

REVIEW ARTICLE

The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease

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Abstract

A common feature of all eukaryotic membranes is the non-random distribution of different lipid species in the lipid bilayer (lipid asymmetry). Lipid asymmetry provides the two sides of the plasma membrane with different biophysical properties and influences numerous cellular functions. Alteration of lipid asymmetry plays a prominent role during cell fusion, activation of the coagulation cascade, and recognition and removal of apoptotic cell corpses by macrophages (programmed cell clearance). Here we discuss the origin and maintenance of phospholipid asymmetry, based on recent studies in mammalian systems as well as in *Caenorhabditis elegans* and other model organisms, along with emerging evidence for a conserved role of mitochondria in the loss of lipid asymmetry during apoptosis. The functional significance of lipid asymmetry and its disruption during health and disease is also discussed.

Keywords: Phospholipid asymmetry; *Caenorhabditis elegans*; programmed cell clearance; mitochondria; phosphatidylserine; phospholipid scramblases; aminophospholipid translocases

Introduction

A common feature of membranes in all eukaryotic cells is the non-random distribution of lipids across the bilayer. Lipid asymmetry in membranes is a consequence of multiple factors, including the biophysical properties of lipids that dictate their ability to spontaneously “flip” their polar headgroups through the hydrophobic membrane interior, and the presence of transporters (enzymes) that assist in active lipid translocation across the bilayer (see van Meer *et al.*, 2008, for an excellent review). Moreover, this asymmetrical distribution of lipids has important functional consequences. For instance, the anionic phospholipid, phosphatidylserine (PS), is exclusively located at the cytoplasmic side of the plasma membrane in quiescent cells and is an essential co-factor for a number of membrane-bound enzymes, such as protein kinase C and Na⁺/K⁺-ATPase (Zwaal *et al.*, 2005). However, when exposed on the cell surface, PS acts as a conserved recognition signal for phagocytes and promotes the blood coagulation cascade (Savill *et al.*, 2002). The present review aims

to discuss the origin and maintenance of phospholipid asymmetry as well as the mechanism and functional significance of its disruption in health and disease.

The origin and maintenance of phospholipid asymmetry in biological membranes

The variations and permutations of headgroups and aliphatic chains, and the occurrence of oxidatively modified lipids, allows for the existence of a multitude of individual molecular species of lipids in any eukaryotic cell (Kagan and Quinn, 2004; Wolf and Quinn, 2008). Importantly, although all lipids appear to be symmetrically distributed between the two leaflets of the endoplasmic reticulum membrane, an asymmetric distribution of lipids is seen in the Golgi, endosomal, and plasma membranes of eukaryotic cells, with sphingomyelin and glycosphingolipids residing predominantly on the non-cytosolic (luminal) side and the anionic

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(Received 15 June 2009; revised 20 July 2009; accepted 20 July 2009)

ISSN 1040-9238 print/ISSN 1549-7798 online © 2009 Informa UK Ltd
DOI: 10.1080/10409230903193307

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phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE) enriched in the cytosolic leaflet (van Meer *et al.*, 2008) (Figure 1). The discovery that this asymmetry results from the adenosine triphosphate (ATP)-dependent translocation of PS and PE between bilayer leaflets underscores the notion that membrane lipid asymmetry is of major physiologic importance, as it demonstrates that cells invest considerable energy in this process (Zwaal and Schroit, 1997). The identification of the specific enzyme(s) responsible for phospholipid translocation in the plasma membrane has attracted considerable attention.

Human erythrocytes have served as a prototypical model to study the structure of the eukaryotic membrane and associated lipid asymmetry. Both energy-dependent and energy-independent transport activities have been reported to play an important role for the generation and maintenance of lipid asymmetry in these cells (Daleke, 2008). At least three types of putative lipid transporter activities have been proposed: a “flippase”, which catalyzes ATP-dependent inward transport of lipids; a “floppase”, which promotes ATP-dependent outward migration of lipids; and a “scramblase”, which stimulates bi-directional movement of lipids between the two membrane leaflets (Daleke, 2008). While the first two activities are thought to generate and maintain lipid asymmetry, bi-directional lipid scrambling promotes the collapse of this asymmetry. Numerous studies have associated a specific class of P-type ATPases (the P4 ATPases) and the ATP-binding cassette (ABC) transporter family, as well as the so-called scramblase family of proteins with each of these three classes of lipid transporters, respectively (for a review and original references, see Zwaal *et al.*, 2005). The discovery of an aminophospholipid translocase (APLT) activity in bovine chromaffin granules led to the cloning of ATPase II (ATP8A1) (Tang *et al.*, 1996) that is homologous to Drs2p, a trans-Golgi network-resident

protein in yeast. ATP8A1 and Drs2p are founding members of a conserved subfamily of P-type ATPases that includes five yeast, six *Caenorhabditis elegans*, four *Drosophila*, and 14 human members (Lenoir *et al.*, 2007; Darland-Ransom *et al.*, 2008; D. Xue, unpublished observation). Approximately at the same time, Sims and colleagues purified a 37-kDa type II single-transmembrane protein from erythrocyte membranes that could mediate Ca^{2+} -dependent movement of phospholipids between membrane leaflets and thus possesses the “phospholipid scramblase” activity (Bassé *et al.*, 1996; Zhou *et al.*, 1997). This phospholipid scramblase, PLSCR1, is highly conserved among different organisms (Wiedmer *et al.*, 2000; Wang *et al.*, 2007). A major roadblock, however, in terms of identifying the specific molecules responsible for the generation or maintenance of lipid asymmetry and for its disruption during cell activation or cell death is that there are numerous members of each family of transporters, which precludes genetic analysis of the *in vivo* role of each of these molecules in mammalian systems due to potential genetic redundancy. On the other hand, our recent studies in *C. elegans*, a model organism especially suited for powerful genetic analyses, have revealed that a worm P4 ATPase homolog, TAT-1, is required for maintenance of PS asymmetry in living cells (Darland-Ransom *et al.*, 2008; Figure 2). These studies also provided evidence that the disruption of phospholipid asymmetry in living cells can result in indiscriminate removal of affected cells by neighboring phagocytes. Moreover, we demonstrated that a worm phospholipid scramblase homolog, SCRM-1, is important for compromising this PS asymmetry and promoting the externalization of PS in apoptotic cells (Wang *et al.*, 2007) (see further discussion below).

