Biochimica et Biophysica Acta – Biomembranes 1862 (2020) 183382

Physiological roles of transverse lipid asymmetry of animal membranes

R. J. Clarke[a,b], K. R. Hossain[a], and K. Cao[a]

[a] *School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia*

[b] *The University of Sydney Nano Institute, Sydney, NSW 2006, Australia*

Address correspondence to Assoc. Prof. Ronald J. Clarke, School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia. Tel.: 61-2-93514406; Fax: 61-2-93513329; E-mail: ronald.clarke@sydney.edu.au

Abstract

The plasma membrane phospholipid distribution of animal cells is markedly asymmetric. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are concentrated in the inner leaflet, whereas phosphatidylcholine (PC) and sphingomyelin (SM) are concentrated in the outer leaflet. This non-equilibrium situation is maintained by lipid pumps (flippases or floppases), which utilise energy in the form of ATP to translocate lipids from one leaflet to the other. Scramblases, which are activated when physiologically required, transport lipids in both directions across the membrane and can abolish lipid asymmetry. Lipid asymmetry also causes imbalances in the areas occupied by lipid in the two membrane leaflets, contributing to membrane curvature. The asymmetry of PS across the plasma membrane plays a crucial signalling role in numerous physiological processes. Exposure of PS on the external surface of blood platelets stimulates blood coagulation. PS exposure by other cells during apoptosis provides an "eat me" signal to surrounding macrophages. Many peripheral and integral membrane proteins have polybasic PS-binding domains on their cytoplasmic surfaces which either provide a membrane anchor or affect activity. These domains can also determine trafficking within the cell and control regulation via an electrostatic switch mechanism, as well as potentially acting as "death sensors" when cytoplasmic PS is transferred to the extracellular leaflet during apoptosis. Apart from these physiological roles, external PS exposure by microorganisms, viruses and cancer cells allows them to mimic the immunosuppressive anti-inflammatory action of apoptotic cells and proliferate, thus providing a strong medical motivation for future research in the field of lipid asymmetry in membranes.

Keywords: phosphatidylserine; flippase; scramblase; blood coagulation; apoptosis; P-type ATPases

1. Introduction

The lipids composing the bilayer of all biological membranes are not uniformly distributed. It is now clear that both their lateral distribution, i.e., within the plane of the membrane, as well as their transverse distribution, i.e., between the two leaflets, vary considerably. This appears to be true for all forms of life and to varying degrees for all membranes, whatever their location, e.g., cellular or organelle membranes. The focus of this review is the transverse lipid asymmetry of animal membranes, in particular the plasma membrane. The aim of our article is primarily to provide an overview of the purposes that this asymmetry serves or could serve for healthy animal cells, rather than to explain how asymmetry originates and is maintained. Nevertheless, before discussing the physiological roles of transverse lipid asymmetry, no review of this topic would be complete without some mention of how the asymmetry was first discovered and the biological systems which are responsible for it. Therefore, we begin with a historical perspective followed by a brief discussion of the different classes of lipid transporters which either produce or modify the transverse lipid asymmetry.

2. Historical overview

In hindsight it would seem justified to refer to the 1970's as a "golden age" of lipid chemistry. An undoubted highlight of this period was the postulation by Singer and Nicolson of the fluid-mosaic model of cell membranes, which was published in *Science* on the 18th of February 1972 [1]. This model, described by the now classical figure (see Fig. 1) from their paper, displays a fluid lipid bilayer matrix in which integral membrane proteins are embedded. Their figure clearly shows a membrane with transverse asymmetry, but the asymmetry is limited to the protein component of the membrane.

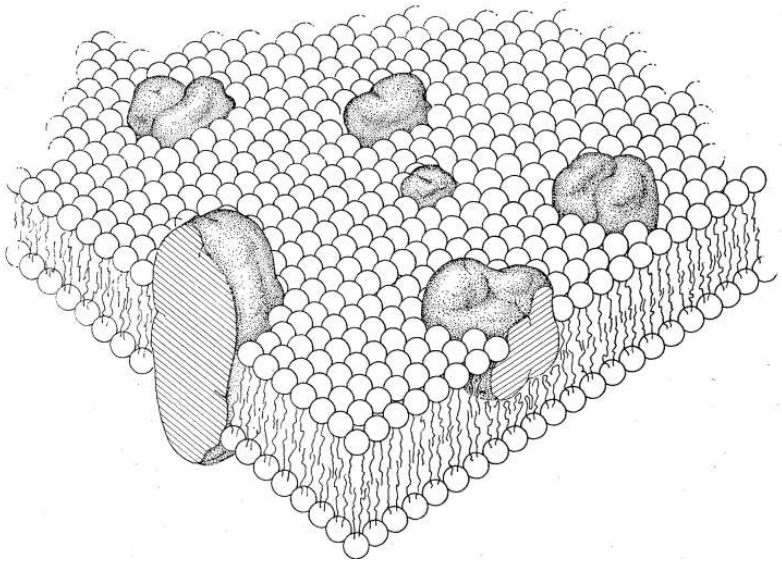


Figure 1: Fluid mosaic model of the cell membrane. From S. J. Singer and G. L. Nicolson, The fluid mosaic model of the structure of cell membranes, Science 175 (1972) 720-731. Reprinted with permission from AAAS.

Less than 2 weeks after the publication of Singer and Nicolson's paper, on the 1st of March another paper appeared, by Bretscher [2], proposing that the lipid component of the membrane is also distributed asymmetrically across the membrane. This hypothesis was based on experiments on red blood cells using chemical reagents capable of labelling the free amino groups of phosphatidylserine (PS) and phosphatidylethanolamine (PE), but not the trimethylamino groups of phosphatidylcholine (PC) or sphingomyelin (SM) (see Fig. 2). Bretscher found that very little labelling of PS or PE occurred for intact red blood cells, but the effectiveness of the labelling increased significantly when red blood cell ghosts were exposed to the same reagents. He concluded that this was due to the accessibility of both sides of the ghost cell membrane to the reagents, and, therefore, that in intact red blood cells PS and PE are primarily located in the cytoplasmic leaflet of the plasma membrane. Bretscher was aware of recently published electron paramagnetic resonance studies of Kornberg and McConnnell [3] using spin-labelled phospholipids showing that the movement of lipids between the two leaflets

of synthetic lipid vesicles, i.e., “flip-flop” is very slow, with a half-life of hours. He reasoned that in a natural membrane containing cholesterol and many different proteins, flip-flop is likely to be much slower, so that lipid asymmetry across the membrane would persist throughout the lifespan of a red blood cell.

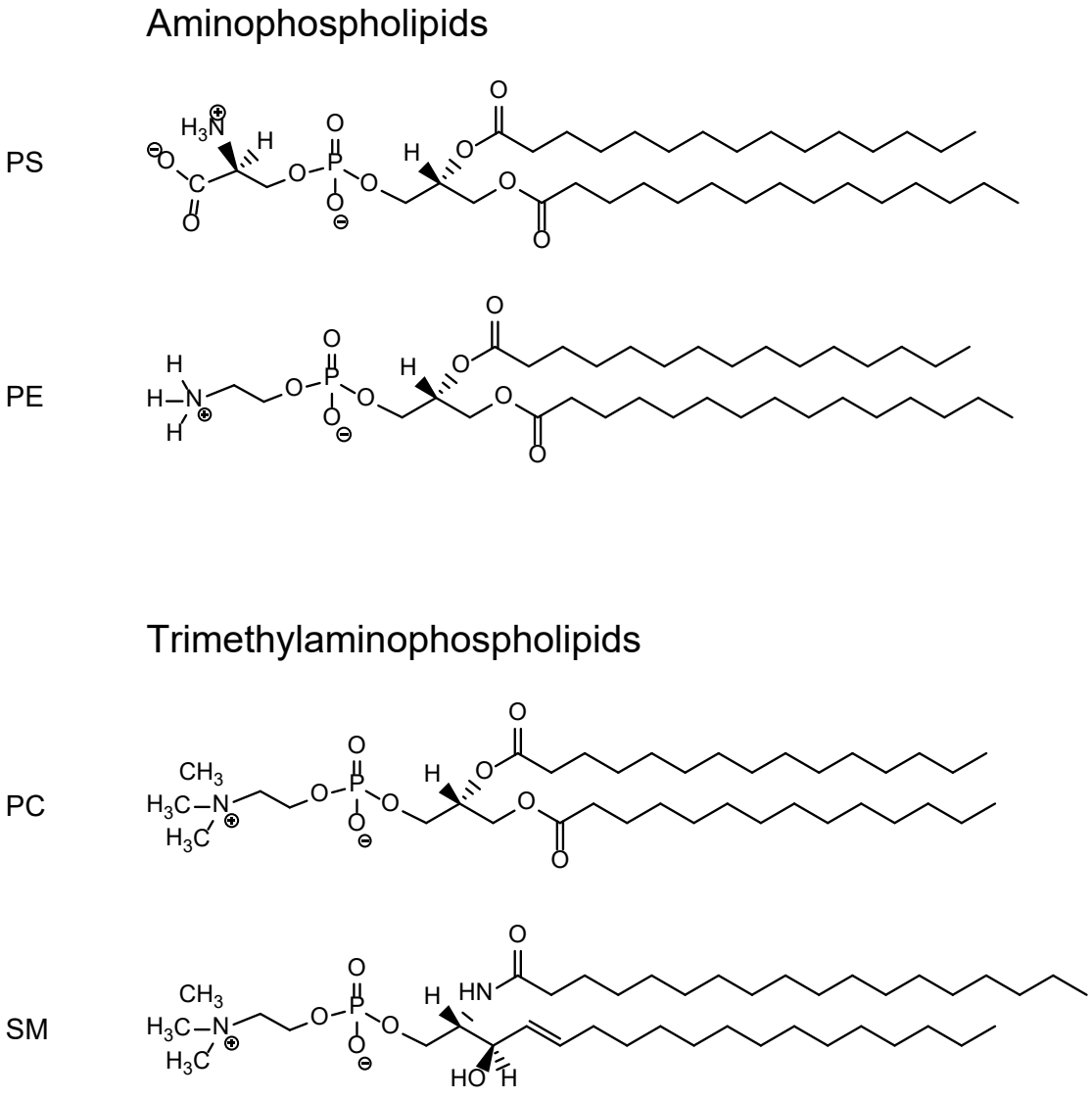
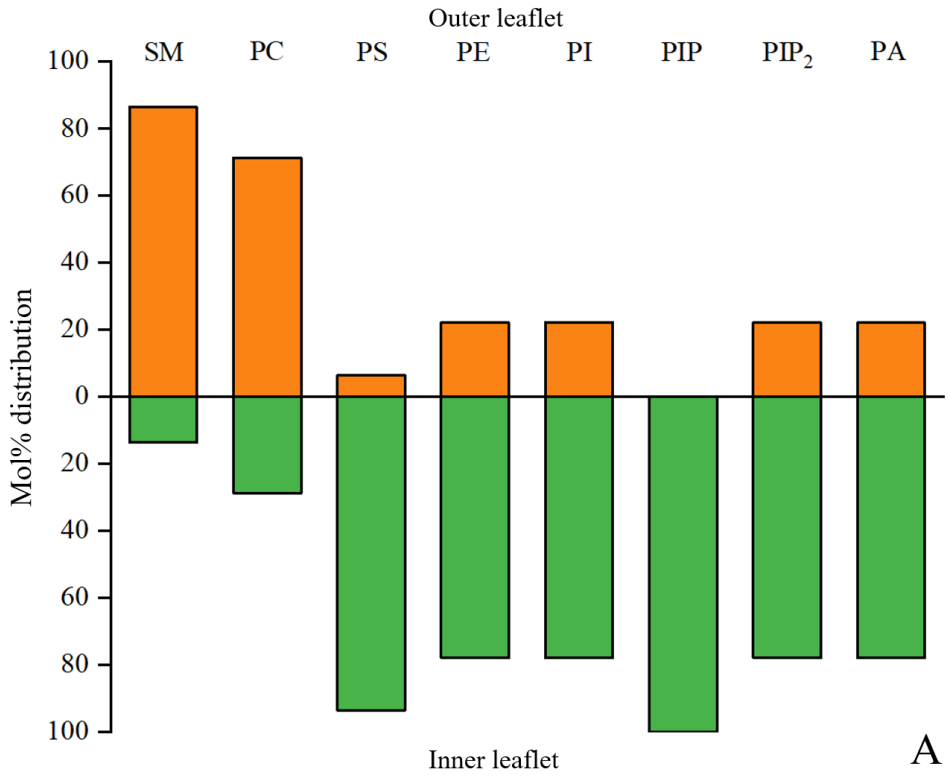


