ATP10A translocates phosphatidylcholine and is involved in plasma membrane dynamics. *J Biol Chem* 2015; **290**: 15004–15017.
124. Takatsu H, Tanaka G, Segawa K, Suzuki J, Nagata S, Nakayama K *et al.* Phospholipid flippase activities and substrate specificities of human type IV P-type ATPases localized to the plasma membrane. *J Biol Chem* 2014; **289**: 33543–33556.
125. Paulusma C, Folmer D, Ho-Mok K, de Waart D, Hilarius P, Verhoeven A *et al.* ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. *Hepatology* 2008; **47**: 268–278.
126. Ray NB, Durairaj L, Chen BB, McVerry BJ, Ryan AJ, Donahoe M *et al.* Dynamic regulation of cardiolipin by the lipid pump Atp8b1 determines the severity of lung injury in experimental pneumonia. *Nat Med* 2010; **16**: 1120–1127.
127. Munchau A, Valente EM, Davis MB, Stinton V, Wood NW, Quinn NP *et al.* A Yorkshire family with adult-onset cranio-cervical primary torsion dystonia. *Mov Disord* 2000; **15**: 954–959.
128. Charlesworth G, Plagnol V, Holmström KM, Bras J, Sheerin U-M, Preza E *et al.* Mutations in ANO3 cause dominant craniocervical dystonia: ion channel implicated in pathogenesis. *Am J Hum Genet* 2012; **91**: 1041–1050.
129. Tsutsumi S, Kamata N, Vokes T, Maruoka Y, Nakakuki K, Enomoto S *et al.* The novel gene encoding a putative transmembrane protein is mutated in gnathodiaphyseal dysplasia (GDD). *Am J Hum Genet* 2004; **74**: 1255–1261.
130. Savarese M, Di Fruscio G, Tasca G, Ruggiero L, Janssens S, De Bleecker J *et al.* Next generation sequencing on patients with LGMD and nonspecific myopathies: findings associated with ANO5 mutations. *Neuromuscular Disord* 2015; **25**: 533–541.
131. Vermeer S, Hoischen A, Meijer RPP, Gilissen C, Neveling K, Wieskamp N *et al.* Targeted next-generation sequencing of a 12.5 Mb homozygous region reveals ANO10 mutations in patients with autosomal-recessive cerebellar ataxia. *Am J Hum Genet* 2010; **87**: 813–819.
132. Balreira A, Boczonadi V, Barca E, Pyle A, Bansagi B, Appleton M *et al.* ANO10 mutations cause ataxia and coenzyme Q(1)(0) deficiency. *J Neurol* 2014; **261**: 2192–2198.
133. Danek A, Rubio JP, Rampoldi L, Ho M, Dobson-Stone C, Tison F *et al.* McLeod neuroacanthocytosis: genotype and phenotype. *Ann Neurol* 2001; **50**: 755–764.