Interestingly, the P4 ATPases belong to the superfamily of P-type ATP pumps whose members usually translocate small cations or metal ions, rather than

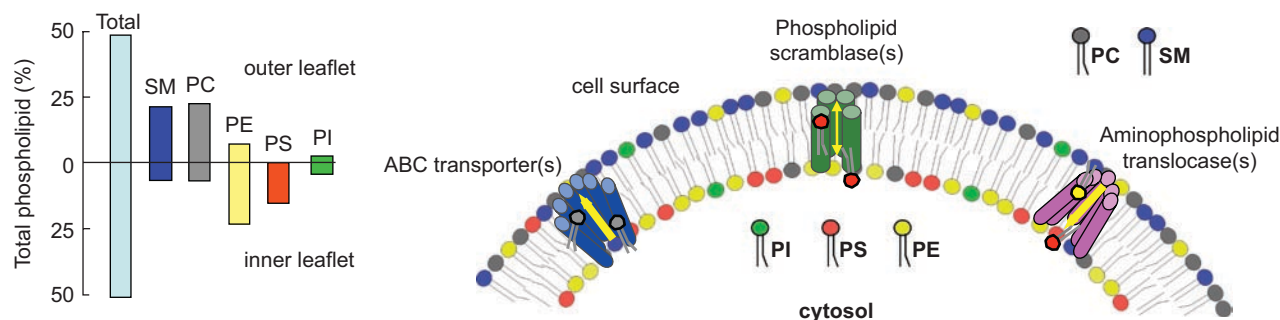


Figure 1. Phospholipid asymmetry and related lipid-translocating enzymes. In the plasma membrane of normal eukaryotic cells, phosphatidylcholine (PC) and sphingomyelin (SM) are present predominantly in the outer leaflet. Phosphatidylethanolamine (PE) and phosphatidylinositol (PI) reside mainly in the inner leaflet, while phosphatidylserine (PS) is located almost exclusively in the inner leaflet of the plasma membrane. Phospholipid asymmetry may be maintained or altered through the action of three classes of proteins, as discussed in the present review: phospholipid scramblases, ATP-binding cassette (ABC) transporters, and aminophospholipid translocases. The graph shows the transbilayer distribution of phospholipids across the human erythrocyte membrane (adapted from Daleke, 2008 with permission from Lippincott Williams & Wilkins, Inc.).

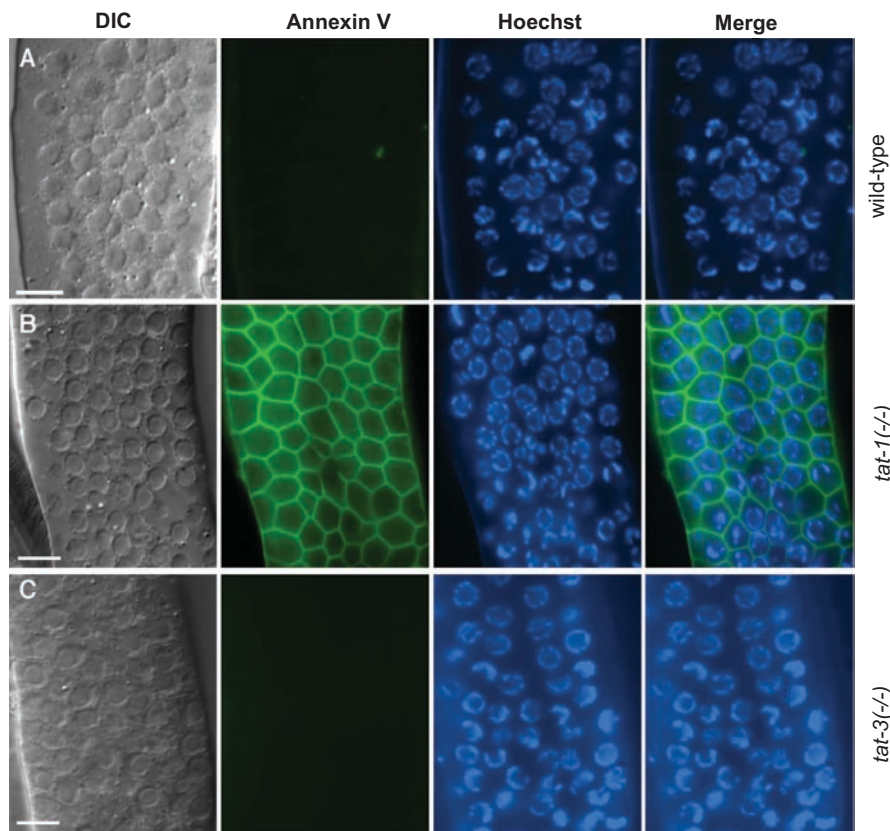


Figure 2. Exposure of PS on the surface of *C. elegans* germ cells in *tat-1*-deficient worms. Exposed gonads of the following hermaphrodite adult worms were stained with the PS-binding protein, annexin V: (A) wild-type, (B) *tat-1(tm1034)*, and (C) *tat-3(tm1275)*, as described in Darland-Ransom *et al.* (2008). Images of differential contrast interference (DIC), annexin V staining, Hoechst 33342 staining, and the merged image of annexin V plus Hoechst 33342 staining are shown. The data show that reduction of *tat-1* activity, but not of related *tat-3* activity is sufficient to disrupt asymmetrical PS distribution on the surface of *C. elegans* germ cells. Scale bars correspond to 6.5 μ m.

lipids (Lenoir *et al.*, 2007). In both cases, the net result of the action of these pumps is to maintain asymmetry or a gradient (of lipids, or ions) across a membrane, a fundamental aspect of cellular physiology that drives numerous processes in the cell. As pointed out by Lenoir and co-workers, a challenging problem is to understand how an ion pump evolved into a flippase, i.e. a protein that “flips” phospholipids. In a very recent study, these investigators provided evidence that the so-called Cdc50 proteins are integral components of the P4 ATPase transport machinery and that the affinity of yeast P4 ATPase Drs2p for its Cdc50 binding partner fluctuates during the transport cycle, with the strongest interaction occurring at a point where the enzyme is loaded with phospholipid ligand (Lenoir *et al.*, 2009). The specific interactions between P-type ATPases and Cdc50 proteins could thus suggest a basis for the transport specificity of P-type ATPases.