Figure 2: Classification of phospholipids based on their terminal basic residue: aminophospholipids (phosphatidylserine (PS) and phosphatidylethanolamine (PE)) and trimethylaminophospholipids (phosphatidylcholine (PC) and sphingomyelin (SM)). The aminophospholipids are located predominantly in the cytoplasmic leaflet of the plasma

membrane, whereas the trimethylaminophospholipids are located predominantly in the extracellular leaflet. Note that although PC and SM are both trimethylaminophospholipids, the purpose here of this classification is purely due to their location, not their chemical properties.

Subsequently, much work on red blood cells, by many other groups using a variety of techniques supported the hypothesis of Bretscher [4-10]. A summary of the distributions of phospholipids on each side of the red blood cell plasma membrane, adapted from the review of Zachowski [11], is shown in Fig. 3. Subsequent measurements on a variety of animal cells have shown that the trends in phospholipid distribution across the plasma membrane of red blood cells seem to have general validity for most other animal cells [11-14]. Shortly after his initial discovery of lipid asymmetry, Bretscher realized, however, that the asymmetry could not be maintained purely by a slow rate of lipid flip-flop. Based on the assumption that in all cells capable of protein synthesis (this excludes red blood cells) all phospholipid synthesis is likely to occur on the cytoplasmic side of a membrane, he concluded [15, 16] that the transfer of choline-containing phospholipids from the cytoplasmic leaflet of the membrane to the outer leaflet must be enzymatically catalysed. He coined the word *flippase* to describe the class of enzymes which catalyse this transfer. Because he was of the opinion, however, that movement of lipids between the two leaflets could not occur at any physiologically relevant rate unless enzymatically catalysed, Bretscher [15, 16] did not mention any energy requirement for flippase activity. It appears that he was envisaging a facilitated diffusion process.


Outer leaflet
SM
PC
PS
PE
PI
PIP
PIP2
PA
100
80
60
40
20
0
20
40
60
80
100
Mol% distribution
Inner leaflet
A

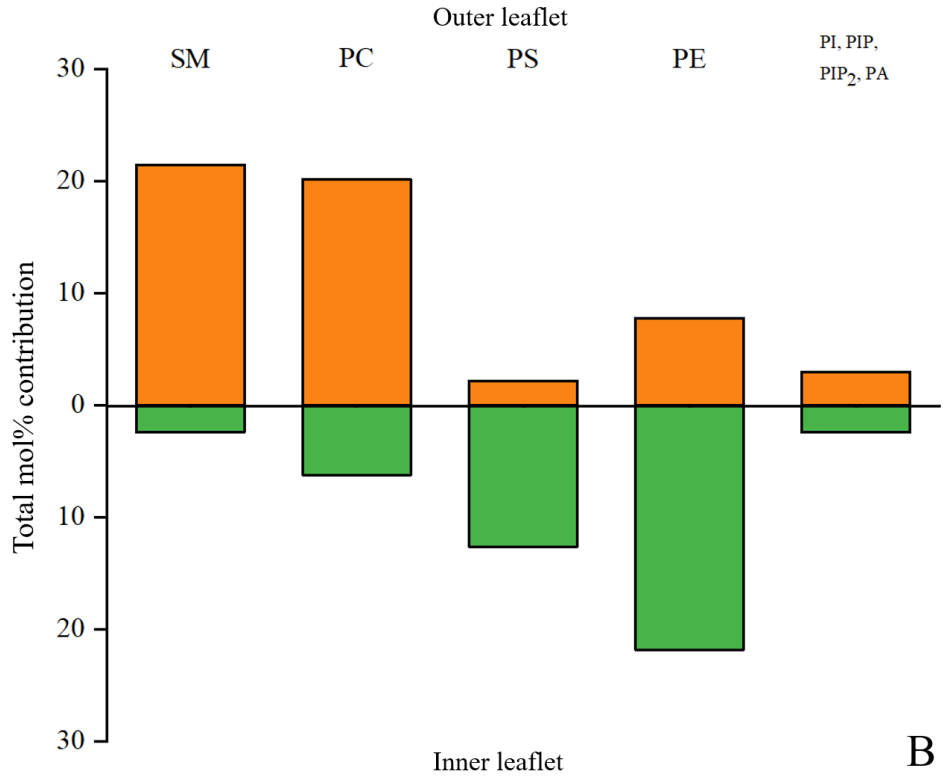

Outer leaflet
SM
PC
PS
PE
PI, PIP,
PIP2, PA
30
20
10
0
10
20
30
Total mol% contribution
Inner leaflet
B

Figure 3: Distribution of phospholipids on each side of the red blood cell plasma membrane (adapted from [11]). The top panel, A, shows the mol percentages of each individual lipid in each leaflet of the membrane, clearly showing that SM and PC are concentrated in the outer leaflet, while all other lipids shown are concentrated in the inner leaflet. The lower panel, B, shows the same data, but expressed as a mole percentage of the total amount of phospholipid in the membrane. This shows that PS is by far the major anionic phospholipid present in the inner leaflet. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PA, phosphatidic acid.

It soon became clear, however, that Bretscher's ideas on the maintenance of lipid asymmetry were in need of further revision. It is certainly true that transmembrane proteins involved in lipid synthesis and cell membrane modelling are oriented asymmetrically across the plasma membrane and that this could give rise to an intrinsic lipid asymmetry [13, 17]. But measurements on both cellular systems and lipid vesicles with reconstituted protein showed [17, 18] that the presence of proteins within the membrane dramatically accelerated lipid flip-flop, with half-times reducing from many hours down into the range of minutes. This has been confirmed by more recent measurements [19, 20]. For example, using small-angle neutron scattering measurements Nguyen et al [19] found a drop in flip-flop half-time for a DMPC/POPC asymmetric bilayer via small-angle from 140 hours to less than 2 hours after the addition of a variety of membrane-binding peptides, such as alamethicin and melittin. As suggested by Devaux [13], this acceleration is most likely due to the proteins acting as "defects" within the membrane, perturbing the packing of the lipid molecules and increasing their dynamics. Recent results [21, 22] comparing the rates of lipid flip-flop between lipid vesicles

and solid-supported bilayers appear to confirm this prediction, with solid-supported bilayers showing faster rates of flip-flop due to disorder induced by the surface of the solid support. Transient pore formation has also been observed in thin DLPC lipid bilayers via molecular dynamics simulations [23], although the simulation of actual flip-flop would be extremely difficult because of the very long simulation time necessary.

If lipid asymmetry across the plasma membrane of animal cells is to persist throughout the lifespan of a cell, it must, therefore, be actively maintained. The situation is analogous to that of the Na^+ concentration gradient across an animal plasma membrane. For most of the first half of the 20th century it was commonly believed (e.g., [24]) that the Na^+ gradient persisted because the membrane was completely impermeable to Na^+. After experiments showed this not to be the case, the existence of a sodium pump was postulated, and in 1957 the enzyme responsible for Na^+ pumping, the Na^+,K^+-ATPase, was isolated by Skou [25]. Thus, in the same way that the Na^+ gradient is maintained by an ion pump, in order to maintain lipid asymmetry lipids must be actively transported across the plasma membrane by lipid pumps, continually expending energy in the form of ATP. In contrast to Bretscher's original definition, the term flippase (e.g., [26, 27]) is now reserved for a protein which actively transports lipid molecules from the extracellular leaflet of the plasma membrane into the cytoplasmic leaflet, whereas the term floppase (e.g., [26, 27]) is used to describe a protein which actively transports lipid molecules from the cytoplasmic leaflet to the extracellular leaflet (see Fig. 4).

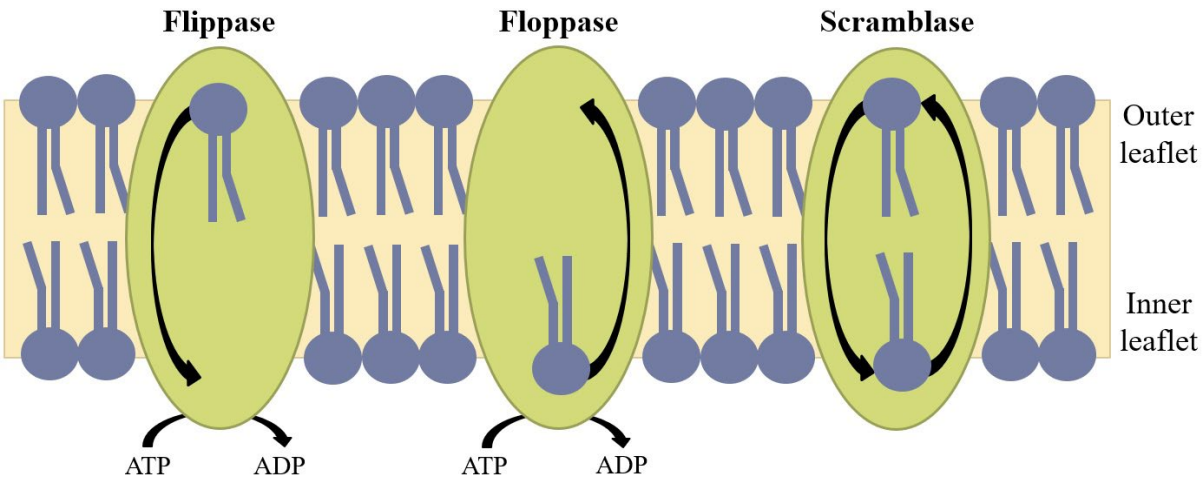


Figure 4: The three classes of phospholipid translocases. Most flippases transport aminophospholipids (PS and PE) from the outer to the inner leaflet of the plasma membrane. Floppases transport a variety of lipids (including PC) from the inner to the outer leaflet. Both flippases and floppases contribute to the maintenance of lipid asymmetry across the membrane, which is a nonequilibrium situation, and hence require energy from ATP hydrolysis to drive the process. Scramblases transport phospholipids in both directions across the membrane, from the inner leaflet to the outer leaflet as well as from the outer to the inner leaflet, hence approaching an equilibrium distribution across the membrane and abolishing lipid asymmetry. Because this is a spontaneous process no energy is required, but scramblases require activation, e.g. by an increase in the cytoplasmic Ca^{2+} concentration or by peptide cleavage. The net directions of lipid transport by scramblases are identical to those of passive lipid flip-flop through the lipid phase of the membrane, but facilitated or catalysed by the scramblase protein.

2. Flippases, floppases and scramblases

Bretscher's prediction [15, 16] of enzyme-mediated lipid facilitated diffusion across a membrane was first demonstrated in 1985 by Bishop and Bell [28, 29] in the case of phosphatidylcholine transport from the cytoplasmic side of the endoplasmic reticulum to the

luminal side. They found that the transport of dibutyroylphosphatidylcholine across the membrane of microsomal vesicles derived from the endoplasmic reticulum was saturable and inhibited by structural lipid analogues and by proteases, all indications of protein-mediated transport [28], but there was no requirement for ATP, suggesting a facilitated diffusion process. Bishop and Bell [28, 29] referred to the endoplasmic reticulum membrane as a *biogenic* membrane, i.e., a membrane involved in lipid biosynthesis, in contrast to the plasma membrane, which is not thought to be heavily involved in lipid biosynthesis. Because the movement of the lipid is not an active transport process, the protein mediating the transport presumably facilitates movement in either direction across the membrane. Proteins which do this are now referred to as *scramblases*.

The first active transport of lipids across the plasma membrane, i.e., requiring ATP, was reported by Seigneurat and Devaux [30]. They found via electron paramagnetic resonance that spin-labelled derivatives of PS and PE (with lower efficiency), but not PC, underwent rapid (i.e., on the timescale of minutes) transverse movement across red cell plasma membranes from the extracellular to the cytoplasmic leaflet. If the cells were depleted of ATP, transport was inhibited, and, if ATP together with its co-factor Mg^{2+} were reintroduced, transport of the aminophospholipids PS and PE into the cytoplasmic leaflet again occurred. Seigneurat and Devaux [30] furthermore suggested that the ATP driven transport of PS and PE could be important in maintaining the discoid shape of red blood cells, which is known to be ATP dependent [31, 32]. They also found that transport is strongly inhibited by orthovanadate, a phosphate analogue, a characteristic common to P-type ATPases, i.e., the same family of enzymes to which the Na^+,K^+-ATPase belongs. In subsequent work, the same group was able to partially purify and characterise [33] a Mg^{2+}-ATPase from the red blood cell membrane which they proposed to be the PS-selective aminophospholipid translocase (or flippase). Conclusive evidence that the aminophospholipid translocation was due to a P-type ATPase was

finally provided by Tang et al. [34], who cloned the gene responsible for aminophospholipid transport activity from bovine chromaffin granules. By comparison of the amino acid sequence of its product with sequences of known members of the P-type ATPase family (e.g., the ion-transporting Na^+,K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase), Tang et al. [34] concluded that the aminophospholipid translocase (flippase) belonged to a previously unrecognized subfamily of P-type ATPases, which may have diverged from primordial P-type ATPases before the evolution of the more well-known metal ion-transporting ATPases. Members of this subfamily are now classified as P4-ATPases, whereas the ion-transporting P-type ATPases belong to the P1-P3 ATPase subfamilies. Cryo-EM structures of a PS-transporting yeast P4-ATPase flippase, Drs2p-Cdc50p, in three different conformations [35] and the human P4-ATPase flippase, ATP8A1-CDC50a, in six different conformational states have recently been published [36], providing atomic level information on the enzyme's mechanism of lipid transport. In all of these structures, the catalytic unit of the flippase, i.e. Drs2p or ATP8A1, exists as a complex with another integral membrane protein of the CDC50 family (CDC = cell division control), which has been found to be necessary for the correct localization and function of the entire complex [37-39].

It appears that *floppases* all belong to the family of ABC (ATP binding cassette) transporters, a completely unrelated family to that of the P-type ATPase flippases. The ABC transporter family consists of many different proteins spread across prokaryotes and eukaryotes which are capable of transporting a wide variety of substrates. They were first recognized in bacteria, where some are involved in nutrient uptake, i.e., as importers. In eukaryotes, ABC transporters were previously thought to function solely as exporters, but some members of the family have now been reported to act as importers even in eukaryotes. A brief historical review of the development of the entire ABC transporter field can be found in [40]. A major focus of ABC transporter research has been their undesired role medically in conferring multi-drug

resistance on cancer cells [41, 42] and allowing infecting bacteria to develop antibiotic resistance [43]. However, the transport of lipids from the cytoplasmic leaflet of the plasma membrane of animal cells to the extracellular leaflet is a crucial physiological role of ABC transporter floppases. Just as the high contents of the aminophospholipids PS and PE in the cytoplasmic leaflet are maintained by P-type ATPase flippases, the high contents of PC and sphingomyelin in the extracellular leaflet are maintained in part by their outward pumping by ABC transporters. The major members of the family implicated in this process are ABCA1, ABCA3, ABCA4, ABCA7, ABCB1 (also known as P-glycoprotein 1 and multidrug resistance protein 1), ABCB4 and ABCC1 [44-46]. The transporter ABCA1 has also been shown to be important in cholesterol homeostasis within the plasma membrane and the secretion of cholesterol to extracellular high-density lipoproteins (HDL), although it appears that the process is mediated by phospholipid transport (possibly PS in this case) across the membrane, causing a change in membrane curvature and the extracellular budding off of vesicles rich in cholesterol, rather than a direct pumping of cholesterol across the membrane by the transporter [47].

While on the topic of maintaining the high level of PC in the extracellular leaflet of the plasma membrane, it is worthwhile mentioning the special case of red blood cells. As mentioned earlier, most cells synthesise the lipids which they incorporate into their membranes on the cytoplasmic side of the biogenic endoplasmic reticulum membrane. However, mature red blood cells contain neither a nucleus nor an endoplasmic reticulum. Although transverse lipid asymmetry was first discovered in red blood cells [2], they are certainly not typical. Thus, instead of incorporating PC into their plasma membrane from the cytoplasm and transporting it into the extracellular leaflet via a floppase, mature red blood cells take up PC extracellularly via high-density lipoprotein in the surrounding blood plasma [48, 49]. Once in the extracellular leaflet, PC can passively flip to the inner leaflet, which would reduce the normal asymmetric

distribution of PC across the membrane and lead to a build-up of the total PC within the red blood cell membrane. This can be pathogenic, and in certain individuals leads to high red cell membrane phosphatidylcholine haemolytic anaemia. Evidence exists that under normal physiological conditions, in addition to the action of membrane-bound floppases to transport PC out again, the level of PC in the inner leaflet of the red blood cell membrane could partly be regulated by the action of phospholipase D from the cell cytoplasm, which cleaves choline from the PC headgroup and converts it into phosphatidic acid [50]. Thus, apart from lipid asymmetry being maintained by the lipid translocating activity of flippases and floppases, this example suggests that lipid-converting reactions of phospholipases potentially play an important contributing role.