Because lipid asymmetry is fundamental to several cellular processes, it is important that this asymmetry is maintained throughout the life-span of the cell. Indeed, as noted above, cells invest considerable amounts of

energy to generate and maintain asymmetric phospholipid distribution. However, lipid asymmetry-sensing proteins or related downstream signaling pathways have been poorly defined. Interestingly, recent studies have indicated that the pH-responsive Rim101 pathway, the protein kinase Mck1, and the transcription factor Mot3 all act in lipid asymmetry signaling in *Saccharomyces cerevisiae* and that the Rim101 pathway is activated in response to changes in lipid asymmetry (Ikeda *et al.*, 2008). These studies are also suggestive of the convergence of lipid asymmetry sensing and pH adaptation through the Rim101 pathway. Whether these pathways are conserved in higher species remains to be elucidated.

Molecular mechanisms underlying the disruption of plasma membrane phospholipid asymmetry

The P4 ATPases flip lipids from the non-cytosolic (outer) leaflet to the cytosolic (inner) leaflet of the membrane,

whereas the so-called ABC transporters work in the opposite direction. Moreover, ABC transporters are capable of expelling a given lipid out of the membrane (van Meer *et al.*, 2008). Most of the mammalian ABC transporters that are involved in lipid transport have been associated with specific diseases or pathologies; however, the mechanism(s) of action and the specific substrates for many of these ABC transporters remain a matter of debate. ABCA1, for instance, is mutated in Tangier disease, a condition characterized by a severe high-density lipoprotein (HDL) deficiency, sterol deposition in tissue macrophages, and prevalent atherosclerosis (Box 1). Early studies suggested a role for ABCA1 in macrophage engulfment of apoptotic cells (Luciani and Chimini, 1996) and subsequent studies implicated ABCA1 in the disruption of phospholipid asymmetry during apoptosis (Hamon *et al.*, 2000). In line with these studies, Wu and Horvitz (1998) reported that the *C. elegans* gene *ced-7*, encoding a protein homologous to ABCA1, functions in the engulfment of apoptotic cell corpses in the worm. They also proposed that CED-7 translocates molecules that mediate homotypic adhesion between the dying and engulfing cells. More recent studies using cells from ABCA1-deficient mice and from Tangier patients with homozygous mutations in the ABCA1 gene showed that mutations in ABCA1 do not measurably alter the rate of transbilayer movements of phospholipids (Williamson *et al.*, 2007). These studies also discounted a role for ABCA1 in the surface externalization of annexins, phospholipid-binding proteins previously reported to be expressed both on the surface of phagocytes and on cells undergoing apoptosis (Arur *et al.*, 2003; Fan *et al.*, 2004). However, these results do not rule out a role for ABCA1 in phospholipid loading to apoA-1. Similarly, there have been conflicting reports regarding the role of *C. elegans* CED-7 in PS externalization in apoptotic cells. In one study (Venegas and Zhou, 2007), CED-7 was found to promote PS externalization in somatic apoptotic cells, whereas in another, CED-7 was suggested to be dispensable (Züllig *et al.*, 2007). Obviously, the role of ABC transporters as potential transbilayer lipid transporters warrants further examination and investigation.

As shown originally for activated platelets, many cell membranes harbor a Ca^{2+} -dependent mechanism that can rapidly move phospholipids back and forth between the two membrane leaflets (lipid scrambling), leading to a loss of membrane phospholipid asymmetry (for a review and original references, see Zwaal *et al.*, 2004; 2005). Scott syndrome, an extremely rare congenital bleeding disorder characterized by a deficiency in platelet procoagulant activity (Miletich *et al.*, 1979; Weiss *et al.*, 1979), is the only known primary pathological consequence caused by an aberration in regulating membrane phospholipid asymmetry (Rosing *et al.*, 1985) (Box 1). Despite its rare occurrence, investigations of cells

Box 1. Examples of human diseases associated with defects in lipid transporters.

Mutations in the *Atp8B1* gene encoding a putative member of the flippase family are the cause of progressive familial intrahepatic cholestasis type I (Klomp *et al.*, 2000), originally described as “Byler disease” in an Amish kindred. The disease primarily manifests itself as a chronic intrahepatic cholestasis, which progresses to severe, end-stage liver disease before adolescence. The patients display impaired hepatobiliary bile salt secretion and normal serum cholesterol. Overexpression of *Atp8B1* was shown to result in energy-dependent PS translocation in a model cell line (Ujhazy *et al.*, 2001). However, it is not known whether the absence of the putative aminophospholipid translocase activity is related to the pathogenesis of this severe disease.

Mutations in the ATP-binding cassette (ABC) transporter, ABCA1, a candidate floppase, are responsible for Tangier disease (Bodzioch *et al.*, 1999; Brooks-Wilson *et al.*, 1999; Rust *et al.*, 1999), an autosomal recessive disorder characterized by the absence of high-density lipoprotein (HDL), reduced levels of low-density lipoprotein (LDL), and mildly elevated triglycerides. Tangier patients are at high risk for developing premature coronary artery disease (atherosclerosis). Mice with a targeted inactivation of ABCA1 are unable to load cholesterol and phospholipids to apoA-1, implicating ABCA1 in the efflux of these lipids from cells (Orso *et al.*, 2000; McNeish *et al.*, 2000).

Scott syndrome is a rare congenital bleeding disorder, characterized by an impaired ability of the patients' platelets to promote blood coagulation (Weiss *et al.*, 1979). Platelets from these individuals are deficient for Ca^{2+} -induced phospholipid scramblase activity in the plasma membrane, resulting in a reduced exposure of procoagulant PS on the outer surface of the cell (reviewed in Zwaal *et al.*, 2004). The molecular lesions responsible for the underlying genetic defect have yet to be determined.

from these patients have shed light on the mechanisms involved in the disruption of phospholipid asymmetry in the plasma membrane (Zwaal *et al.*, 2004). The disorder was originally described as an isolated deficiency of platelet procoagulant activity, but the underlying defect in Ca^{2+} -induced lipid scrambling is also evident in other cell types. Interestingly, studies in lymphocytes have shown that there are differences between Ca^{2+} -induced lipid scrambling that occurs during cell activation and the egress of PS that occurs when cells are undergoing apoptosis. Indeed, whereas Scott syndrome cells fail to expose PS following Ca^{2+} influx, PS externalization is normal in apoptotic cells from these patients