In the previous section it was pointed out that flippases, floppases and scramblases allow much faster movement of lipid between the two leaflets of the plasma membrane than passive flip-flop. However, apart from this, there are important kinetic differences between the different classes of lipid-translocating proteins themselves. Both flippases and floppases transport lipids against a lipid concentration gradient within the membrane. This requires energy, which they derive from the hydrolysis of ATP. Furthermore, because they transport lipids in a direction uphill in energy, for them to function efficiently their molecular mechanisms must somehow inhibit the spontaneous movement of lipids downhill in energy back to where they originated. This must involve significant protein conformational changes, allowing alternating access of lipids to each leaflet of the plasma membrane, but not to both leaflets simultaneously. The situation is analogous to a river boat moving to a higher water level in the river by passing through a lock with gates which open and close sequentially on the low and then the high water side. Because of these significant protein conformational changes, flippases and floppases have relatively low turnover numbers of not more than ~100 s^{-1} [51], i.e., similar to the turnovers of ion pumps. In contrast, scramblases can have turnover

numbers of >1,000 s^{-1}. For example, Watanabe et al. [52] carried out a single-molecule analysis of the kinetics of lipid transport via the scramblase transmembrane protein 16F (TMEM16F) incorporated into an asymmetric membrane mounted within a microarray platform which yielded a transport rate of 4.5×10^4 lipids per second at 25°C. The reason for the high lipid transport rate of scramblases is that they allow lipids to move down a concentration and down an energy gradient, which requires much less significant protein conformational motion. Whereas flippases and floppases can be considered as analogous to ion pumps, scramblases can be considered as analogous to ion channels, where the opening of a single gate allows spontaneous movement of the ions through the channel. This difference in kinetics has significant consequences for the action of flippases, floppases and scramblases. Because of their slow kinetics many flippase and floppase molecules need to be continually pumping their lipid substrates to maintain lipid asymmetry. But the rapid abolition of lipid asymmetry as a signal to initiate physiological processes such as blood coagulation or apoptosis (both described in detail in section 4), only requires the activation of a much smaller number of scramblase molecules. Therefore, one would expect membrane expression levels of flippases and floppases to be much greater than those of scramblases,

3. Effect of lipid asymmetry on membrane curvature

Not all living cells are spherical. They exist in a variety of forms. One specific example is the discoid shape of red blood cells, which facilitates reversible elastic deformation of the cells, crucial to effective oxygen transport, as they pass through small capillaries. Even in the case of a single cell, local changes in plasma membrane structure occur, i.e. inward or outward budding (endo- and exo-cytosis), which allow the transfer of material between the cytoplasm and the extracellular medium. These different shapes laterally across the surface of the plasma membrane can, at least in part, also be explained by the transverse lipid asymmetry of the

membrane. Another major contributor to cell shape is, of course, the cytoskeleton, but here, because the aim of our paper is to review the physiological roles of lipid asymmetry, we focus on the contribution of lipid to the determination of shape.

Consider first the case of the discoid red blood cell. If red blood cells are stored for long periods of time (i.e., weeks), they undergo shape changes, transitioning from discocytes to what are termed echinocytes or spherocytes, i.e., sea-urchin-shaped or spherical cells [53]. This indicates that the discocyte structure is not an equilibrium state. It is maintained by the input of energy in the form of ATP [32]. The way that the ATP is utilized is thought to be in powering a PS flippase within the plasma membrane to maintain the asymmetrical lipid distribution across the membrane [53]. The logic in this conclusion is not immediately obvious, but will become clear from a simple theoretical consideration of the lipid packing in both leaflets of a lipid bilayer and the total areas covered in each leaflet.

The movement of lipids between the two leaflets of a membrane causes a mismatch in the total surface area of the two leaflets. If the total areas of each leaflet are equal, i.e. $A^{in} = A^{out}$, then the bilayer will be completely flat. However, if the number of lipids in the outer leaflet increases and the number in the inner leaflet decreases due to the movement of lipid molecules from the inner to the outer leaflet, to avoid lateral tension the lipids in the outer leaflet must space themselves out and those in the inner leaflet must condense to achieve the same lipid packing density in both leaflets (see left hand side of Fig. 5). This leads to a mismatch in the areas of the two leaflets, i.e. $A^{out} > A^{in}$. The only way for the membrane to maintain a stable bilayer structure with such an area mismatch is for the membrane to bend outwards. Similarly, if the number of lipids in the inner leaflet increases and the number in the outer leaflet decreases due to movement of lipid molecules from the outer to the inner leaflet, so that $A^{in} > A^{out}$, the membrane must bend inwards (see right hand side of Fig. 5). This method of generating curvature is termed the bilayer-couple mechanism [54, 55]. This purely theoretical

consideration was elegantly supported by some relatively simple experiments conducted by Müller et al. [56] on red cell ghosts. They found that the external addition of PS to ATP-containing ghosts caused an inward bending, similar to that which occurs in the process of endocytosis. In contrast, if they added PC the reverse occurred, i.e. an outward bending or budding. These results can be explained by the presence of a PS-specific flippase in the plasma membrane. Thus, flipping of PS to the inner leaflet caused the inner leaflet to expand and bend inwards. However, when PC was added, it remained in the outer leaflet, thus causing it to expand and bend outwards.

Now returning to the observation of shape changes in red cells stored for long periods; it is clear that if a red cell is starved of oxygen and hence of ATP, its flippase activity would be inhibited. In time, due to passive flip-flop of PS into the external leaflet, this would lead to an increase in the number of lipids molecules in the outer leaflet relative to the inner, inducing a shape change away from the physiological discoid structure.

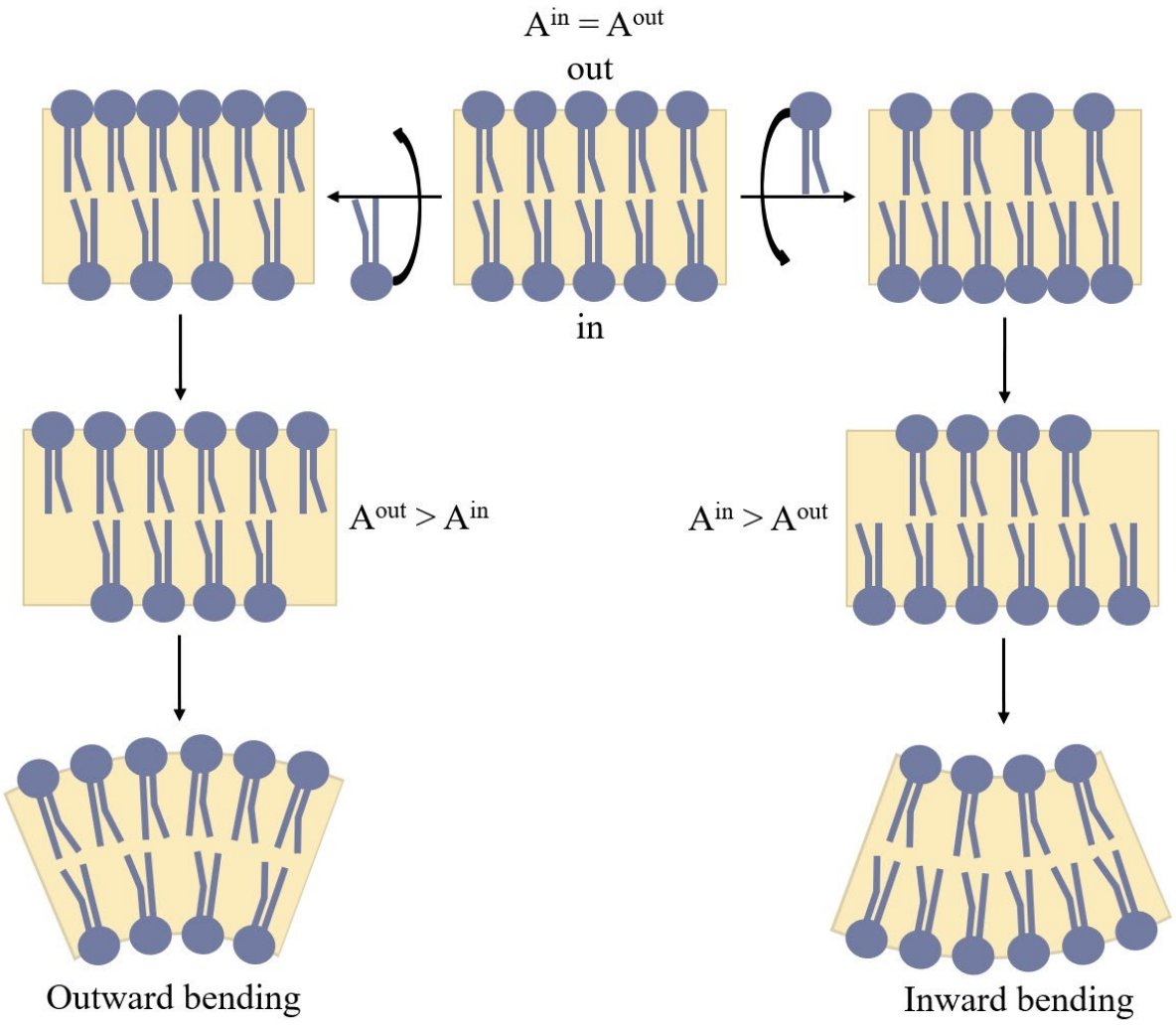


Figure 5: Formation of membrane curvature by lipid translocation via the bilayer-couple mechanism. If the areas occupied by lipid in each membrane leaflet were equal, $A^{in} = A^{out}$, the membrane would be flat (top row, centre). The left hand side (LHS) and right hand side (RHS) of the figure show the effects of translocation of lipid from the inner to the outer leaflet and from the outer to the inner leaflet, respectively.

LHS Top row: Movement of lipid from the inner to the outer leaflet causes a transient increase in lipid packing density in the outer leaflet and a transient decrease in packing density in the inner leaflet. Middle row: To reduce lateral tension the lipid molecules relax back to the same packing density in each leaflet, causing a mismatch in the areas of the two leaflets, i.e. $A^{out} >$

A^{in}. Bottom row: To maintain a stable bilayer with equal lipid packing densities in each leaflet, the membrane bends out.

RHS Top row: Movement of lipid from the outer to the inner leaflet causes a transient decrease in lipid packing density in the outer leaflet and a transient increase in packing density in the inner leaflet. Middle row: To reduce lateral tension the lipid molecules relax back to the same packing density in each leaflet, causing a mismatch in the areas of the two leaflets, i.e. $A^{in} > A^{out}$. Bottom row: To maintain a stable bilayer with equal lipid packing densities in each leaflet, the membrane bends in.

As mentioned earlier, of course many cell types also possess a cytoskeleton which, in addition to lipid asymmetry, plays an important role in determining cell shape, in particular in the case of red blood cells when they undergo the transition to echinocytes [53, 57]. Nevertheless, the above discussion indicates that the activation of flippases, floppases or scramblases within a membrane could potentially play an important role in triggering both the processes of endocytosis and cell budding [58], i.e., with net inward transport of lipid by a flippase favouring endocytosis and a net outward transport of lipid by a floppase favouring budding. Activation of a scramblase could potentially favour endocytosis or budding depending on the direction of the asymmetry prior to its activation. Investigation of this relatively physicochemical mechanism by which lipid asymmetry could affect membrane curvature would seem to lend itself to study by modern molecular dynamics simulations. An example is a recent study [59] showing the spontaneous formation of a vesicular bud in a simple model system composed purely of PC but with different numbers of lipids in each membrane leaflet and the subsequent disappearance of the bud after insertion of a scramblase protein into the membrane.

4. Phosphatidylserine signalling on the extracellular membrane surface

PS is normally concentrated in the cytoplasmic leaflet of the cell membrane (see Fig. 3). When this is not the case, this has important physiological consequences for the cell. Here we describe two processes, blood coagulation and apoptosis, where PS exposure provides the signal to "clot" or "eat me", respectively.

4.1 Blood coagulation

When vascular injury occurs, blood coagulation is vital in reducing blood loss and beginning repair. Initially, in primary haemostasis (i.e., the process of preventing and stopping bleeding), interaction of discoid-shaped resting blood platelets with exposed subendothelial factors such as collagen on the interior surface of a blood vessel initiate an activation process. This involves a shape change of the platelets, whereby they develop pseudopodia, i.e., projections of the plasma membrane, and release coagulant mediators (including fibrinogen) from cytoplasmic α-granules into the bloodstream [60-63]. The platelets then aggregate to form a temporary haemostatic plug to stop bleeding [64].

In secondary haemostasis, loss of lipid asymmetry and PS exposure on the extracellular membrane surface of activated platelets by the stimulation of a scramblase in the platelet plasma membrane (described in more detail below) provides a catalytic surface in the coagulation cascade [65]. The coagulation cascade involves coagulation factors that normally circulate in the bloodstream but with an activity too low for efficient clot formation [66]. The PS-containing extracellular membrane surface of activated platelets attracts clotting factors, increasing their local concentration so that they bind and assemble procoagulant enzyme complexes, catalysing the conversion of prothrombin to thrombin [67-70]. Thrombin is the key procoagulant enzyme that converts soluble fibrinogen into an insoluble fibrin clot that strengthens the haemostatic plug [64].

The key factor which allows secondary haemostasis to occur is the loss of lipid asymmetry via scramblase activation in the platelet plasma membrane. The specific scramblase involved is now known to be transmembrane protein 16F (TMEM16F), whose activity is dependent on the cytoplasmic Ca^{2+} concentration [71-73]. Both X-ray-crystallographic [74] and recent cryo-electron-microscopy structures of fungal members of the TMEM16F family [75], combined with biochemical assays, site-directed mutagenesis and molecular dynamics simulations [76, 77], are now providing molecular detail on the lipid transporting mechanism of this class of scramblase.

A further consequence of the activation of platelets by the initial interaction with collagen is that a signalling pathway is triggered, mediated by glycoprotein VI, phospholipase C and inositol 1,4,5 trisphosphate (IP_3) [61, 78], whereby Ca^{2+} is released from cytosolic stores (i.e. endoplasmic reticulum (ER)) through the activation of IP_3-receptor Ca^{2+}-channels in the ER membrane. At the same time Ca^{2+} also flows into the cell from the extracellular medium by the opening of Ca^{2+} channels in the plasma membrane, with ATP-gated P2X1 channels [79], Orai1 store-operated channels [79] and, under shear stress conditions, mechanosensitive Piezo1 channels [80] all being implicated. Both extracellular influx and release from cytosolic stores cause a sustained increase in the cytoplasmic Ca^{2+} concentration, thus activating TMEM16F to scramble the platelet's lipid distribution and expose PS on the cell's outer membrane leaflet [78, 81]. This provides an optimal anionic procoagulant membrane surface with 10-15 mol% PS [61, 65, 67, 82], increasing the rate of thrombin formation almost a millionfold [66, 83] and allowing clotting to proceed. The process is summarized schematically in Figure 6. Since only activated platelets provide PS-containing surfaces, fibrin formation is localised to the site of damage, reducing coagulation occurring elsewhere. Furthermore, in resting platelets the cytoplasmic Ca^{2+} concentration is kept low by the combined action of plasma membrane and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases [78], which actively pump Ca^{2+} from the

cytoplasm into the extracellular medium and into the endoplasmic reticulum, respectively, thus preventing PS scrambling and platelet activation in the absence of any vascular injury.

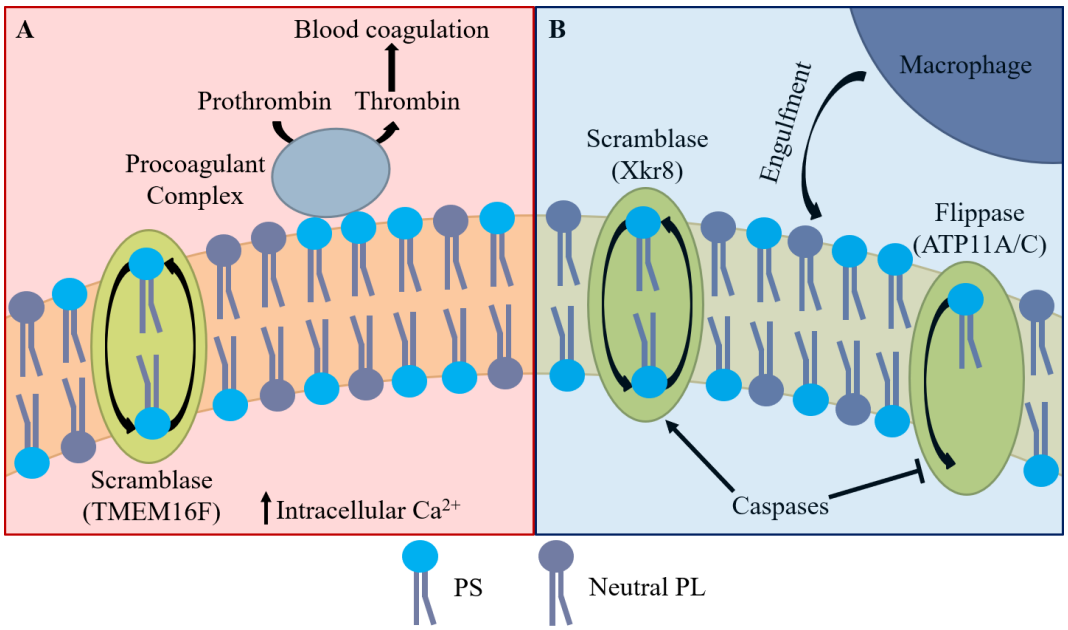


Figure 6: Summary of the processes involved in blood coagulation (A) and apoptosis (B) (adapted from [72]). In both processes phosphatidylserine (PS) is shown with a blue lipid headgroup, whereas uncharged neutral phospholipids (PL) are shown with a grey headgroup. Blood coagulation (A): An increase in the intracellular Ca^{2+} concentration activates a scramblase (TMEM16F), which causes the exposure of PS on the cell surface. This provides a negatively charged surface, which attracts clotting factors, forming a procoagulant complex and promoting the conversion of prothrombin to thrombin. Thrombin catalyses the conversion of fibrinogen to fibrin, which creates a stable clot.

Apoptosis (B): The activation of caspases within the cell cytoplasm inhibits the translocation of PS from the outer to the inner leaflet of the plasma membrane by the flippases ATP11A and ATP11C, and activates a scramblase (Xkr8). Both of these caspase-mediated effects cause the exposure of PS on the external surface of the cell, providing an “eat-me” signal for the cell to

be recognised and engulfed by a macrophage, where the cell is disposed of within the macrophage's lysosomes.