(Williamson *et al.*, 2001; Martinez and Freyssinet, 2001). The identification of PLSCR1 as a protein that exhibits Ca^{2+} -activated phospholipid scrambling activity has provided plausible candidates for studying the disruption of lipid asymmetry (Zhou *et al.*, 1997). Indeed, PLSCR1 has been implicated in promoting PS externalization in apoptotic cells or PS exposure in neutrophils stimulated by a chemotactic peptide (Frasch *et al.*, 2000; 2004). However, in other studies, overexpression of exogenous PLSCR1 or induction of endogenous PLSCR1 expression by interferon- α failed to promote PS externalization in human lymphoma cells undergoing apoptosis (Fadeel *et al.*, 1999). Moreover, mice deficient in PLSCR1 do not display the hemostatic abnormalities characteristic for Scott syndrome or a defect in PS externalization in activated cells (Zhou *et al.*, 2002). In fact, recent data suggest a considerably more complex biology for PLSCR1 (see, for instance, Ben-Efraim *et al.*, 2004; Huang *et al.*, 2006). Nevertheless, since multiple phospholipid scramblases exist in mammals and in other organisms (Wiedmer *et al.*, 2000; Wang *et al.*, 2007), these results do not exclude a role for other phospholipid scramblase(s) in the process of PS externalization in the plasma membrane.

Two recent studies have provided evidence that some *C. elegans* phospholipid scramblases indeed are involved in PS externalization in apoptotic cells (Venegas and Zhou, 2007; Wang *et al.*, 2007). We have found that inactivation of *scrm-1*, which encodes one of the eight *C. elegans* phospholipid scramblase homologues (named SCRM proteins), significantly reduces PS exposure on the surface of *C. elegans* apoptotic germ cells and compromises cell corpse engulfment (Wang *et al.*, 2007) (Figure 3),

suggesting that surface-exposed PS also serves as an “eat-me” signal for removal of apoptotic cells in *C. elegans*. In a similar study, Venegas and Zhou (2007) found that another *C. elegans* scramblase, SCRM-3 (or PLSC-1) also mediates PS externalization in apoptotic germ cells and affects their removal by phagocytes. However, loss of *scrm-1* or *scrm-3* only partially reduces PS exposure on the surface of apoptotic cells, indicating that additional phospholipid scramblases or lipid transporters may be involved in mediating PS externalization in *C. elegans* apoptotic cells. Moreover, as lipid scrambling enzymes, the activities of these scramblases need to be tightly controlled so that their activities are activated only in apoptotic cells. What could be the activation signals or mechanisms? Interestingly, we found that the *C. elegans* mitochondrial factor, WAH-1, a homolog of human apoptosis-inducing factor (AIF), also affects cell corpse engulfment (Wang *et al.*, 2007). Further analysis indicated that loss of *wah-1* markedly reduces PS externalization in apoptotic germ cells. However, WAH-1 by itself had no lipid scrambling activity and therefore probably needs to act through a lipid transporter. Indeed, we found that WAH-1 and SCRM-1 act in the same pathway to promote PS externalization in *C. elegans* apoptotic cells and that they interact with each other *in vitro*. Strikingly, in proteoliposome assays, WAH-1 could activate the PS scrambling activity of SCRM-1 10-fold and this SCRM-1-activating activity depends on specific interaction between SCRM-1 and WAH-1. Therefore, WAH-1 is an apoptotic activator and SCRM-1 is a downstream lipid-flipping enzyme that mediates PS externalization during apoptosis (Figure 4). Since the conserved BH3-only cell

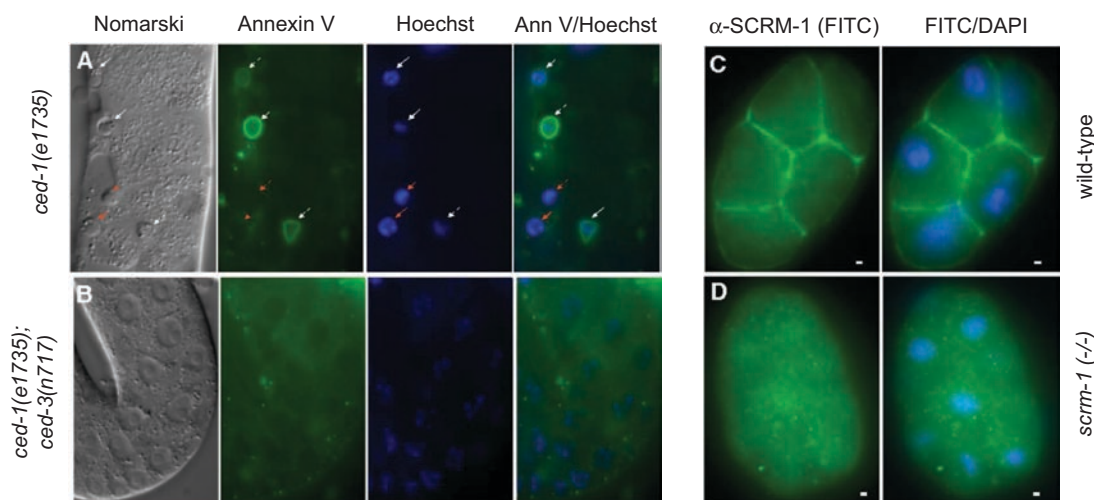


Figure 3. *wah-1* and *scrm-1* promote PS exposure on the surface of apoptotic germ cells in *C. elegans*. (A, B) The exposed gonad of a *ced-1(e1735)* (A) hermaphrodite animal or a *ced-1(e1735); ced-3(n717)* (B) animal was stained with annexin V and Hoechst 33342. Germ cell corpses were identified by their raised-button-like morphology under Nomarski optics and the condensed Hoechst 33342 staining pattern, as detailed in Wang *et al.* (2007). Germ cell corpses stained with both Hoechst and annexin V are indicated by white arrows, and those that were stained with Hoechst but not with annexin V are indicated with red arrows. The scale bar represents 6.5 μm . (C, D) SCRM-1 localizes to the plasma membrane. Images of FITC (SCRM-1 antibody staining) and FITC-DAPI *C. elegans* 16-cell stage wild-type (C) or *scrm-1(tm805)* (D) embryos are shown. The scale bars correspond to 1 μm .