A further process which occurs on platelet activation is bleb formation, which results in the release of lipid vesicles or microparticles from the platelet membrane surface [61, 84]. Microparticles provide further catalytic PS-containing surfaces which have additional clotting factor binding sites and facilitate procoagulant complex assembly and thrombin formation [85]. An important factor in the recognition of the role of lipid asymmetry and scramblase involvement in blood coagulation has been research into the molecular origin of Scott syndrome, an extremely rare bleeding disorder, first described in 1979 [86], which is named after the first diagnosed patient. This disease is characterised by severe bleeding, caused by deficient platelet procoagulant activity. Research on platelets of Scott syndrome patients showed that their activated platelets show no increase in PS in the outer leaflet, indicating a lack of scramblase activity [66, 69]. Furthermore, analysis of the platelet scramblase of a Scott syndrome patient allowed a genetic mutation in the TMEM16F protein to be discovered, thus allowing the scramblase to be definitively identified [71].

Before proceeding, it is important to point out that blood coagulation is not the only physiological process in which proteins of the TMEM16F family are involved [87, 88]. For example, it was found [89] that deletion of the TMEM16F gene in mice resulted in skeletal deformities and that Ca^{2+}-dependent PS scrambling was impaired in their osteoblasts. This data supports a function of PS exposure by TMEM16F in bone mineralisation, and could be explained by the negative surface charges of PS attracting Ca^{2+} ions from the extracellular medium, thus promoting the deposition of hydroxyapatite crystals, $Ca_{10}(PO_4)_6(OH)_2$. Further physiological processes in PS exposure by TMEM16F proteins is likely to play an important signalling role are discussed in recent reviews [87, 88].

4.2 Apoptosis

Apoptosis, or programmed cell death, is incorporated into a cell's life cycle. To eliminate old cells before they die spontaneously, i.e. via necrosis, and release toxic substances such as cytoplasmic enzymes to the surroundings, it is beneficial for the health of the entire organism if they are disposed of in a controlled fashion. Apoptosis can follow two different pathways, termed extrinsic and intrinsic. The extrinsic pathway or death factor pathway is initiated by the binding of death factors of the tumour necrosis (TNF) family to receptors on the plasma membrane. The intrinsic pathway or mitochondrial pathway is cell-internal and is associated with an enhanced permeability of mitochondrial membranes, causing the release of cytochrome c into the cytoplasm. Both pathways, however, finally converge, resulting in the activation of caspases, i.e., cysteine-dependent aspartate-specific peptidases, which have a cysteine within the active site, recognise a specific 4-amino acid residue sequence in proteins and cleave after an aspartate residue [90-92]. Apoptosis ensures a balance between cell proliferation and cell death, thus keeping the number of cells in an organism fairly constant and maintaining tissue homeostasis. Cells undergoing apoptosis display morphological changes such as cytoplasmic and organelle condensation and DNA fragmentation, ultimately forming apoptotic bodies – remnants of apoptotic cells [90]. In the early stages of apoptosis, apoptotic cells expose PS on their extracellular surface [72, 73, 81, 93-97]. This functions as an "eat-me" signal which is recognised by macrophages, finally leading to their ingestion and degradation of the apoptotic bodies by phagolysosomes. As in the case of blood coagulation, the exposure of PS on the extracellular surface of apoptotic cells is caused by the activation of a scramblase in the cell's plasma membrane. However, instead of scrambling via TMEM16F, as occurs in platelets during clotting, the scramblase Xkr8 has been identified as the protein responsible for producing PS exposure in apoptosis [72, 73, 97, 98].

An important difference between TMEM16F and Xkr8 is the mechanism of activation. As described in the previous section, TMEM16F is activated by an increase in cytoplasmic Ca^{2+}. Xkr8, on the other hand, is activated by the action of caspases, the end result of the apoptosis initiation pathway. Xkr8 possesses a conserved Asp-Glu-Val-Asp-Gly caspase 3/7 cleavage site motif at its C-terminus, which on cleavage releases an inhibitory sequence, thus activating scramblase activity [72, 73, 97]. The kinetics of scrambling by TMEM16F and Xkr8 are also very different. PS exposure by TMEM16F is very rapid, i.e., within minutes, and is reversible on reduction of the platelet's cytoplasmic Ca^{2+} concentration back to its resting value, whereas PS exposure mediated by Xkr8 is much slower, on the hour timescale, and is irreversible [73, 99]. It is important to bear in mind, however, that the measured kinetics of lipid scrambling is a combination of the actual lipid transfer kinetics together with the kinetics of scramblase activation. Thus, the slow scrambling via Xkr8 is likely due to the time required to activate caspase 3, the most downstream caspase in the signalling pathway. This conclusion is supported by recent results [100] showing that Xkr8 can also be activated via phosphorylation, producing lipid scrambling in minutes rather than hours in the case of caspase activation. Thus, the actual lipid transfer rates of TMEM16F and Xkr8 may in fact not be so different.

Perhaps because the kinetics of the Xkr8 scrambling pathway are slow, to further enhance exposure of PS as an "eat me" signal on the cell surface during apoptosis an inactivation of flippases ATP11C and ATP11A occurs [101]. These flippases would otherwise transport PS back to the cytoplasmic leaflet. Similar to Xkr8, ATP11C and ATP11A also possess caspase cleavage sites, but they undergo inactivation rather than activation on cleavage [72, 73, 102, 103]. The inhibition of ATP11C and ATP11A could explain why PS exposure in apoptosis is irreversible. A schematic summary of the entire process is shown in Figure 6B.

There is also some evidence that the strength of the "eat-me" signal produced by the external exposure of PS during apoptosis depends on the exact chemical structure of the PS molecules

exposed, i.e., not just on the headgroup of the PS molecule but also on the nature of the hydrocarbon tails to which it its attached. In particular, the exposure of PS with peroxidized acyl chains has been found to promote phagocytosis [104]. Differing structural forms of PS leading to apoptosis could perhaps explain why phagocytes possess a number of different receptors on their surface for the recognition of apoptotic cells [75, 92, 104, 105]. A likely mechanism for the peroxidation of PS is via the action of cytochrome c, which is released from mitochondria during apoptosis and could diffuse to the cytoplasmic leaflet of the plasma membrane [106-108]. Peroxidized PS could passively diffuse to the outer leaflet of the plasma membrane via flip-flop or its diffusion could be facilitated by a scramblase [106]. Once in the external leaflet its flipping back to the cytoplasmic leaflet by a membrane bound flippase, such as ATP11C, could be inhibited simply because peroxidized PS is a poorer substrate for the enzyme than non-peroxidized PS, or peroxidized PS could directly inhibit flippase activity by itself or via oxidation products that it generates [106].

Apoptosis is a vital naturally-occurring cellular physiological process. However, finally in this section we would like to mention some disease states in which it plays an important role in disease progression. To understand a variety of apoptosis-related diseases it is first important to realise that the events involved in apoptosis are genetically programmed in such a way that they avoid any inflammatory response or tissue damage to the surroundings. This has been found to depend on an immunosuppressive action of apoptotic cells [73, 109], i.e., apoptotic cells promote the secretion of the anti-inflammatory and immunoregulatory cytokine interleukin 10 and decrease the secretion of pro-inflammatory cytokines. Unfortunately, this allows PS-dependent recognition to be hijacked by viruses, microorganisms and parasites to evade the body's natural defense systems. The general term for this phenomenon is "apoptotic mimicry" [73, 110]. A prime example is infection by the HIV-1 virus [73, 111]. When exiting a cell the HIV-1 virion obtains PS and exposes it on its outer membrane, thus mimicking an

apoptotic cell and allowing it to evade an inflammatory immune response. The virus is then engulfed by a phagocyte as if it were an apoptotic cell. Phagocytic infection by HIV-1 causes the infected phagocytes to expose an elevated level of PS on their surfaces [73, 111], resulting in apoptosis of the infected phagocytes, leading eventually to the failure of the host's immune system.

Without apoptosis, the balance between cell proliferation and death would be disturbed. Thus, cancer is characterised either by excessive cell proliferation or insufficient cell death, or presumably both, leading to tumour growth. Many cancer cell types have been found to expose high levels of PS on their surface, similar to apoptotic cells, thus reducing an inflammatory immune response, and allowing them to proliferate. In the case of noncancerous cells, the exposure of PS would cause engulfment and elimination by macrophages due to the "eat-me" signal provided by PS. However, in addition to exposing PS, many cancerous cells over-express a transmembrane glycoprotein CD47 within their plasma membrane, which for phagocytes functions as a "don't eat me" signal [112-115]. Thus, the cancer cells are protected from attack by both phagocytes and the rest of the immune system, and proliferate unhindered. The development of anti-cancer drugs which specifically target the external PS of cancer cells is currently an active area of research providing promising results in pre-clinical and clinical trials [73, 115-118]. An immunotherapeutic approach which is also showing promise involves blocking CD47 with anti-CD47 antibodies, thus preventing CD47 from interacting with its inhibitory receptor SIRPα (signal regulatory protein) on the macrophage's surface [119]. This approach was further enhanced by binding opsonizing antibodies to the tumour cell which engage activating Fc receptors on the macrophage and stimulate phagocytosis [119]. This immunotherapeutic strategy does have the safety concern of potentially causing the clearance of "self" cells, but results suggest some success on solid and haematological (liquid) cancer forms.

Evidence also exists, however, that the over-expression of CD47 is not entirely sufficient to prevent the engulfment of cancer cells by macrophages [120]. Whereas PS exposure in apoptotic cells is irreversible, it may be the case that in cancer cells PS can still move between the extracellular and cytoplasmic leaflets of the membrane via the action of the flippases ATP11A and ATP11C, thus allowing them to elude macrophagocytic engulfment. Results suggesting such a role of PS transfer kinetics in promoting cancer cells' "don't eat me" capability were found by Segawa et al [120] using precursor B cells, which proliferate in precursor B-cell lymphoblastic leukaemia. Thus, mutated precursor B cells lacking ATP11C were found to still be engulfed by macrophages.