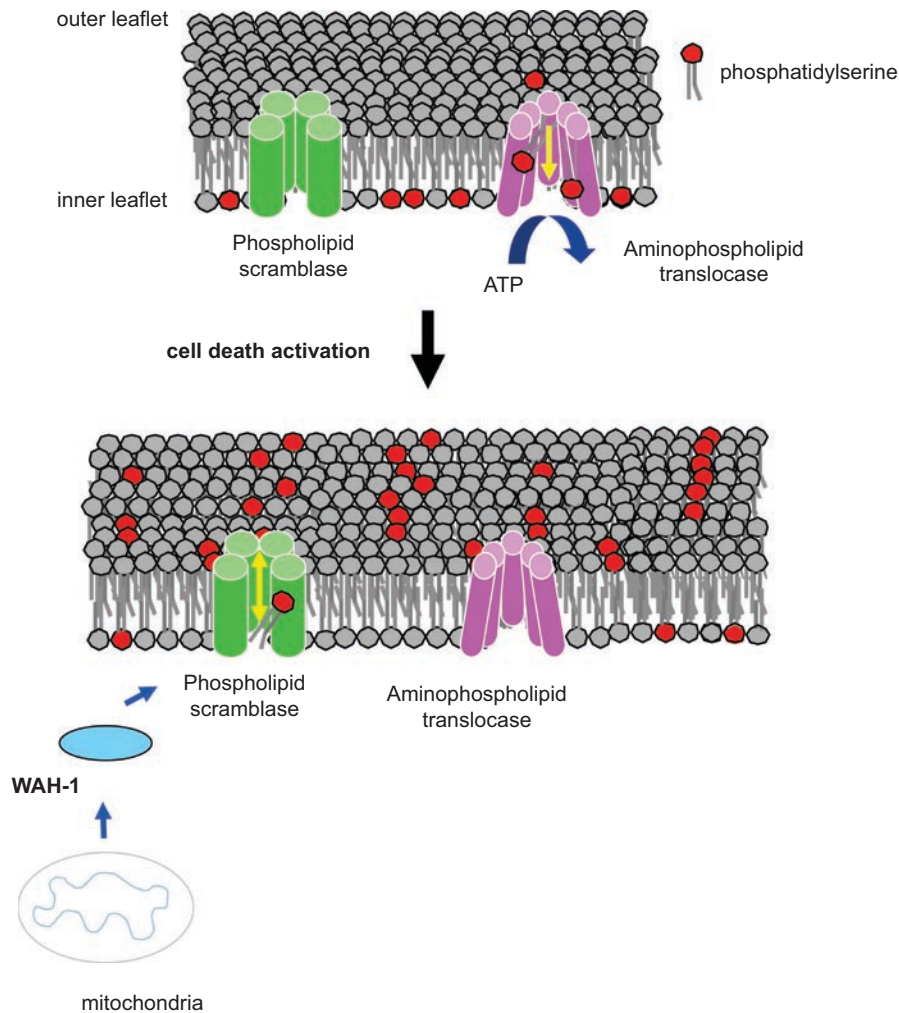


Figure 4. Plasma membrane lipid transporters regulate the distribution of the phospholipid, PS. In quiescent cells, energy (ATP)-dependent translocation of PS from the outer to the inner leaflet of the plasma membrane serves to maintain the absolute asymmetry of PS distribution. Upon activation of the cell death program, the mitochondrial apoptogenic factor, WAH-1 (worm homolog of AIF) exits from mitochondria and activates the lipid scrambling activity of the plasma membrane phospholipid scramblase, SCRM-1. The bi-directional scrambling of phospholipids destroys the asymmetrical distribution of PS. Externalized PS serves as a signal to trigger engulfment by phagocytes through its binding to the PS receptor, PSR-1 (not shown).

death initiator, EGL-1 induces the release of WAH-1 from mitochondria during apoptosis (Wang *et al.*, 2002), this finding delineates a novel mitochondrion-to-plasma membrane signaling pathway that promotes apoptotic PS externalization. Interestingly, previous studies demonstrated that the microinjection of AIF into the cytosol of human cells induces dissipation of the mitochondrial transmembrane potential and PS externalization on the cell surface (Susin *et al.*, 1999). Furthermore, numerous studies support the view that PS externalization occurs downstream of mitochondria in cells undergoing apoptosis (Zhuang *et al.*, 1998; Uthaisang *et al.*, 2003; Blom *et al.*, 2003; Ricci *et al.*, 2004). It will be interesting to see whether the WAH-1/SCRM-1 PS externalization pathway is conserved in mammals. Further dissection of the molecular pathways that mediate PS externalization

is now possible based on the studies performed in the nematode system.

The expanding role of phospholipid scrambling: implications for mitochondrial apoptosis signaling

Mice lacking PLSCR3, a member of the mammalian phospholipid scramblase family, display adiposity, dyslipidemia, and insulin resistance (Wiedmer *et al.*, 2004), suggesting that defects in lipid scrambling may contribute to the dysregulation of lipid metabolism and the development of metabolic syndromes. PLSCR3 has also been implicated in the translocation of a phospholipid, cardiolipin (CL), from the inner to the

outer mitochondrial membrane (Liu *et al.*, 2003). CL integrates a variety of apoptosis-inducing signals at the level of mitochondria (Schug and Gottlieb, 2009). For instance, apoptosis induced by tumor necrosis factor (TNF)- α and TNF-related apoptosis-inducing ligand (TRAIL) is potentiated by PLSCR3, through its effects on the distribution of CL on the mitochondrial surface (Liu *et al.*, 2008; Ndebele *et al.*, 2008). The latter serves as an illustrative example of the importance of the specific membrane distribution of a phospholipid for the regulation of cellular processes (apoptosis).

Further studies on the regulation of plasma membrane phospholipid asymmetry by mitochondria

Calcium has been implicated in the regulation of both cell survival and cell death (apoptosis) pathways and mitochondria participate actively in intracellular Ca^{2+} compartmentalization and signaling (Orrenius *et al.*, 2003). Ca^{2+} ions are thought to play a role in the regulation of phospholipid asymmetry, as the activity of some scramblases is dependent on Ca^{2+} , at least in platelets, whereas aminophospholipid translocase activity is also inhibited by Ca^{2+} (Zwaal and Schroit, 1997). Furthermore, in platelets, elevation of cytosolic Ca^{2+} with thapsigargin, an inhibitor of the sarco-endoplasmic reticulum Ca^{2+} -ATPase, can trigger PS exposure in minutes (Dachary-Prigent *et al.*, 1995). In thapsigargin-treated Jurkat cells, however, far fewer cells exposed PS, and the process took up to six hours (Hampton *et al.*, 1996). In addition, removal of extracellular Ca^{2+} , but not chelation of intracellular Ca^{2+} , inhibited PS exposure in Fas-treated Jurkat cells by approximately 50%. Therefore, it appears that PS exposure in cells undergoing death receptor-mediated apoptosis is Ca^{2+} -dependent, but not Ca^{2+} -regulated, whereas the same process in platelets is Ca^{2+} -regulated. Interestingly, antibodies to CD9, a member of the tetraspan family of membrane proteins, inhibits the prompt (within minutes) PS exposure in Jurkat cells stimulated by the calcium ionophore, A23187, but fails to block the delayed PS exposure induced by various apoptotic treatments (Li and Tait, 1998). Moreover, in lymphocytes from patients with Scott syndrome, the scramblase activity is not activated by elevated internal Ca^{2+} levels but can still be induced by apoptosis (Williamson *et al.*, 2001; Martinez & Freyssinet, 2001), suggesting that at least two different pathways, one of which is Ca^{2+} -dependent and one of which is Ca^{2+} -independent but apoptosis-dependent, independently activate the scramblase activity in the cell. Consistent with this observation, our recent studies in *C. elegans* have demonstrated that the mitochondria-derived factor, WAH-1 can activate the phospholipid scrambling activity of SCRM-1 in a Ca^{2+} -

independent manner (Wang *et al.*, 2007). These findings further support the view that different pathways of PS externalization are initiated in cells undergoing apoptosis and following Ca^{2+} -dependent activation.

Interestingly, a sustained intracellular calcium level in Jurkat cells mediated through the receptor-operated calcium channel was shown to induce mitochondria-dependent PS exposure in the absence of caspase activation and without evidence of mitochondrial release of cytochrome c or AIF (Jambrina *et al.*, 2003). However, the process required oxidative/nitrosative stress. One may thus speculate that the PS exposure evidenced in the latter model is related to oxidative/nitrosative inactivation of aminophospholipid translocase activity, as demonstrated recently in HL-60 cells (Tyurina *et al.*, 2007). In this context, it is also interesting to note that neutrophils from patients with Barth syndrome, a rare, metabolic (mitochondrial) disorder characterized by neutropenia and cardioskeletal myopathy, expose low amounts of PS on the surface, yet do not display other features of apoptosis (Kuijpers *et al.*, 2004). The mechanism that underlies the PS exposure in Barth syndrome neutrophils remains elusive but it has been suggested that mitochondria-derived reactive oxygen species (ROS) may act as signaling intermediates in this process (van Raam and Kuijpers, 2009). Detailed assessment of phospholipid scramblase and aminophospholipid translocase activities in neutrophils from these patients versus healthy controls could shed some light on the link between mitochondrial ROS production and the loss of phospholipid asymmetry in the plasma membrane.

Evidence has also been presented for *de novo* synthesis of PS during apoptosis, suggesting yet another potential role for mitochondria in regulating PS asymmetry in the plasma membrane. PS synthesis takes place in the endoplasmic reticulum (ER) and results from replacement of the polar head group of preexisting phospholipids (either PC or PE) by a serine (Kuge and Nishijima, 2003). The exchange of the polar head group is catalyzed by the Ca^{2+} -dependent serine-base exchange enzyme system. Newly synthesized PS migrates either to the inner leaflet of the plasma membrane or to mitochondria where it is decarboxylated to PE. Aussel *et al.* (1998) first reported a robust yet transient neosynthesis of PS in Jurkat cells triggered to undergo Fas-mediated apoptosis. PS decarboxylation, a mitochondria-specific process, was strongly inhibited in this model, and the newly synthesized PS was detectable on the cell surface. Subsequent studies from the same investigators suggested a relationship between PS synthesis through the serine-base exchange enzyme system in the ER and PS exposure at the cell surface during Fas-mediated apoptosis (Pelassy *et al.*, 2000). Similarly, treatment of mouse thymocytes with the apoptotic stimulus, dexamethasone, enhances the activity of the serine-base exchange

enzyme (Burrata *et al.*, 2000). However, the decarboxylation of newly synthesized PS to PE was almost negligible in mouse thymocytes, indicating that while different apoptotic stimuli may trigger an increase of PS synthesis, with subsequent PS exposure they may have specific effects on the enzymes and organelles involved in PS metabolism.

Role of plasma membrane phospholipid asymmetry for intrinsic membrane potential

The intrinsic membrane potential affects or governs a variety of biological phenomena, and it has been generally accepted that the difference in membrane potential arises from the salt ion imbalance across the plasma membrane. However, recent atomic-scale molecular dynamics simulations show that a non-zero transmembrane potential can be observed in phospholipid membranes in the absence of ions, and suggest that the asymmetric distribution of lipids across the membrane and the intrinsic membrane potential are coupled phenomena (Gurtovenko and Vattulainen, 2007). The latter findings are in agreement with classical experimental data on dipole potential measurements in asymmetric lipid membranes (Latorre and Hall, 1976). It is of interest to note that the negative charge of the surface of apoptotic cells associated with externalization of PS has been shown to trigger endothelial cell membrane hyperpolarization and cytoskeleton reorganization, events that are important for angiogenesis and vascular remodeling (Weihua *et al.*, 2005). Moreover, the negative charge associated with the presence of PS on the cytosolic leaflet of the membrane in living cells has been shown to direct proteins with a moderately positive charge to the endocytic pathway (Yeung *et al.*, 2008). Recent studies have shown that PS persists on phagosomes of murine macrophages as the plasma membrane invaginates and that by conferring a considerable negative charge onto the phagosomal surface, PS contributes to the recruitment of cationic proteins such as the c-Src tyrosine kinase (Yeung *et al.*, 2009).

Loss of phospholipid asymmetry and PS externalization regulate important biological processes

The loss of lipid asymmetry in the plasma membrane and the appearance of the anionic phospholipids such as PS and PE on the cell surface have been implicated in numerous biological processes, including blood coagulation, myotube formation, vesicle fusion, cell division, sperm capacitation, and phagocytic recognition and

clearance of apoptotic cell corpses (for a review, see Witasz *et al.*, 2008). Moreover, loss of PS asymmetry in the plasma membrane may also be important for some viral infections. Viruses budding off an apoptotic host cell acquire a viral envelope that also exposes PS on its external surface, and entry of vaccinia virus through macropinocytosis was recently shown to be dependent on the presence of exposed PS on the viral membrane, suggesting that certain viruses may employ apoptotic "mimicry" to enter cells (Mercer and Helenius, 2008). Apoptotic mimicry by another obligate intracellular parasite was demonstrated previously (de Freitas Balanco *et al.*, 2001; Wanderley *et al.*, 2006). These studies showed that exposure of PS on the surface of the disease-propagating amastigotes of *Leishmania amazonensis* inhibits macrophage activity and increases susceptibility to intracellular leishmanial growth. Hence, while the protozoan *Trypanosoma cruzi* has been reported to use apoptotic host T lymphocytes for macrophage inactivation and consequent persistence in the mammalian host (Freire de Lima *et al.*, 2000), *Leishmania amazonensis*, an intramacrophagic parasite, appears to use an apoptotic-like feature of its own cells for a similar purpose.