5. Phosphatidylserine signalling on the cytoplasmic membrane surface

As explained in the previous section, exposure of PS on the extracellular surface of the plasma membrane due to scramblase activation and flippase inhibition and its role in blood coagulation and apoptosis have been already extensively investigated and, although much is still to be discovered, great advances have already been made. In this section we concentrate on the role of PS in its normal location on the cytoplasmic face of the plasma membrane. Much less is known about its function on this side of the membrane. However, it seems very unlikely that the ATP-driven flippase activity of a cell to maintain PS on the cytoplasmic surface purely occurs to prevent external PS exposure and stop the cell undergoing apoptosis. There must also be important roles for PS on the cytoplasmic surface.

5.1 Trafficking of peripheral membrane proteins

It now seems clear that one important function of PS in the cytoplasmic leaflet is to control the trafficking of peripheral membrane proteins between the plasma membrane and other locations within the cell cytoplasm. Peripheral membrane proteins are those which bind

temporarily to a membrane surface via relatively weak interactions, in contrast to transmembrane proteins, which are permanently embedded in the membrane. Breaking of the interactions allows peripheral proteins to move around a cell and perform functions in different locations. Two main forces are responsible for their association: hydrophobic (or nonpolar) interactions and electrostatic interactions, often in combination with one another [121-125]. Many peripheral membrane proteins possess a post-translational modification to either their N- or C-termini which consists of a myristoyl- or palmitoyl chain. This inserts itself into the lipid bilayer and acts as a hydrophobic anchor to the membrane. A prime example is the MARCKS protein (myristoylated alanine-rich C-kinase substrate) [126, 127]. In addition to a hydrophobic anchor, many peripheral membrane proteins also have clusters of basic amino acid residues, i.e., lysine (K) or arginine (R), at their N-or C-termini. Because these residues are positively charged at physiological cytoplasmic pH, the clusters can interact with negatively charged phospholipid headgroups, in particular PS, on the membrane surface, providing an electrostatic component to membrane anchoring (see Fig. 7). This also occurs in the case of the MARCKS protein [126, 127]. Other examples include the Src kinase [128], G proteins [129], the SH2B1β gene product [130] and the Gag protein of retroviruses such as HIV [131].

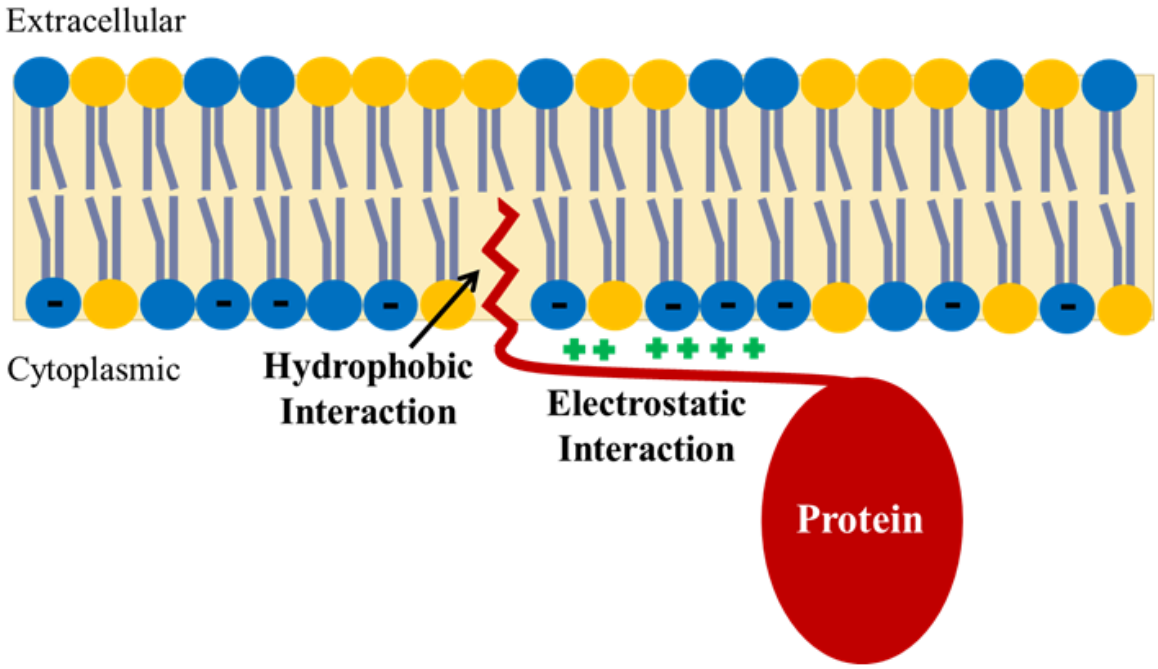


Figure 7: Membrane anchoring of peripheral membrane proteins. Many peripheral membrane proteins have two thermodynamic contributions to their membrane anchoring: 1) a hydrophobic (entropy-driven) interaction arising from insertion of a hydrocarbon chain (myristoyl- or palmitoyl) into the lipid bilayer, whereby the hydrocarbon chain is a post-translational modification to the protein; and 2) an electrostatic (enthalpy-driven) interaction between positively charged basic amino acid residues of the protein (lysine or arginine) and negatively charged anionic lipid headgroups (predominantly phosphatidylserine) on the cytoplasmic face of the membrane. Membrane anchoring thus relies in part on the asymmetric transverse distribution of PS across the membrane, which is maintained by a flippase.

There is now much evidence that the release of peripheral membrane proteins of this type from the plasma membrane occurs via an electrostatic-switch mechanism [121, 130, 132] (see Fig. 8). Interspersed within the basic clusters at the N- or C-termini are usually serine residues, which are targets for phosphorylation by protein kinase C [123]. Phosphorylation of these residues thus introduces negative charge, partially neutralizing the positive charges of the basic

clusters and weakening their interaction with the PS headgroups of the membrane. The hydrophobic myrisotyl or palmitoyl anchors alone are not strong enough to hold the protein onto the membrane and hence it's released to move through the cytoplasm to a new location, e.g. the nucleus in the case of the SH2B1β gene product [130]. Another variation of the electrostatic switch mechanism is that in some cases the electrostatic interaction could be weakened by the influx of Ca^{2+} which binds to the PS headgroup thus neutralizing its negative charge [132].

In principle one could imagine a further variation on the electrostatic switch mechanism. If the concentration of PS on the cytoplasmic face of the membrane were reduced by the activation of a floppase or scramblase, this would also decrease the strength of electrostatic interaction, allowing release of peripheral membrane proteins. However, at this stage the authors are not aware of any experimental evidence supporting such a mechanism.

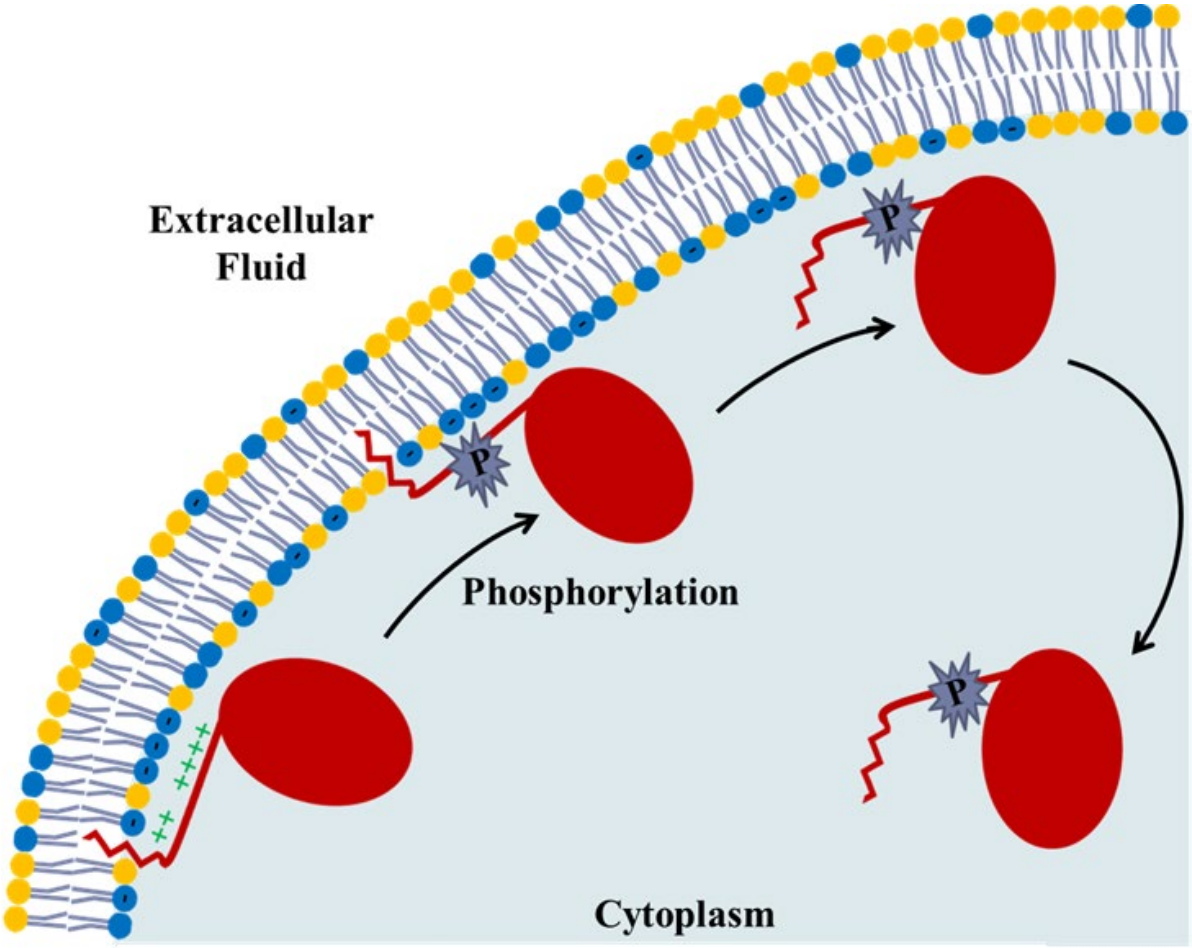


Figure 8: Electrostatic switch mechanism of peripheral membrane protein trafficking. The left side of the figure shows a peripheral membrane protein anchored to the cytoplasmic membrane surface via hydrophobic and electrostatic interactions of its N- or C-terminus. Phosphorylation of a serine (or possibly tyrosine) located within the protein's positively-charged polybasic region of clustered lysine and arginine residues by a protein kinase introduces negative charge, thus weakening the electrostatic attraction for the membrane. The hydrophobic interaction alone is not strong enough to hold the protein onto the membrane and it, therefore, dissociates and moves away from the membrane into the cytoplasm. Dephosphorylation of the serine (or tyrosine) by a phosphatase would remove the negatively charge and allow the protein to re-associate with the cytoplasmic membrane surface.