Binding of blood clotting enzyme complexes to phospholipid membranes is a characteristic feature of blood coagulation (Zwaal *et al.*, 2004). PS externalization on the surface of activated platelets produces a nearly million-fold increase in the rate of thrombin formation, the key enzyme of blood coagulation. Surface exposure of PS is brought about by the activation of Ca^{2+} -dependent plasma membrane scramblases. Moreover, and as noted above, Scott syndrome is a hematological disorder in which blood clotting does not take place due to defective phospholipid scrambling in the plasma membrane. Schoenwaelder *et al.* (2009) recently reported the existence of two distinct pathways regulating the procoagulant function of platelets *in vitro*: the classical, Ca^{2+} -dependent pathway induced by physiological agonists, and a Bak/Bax-dependent and caspase-mediated (apoptotic-like) pathway independent of platelet activation. Whether the apoptosis-related pathway participates in externalizing PS on the surface of platelets to generate thrombin *in vivo* or whether it merely acts to produce a signal for the clearance of platelets by phagocytic cells remains to be investigated. Platelets are anuclear cells generated through the compartmentalized, caspase-directed death of their progenitors, megakaryocytes (de Botton *et al.*, 2002). Interestingly, this aborted apoptosis process yields functional platelets that retain their plasma membrane phospholipid asymmetry (Clarke *et al.*, 2003). The latter findings thus reinforce the notion that plasma membrane changes of apoptosis can be dissociated from the caspase-directed program of nuclear condensation and fragmentation (Zhuang *et al.*, 1998).

Cell surface exposure of PS has also been shown to be part of normal physiology of skeletal muscle development and to mediate myotube formation (van den Eijnde *et al.*, 2001). PS exposure in myoblasts is caspase-independent, occurs mainly at cell-cell contact areas, and takes place at a stage when the structural organization of the sarcomeric protein titin is initiated, prior to actual fusion of individual myoblasts into multinucleated myotubes. PS exposure is viewed as a common signal for engulfment, yet studies in mouse embryos showed that no accumulation of phagocytes occurred in areas of myoblast differentiation. As suggested by van den Eijnde *et al.* (2001), this may potentially be explained by the lack of secretion of chemoattractant factors for phagocytes.

Loss of phospholipid asymmetry and cell surface externalization of PS is a general feature of the phagocytosis of apoptotic cells by macrophages (Fadok *et al.*, 1992; Martin *et al.*, 1995; Krahling *et al.*, 1999) and has been linked to the activation of caspases (Martin *et al.*, 1996; Vanags *et al.*, 1996). Apoptotic cells that fail to express PS are not efficiently engulfed by macrophages, and the clearance defect can be restored by repleting the plasma membrane of target cells with exogenous PS (Fadok *et al.*, 2001; Kagan *et al.*, 2002). In addition, oxidized phospholipids including oxidized PS (PS-OX) may mediate macrophage recognition (Chang *et al.*, 1999; Matsura *et al.*, 2002; Kagan *et al.*, 2002; Arroyo *et al.*, 2002; Tyurina *et al.*, 2004). Furthermore, Kagan and associates have demonstrated that cytochrome c released from mitochondria is involved in the selective catalysis of PS oxidation during programmed cell clearance (Jiang *et al.*, 2004), underscoring the role of mitochondria-derived factors in apoptosis-related alterations of the plasma membrane.

Phosphatidylserine receptors: lessons emerging from studies in mice and other model organisms

PS externalization serves as an important and conserved recognition signal during apoptosis (Fadeel and Xue, 2006), and recent studies have identified several different PS receptors on macrophages, including T cell immunoglobulin- and mucin-domain-containing molecule-4 (Tim-4) and Tim-1 (Miyamishi *et al.*, 2007; Kobayashi *et al.*, 2007; Ichimura *et al.*, 2008), the G protein-coupled receptor, brain-specific angiogenesis inhibitor 1 (BAI1) (Park *et al.*, 2007), and stabilin 2 (Park *et al.*, 2008), in addition to the original PS receptor (PSR) that was cloned almost 10 years ago by Fadok *et al.* (2000). Importantly, studies in mice that are deficient for PSR (Li *et al.*, 2003; Kunisaki *et al.*, 2004) or the PS-binding protein, milk fat globule-epidermal growth factor 8

(MFG-E8) (Hanayama *et al.*, 2004) have demonstrated the occurrence of numerous unengulfed apoptotic cells and autoimmune disease, thus providing compelling evidence that PS-dependent programmed cell clearance is required for maintenance of tissue homeostasis. Injection of an MFG-E8 mutant protein that binds to PS but fails to bridge the apoptotic cells to macrophage receptors also results in impairment of apoptotic cell engulfment and production of autoantibodies in mice (Asano *et al.*, 2004). Furthermore, inactivation of the *C. elegans* PSR homolog, PSR-1, also results in an engulfment defect and PSR-1 is found to act upstream of the CED-5/CED-12 engulfment pathway and may transduce the PS "eat-me" signal through its interacting proteins, CED-5 and CED-12 (Wang *et al.*, 2003). Similarly, knockdown of the zebrafish PSR homolog (zfpsr) by a PSR morpholino oligonucleotide led to accumulation of a large number of dead apoptotic cells during development (Hong *et al.*, 2004). Moreover, the *Drosophila* PSR homolog (dPSR) plays an important role in mediating the removal of growth-disadvantaged cells that have reduced ribosomal protein gene dose by neighboring wild-type cells through a phagocytic mechanism (Li and Baker, 2007), although in a different study no obvious defect in engulfment of apoptotic cells was found in dPSR-deficient flies and instead dPSR appeared to function to inhibit apoptosis (Krieser *et al.*, 2007). In a third study involving PSR-deficient mice, Böse *et al.* (2004) reported that PSR-deficient macrophages were impaired in pro- and anti-inflammatory cytokine signaling after stimulation with apoptotic cells, but they could not document any impairment of apoptotic cell engulfment. Therefore, one may conclude that PSR plays a conserved role in phagocytosis of apoptotic or non-apoptotic cells in multiple organisms but may not be the dominant receptor for phagocytosis. It also should be noted that PSR is so far the only conserved candidate PS receptor, as the other proposed PS receptors discussed above are not found in worms or fruit flies. Further analysis of PSR, and in particular the cellular localization pattern of the endogenous PSR protein, will be critical for understanding how PSR is involved in phagocytosis and animal development.

Despite the prevailing view that PS exposure on the plasma membrane induces phagocytosis, recent studies of TAT-1-deficient worms have shown that the loss of living cells with ectopic exposure of PS appears to be random and non-exhaustive but is completely dependent on the activity of PSR-1 and another worm phagocyte receptor CED-1, which acts in a different engulfment pathway from PSR-1 (Darland-Ransom *et al.*, 2008). This raises the possibility that engagement of a single cell surface receptor for PS may not suffice to trigger phagocytosis, and that other "eat-me" signals or multiple receptors or pathways need to be engaged

for efficient removal of apoptotic cells. This observation may also explain the weak engulfment defect observed in PSR-deficient animals, as discussed above. Our previous studies in mammalian model systems have suggested that oxidation of PS may be required for efficient macrophage clearance of apoptotic cell corpses (Kagan *et al.*, 2002). Interestingly, the murine scavenger receptor, CD36, was shown to specifically recognize oxidized forms of PS (Greenberg *et al.*, 2006) and the CD36-related receptor, croquemort, is also implicated in apoptotic corpse clearance in *Drosophila* (Franc *et al.*, 1999). It is thus possible that different macrophage receptors may have very specific preferences for certain species of lipid generated during apoptosis (Fadeel *et al.*, 2007). Alternatively, or additionally, other engulfment signals or pathways may also be required for efficient engulfment of apoptotic cell corpses. We recently reported that PS externalization alone is not sufficient for macrophage disposal of neutrophils (Jitkaew *et al.*, 2009). However, addition of recombinant MFG-E8, a PS-binding protein, restored engulfment of target cells, thus enforcing the view that bridging molecules such as MFG-E8 may activate a different signaling pathway that is required for opsonization and efficient engulfment of target cells. Besides being a binding partner for the integrin receptor, MFG-E8 may also bind transglutaminase 2 (TG2) on macrophages (Toth *et al.*, 2009). Indeed, the presence of TG2 was shown to be important for the formation of an efficient phagocytic "portal" for internalization of apoptotic cells. Therefore, a single recognition signal may trigger the activation of multiple pathways through different receptors, which coordinately may lead to efficient engulfment of cells with surface exposed PS.

Novel pathways of phosphatidylserine externalization: role in the resolution of inflammation

While the externalization of PS has been linked to the apoptotic program and is frequently employed as a marker of apoptotic cell death (van Genderen *et al.*, 2006), several studies have demonstrated that caspase-independent and/or non-apoptotic pathways of PS exposure also exist. For instance, PS exposure is seen during B cell activation (Dillon *et al.*, 2000) and mast cell degranulation (Martin *et al.*, 2000) and transient PS exposure is also observed during stimulation of neutrophils with the chemotactic peptide formylated Met-Leu-Phe (fMLP) (Frasch *et al.*, 2004). Our previous work demonstrated that neutrophils possess two different pathways of PS exposure: a caspase-dependent pathway associated with induction of constitutive apoptosis, and a ROS-dependent, caspase-independent pathway following neutrophil activation (Fadeel *et al.*, 1998). More

recently, we have identified an additional, novel pathway of PS externalization in primary human neutrophils that is neither caspase-dependent nor ROS-dependent but is induced by neighboring phagocytes in a manner that is dependent on cell-cell contact (Jitkaew *et al.*, 2009). In another recent study, activated macrophages were shown to cause inhibition of aminophospholipid translocation in the plasma membrane of neighboring, "innocent" target cells via a nitrosative stress-dependent mechanism, resulting in PS exposure on the surface of the latter cells and subsequent engulfment by macrophages (Tyurina *et al.*, 2007). PS-dependent clearance of apoptotic cells by macrophages plays an active role in the resolution of inflammation, through production of anti-inflammatory cytokines such as transforming growth factor- β and down-regulation of pro-inflammatory mediators such as tumor necrosis factor- α (Voll *et al.*, 1997; Fadok *et al.*, 1998). The novel observation of macrophage-induced PS exposure in bystander neutrophils (Jitkaew *et al.*, 2009) and other target cells (Tyurina *et al.*, 2007) suggests that activated macrophages may engage in a PS-dependent feedback mechanism that potentially could serve to limit excessive inflammatory responses.

Concluding remarks

Several putative lipid transporter activities have been implicated in the generation, maintenance, and alteration of phospholipid asymmetry in the plasma membrane, and are critical for maintaining cell integrity and physiology and for regulating multiple important cellular events. However, it has been difficult to assign these activities to specific molecular entities. Recent studies in the model organism *C. elegans* have revealed a role for the P4 ATPase homolog, TAT-1, in the maintenance of phospholipid asymmetry in living cells, and have unearthed a novel mitochondria-to-plasma membrane signaling pathway that promotes the disruption of lipid asymmetry in apoptotic cells. The latter pathway involves the WAH-1 (worm homolog of AIF)-dependent activation of SCRM-1, a homolog of the mammalian phospholipid scramblases, in the plasma membrane (see Figure 4 for a schematic diagram). The mechanisms of lipid asymmetry and its disruption are thus beginning to unravel. Collectively, studies in this exciting field of research have served to elucidate how crucial biological functions are encoded into the vectorial distribution of plasma membrane phospholipids.

Acknowledgement

This work was supported by grants from the Swedish Research Council, National Institutes of Health (GM059083

and GM079097), and Human Frontier Science Program (RGP0016/2005-C). B.F. is the recipient of a Senior Investigator Award from the Swedish Cancer Foundation. D.X. is a Foreign Adjunct Professor of Karolinska Institutet. We thank our long-term collaborators, Prof. Valerian Kagan, University of Pittsburgh, and Prof. Peter Quinn, King's College London, for valuable discussions.

Declaration of interest: The authors report no conflicts of interest.

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Editor: Michael M. Cox