5.2 Regulation of integral membrane proteins

Integral membrane proteins are permanently anchored in the membrane by large hydrophobic domains. Once inserted into the membrane they do not undergo any trafficking through the cell. However, the asymmetry of the plasma membrane could play an important role in determining their activity or regulation. This is still an area of current research and it is, therefore, not yet possible to make definitive statements. Nevertheless, there are certain integral membrane proteins which show clear structural similarities to the peripheral membrane proteins just discussed, which would seem to suggest that an electrostatic switch mechanism could also be operating here.

Two candidates are the closely related P-type ATPases, the Na^+,K^+-ATPase and the gastric H^+,K^+-ATPase. Both of these ion pumps have lysine-rich N-termini which are located on the cytoplasmic surface of the protein [133]. Close to the lysine clusters both proteins have a serine residue, which in the case of the Na^+,K^+-ATPase is conserved across all vertebrates and, in the

case of the H^+,K^+-ATPase, is conserved across all mammals. Phosphorylation of the serine residues by protein kinase C has been demonstrated experimentally [134-138]. In the case of the Na^+,K^+-ATPase there is, furthermore, a large body of experimental evidence implicating the N-terminus in controlling the enzyme's conformational transition between the K^+-specific E2 state and the Na^+-specific E1 state [139-145], which is a major rate-determining step of the ion pumping cycle [146, 147]. More recently it was shown that an increase in the ionic strength of the medium induces a shift from the E2 to the E1 conformation and that the shift could be explained by the breaking of an electrostatic interaction which stabilises the E2 conformation [148] and that peptides analogues of the Na^+,K^+-ATPase N-terminus preferentially interact with membranes containing PS [149]. All of this data would seem to support the idea that an electrostatic switch is involved in Na^+,K^+- and H^+,K^+-ATPase activity and regulation.

There are, however, as yet no X-ray crystallographic data available on the N-terminus of either the Na^+,K^+-ATPase or the H^+,K^+-ATPase, because it could either not be resolved [150-153] or it was removed prior to crystal formation [154]. The fact that the N-terminus could not be resolved suggests that is probably undergoing dynamic motion on the timescale necessary for X-ray data collection, which is consistent with the prediction that it is an intrinsically disordered region of the protein [133]. Intrinsic disorder could in fact facilitate membrane interaction, because it allows greater flexibility than structured α-helices or β-pleated sheet. Experimental data on the Na^+,K^+-ATPase and H^+,K^+-ATPase exogenously expressed in cell lines, however, do not support a role of the N-terminus in determining ion pumping activity. Thus, although deletion of the N-terminus of purified Na^+,K^+-ATPase preparations caused significant changes in the kinetics of the enzyme's partial reactions [144], after expression in a cell line N-terminal deletion was found to have no effect on Na^+,K^+-ATPase activity [155] and, in the case of the H^+,K^+-ATPase, mutating all of the N-terminal lysines to alanines had no effect on the enzyme's kinetic properties [156]. It is possible, however, that if the N-terminus

effect is mediated by the membrane, and the membrane environment of the exogenously expressed protein does not adequately reproduce the native environment, the N-terminal effect could be lost.

A polybasic PS-binding domain with multiple lysine residues has also been identified on the cytoplasmic side of the plasma membrane Ca^{2+}-ATPase (PMCA) [157]. Rather than being at the N-terminus, the PS-binding domain of the PMCA is in the first cytoplasmic loop, but it is also thought to be involved in ion pump regulation. As discussed earlier, in the resting state blood platelets have a low cytoplasmic Ca^{2+} concentration, which is maintained in part by the PMCA. When the platelets become activated and the cytoplasmic Ca^{2+} concentration increases, PS is transferred to the extracellular membrane surface by TMEM16F. It seems likely that the loss of PS on the cytoplasmic side of the membrane could inhibit the PMCA due to a disruption of its cytoplasmic PS-binding domain and hence prevent activated platelets from reverting to their resting state.

It is interesting to note that the P-type ATPases with polybasic regions, i.e. the Na^+,K^+-ATPase, the H^+,K^+-ATPase and PMCA, are all located in the asymmetric plasma membrane, whereas the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), located in the relatively symmetric sarcoplasmic reticulum membrane has no polybasic region. One could speculate, therefore, that the polybasic regions could provide a general mechanism for sensing changes in plasma membrane asymmetry, not just in blood coagulation. Thus, the polybasic regions could also allow a plasma membrane pump to detect loss of PS in the cytoplasmic leaflet caused by activation of the scramblase Xkr8 during apoptosis. In this context the polybasic regions could serve the function of a “death sensor”, providing the signal to decrease ion pumping activity in apoptotic cells and avoiding the wastage of energy in the form of ATP. Of course a decrease in ion pumping activity cannot be occurring in cancer cells, which also expose PS to the extracellular medium, because this would prevent their proliferation. However, an over-

expression of the Na^+,K^+-ATPase has been reported in the progression of a variety of cancers [158], which could potentially cancel out any reduction in ion pumping activity at the single molecule level.

Another integral membrane protein whose activity could be dependent on lipid asymmetry is the dopamine receptor. This protein also has a lysine-rich N-terminus which, in a similar fashion to the Na^+,K^+- and H^+,K^+-ATPases, has been proposed to interact electrostatically with negatively charged lipids on the plasma membrane [159], in this case phosphatidylinositol (4,5)-bisphosphate (PIP_2), although an interaction with PS cannot be excluded. Hamilton et al. [159] showed that mutation of the lysines to uncharged amino acid residues had clear *in vivo* functional significance, with a change in amphetamine-induced locomotion of *Drosophila melanogaster*.

Some K^+-channels are also thought to be regulated by an electrostatic interaction of a basic domain with the cytoplasmic side of the membrane. Inward rectifier K^+ (Kir) channels of some bacteria possess an RCK (regulates conductance of K^+) domain containing many lysine residues which would promote interaction with anionic lipids in the cytoplasmic leaflet of the plasma membrane. Experiments at increasing ionic strength showed a clear reduction in membrane binding affinity, supporting the electrostatic nature of the interaction [160].

To experimentally demonstrate the effect of lipid asymmetry on the function of a membrane protein it would be highly desirable to reconstitute the protein into asymmetric vesicles. The synthesis of asymmetric vesicles is, however, not a trivial matter. One method that has proved successful involves the exchange of lipids between the vesicular outer leaflet and a methyl-β-cyclodextrin-lipid carrier complex [161-163]. Another approach which has been used and which is particularly useful for varying the PS composition of the outer leaflet is to treat vesicles with enzymes such as phosphatidyl serine decarboxylase [164] or phospholipase D [165] to produce asymmetric vesicles as models of the animal plasma membranes or

intracellular vesicles, respectively. As mentioned earlier, asymmetric vesicles have been used to measure passive flip-flop rates in a probe-free manner via small-angle neutron scattering and investigate the effects of peptide insertion [19] and the solvent methanol [166]. These measurements show that peptides dramatically increase the rate of flip-flop. A major technical problem in the development of asymmetric vesicles incorporating membrane proteins is, therefore, that in many cases the introduction of the protein accelerates flip-flop to such a degree that asymmetry is lost in the time range of minutes to hours. Markones et al. [167] recently reported a method for developing vesicles whose asymmetry was maintained for weeks and into which they successfully reconstituted a bacterial Na^+/H^+ antiporter. In such a system it is likely that the orientations of the reconstituted proteins are not uniform, e.g. both outside-out and inside-out populations of the protein may be both be present. However, activating the protein by the external addition of the appropriate substrate ensures that only one population of membrane proteins are activated. Rather than use reconstituted vesicles, Watanabe et al [52] developed an asymmetric lipid bilayer within a microarray system and, as discussed earlier, succeeded in determining the lipid transfer rate of the scramblase TMEM16F. To show that lipid asymmetry is crucial to a transporter's activity in such model systems it would be necessary to determine changes in activity after asymmetry is abolished by the addition of a scramblase. To our knowledge this has not yet been achieved.

6. Conclusion

Since its discovery in the 1970's, transverse lipid asymmetry of animal membranes and its role in physiological processes has been an active field of research. Early research concentrated on identifying the extent of the asymmetry and the mechanisms by which it is generated and maintained, i.e., by flippases, floppases and scramblases. More work still needs to be done to

understand the molecular detail of how these enzymes work, but with the recent revolutionary developments in the capabilities of cryo-electron-microscopy the pace of structural studies in the field is significantly accelerating. Looking to the future, it seems certain that research into the role of lipid asymmetry in both health and disease will intensify. This is particularly so because of the now recognized role of PS as a signalling molecule in apoptosis and the medical relevance of apoptotic mimicry, which is providing an impetus for the development of new therapies for both infectious diseases and cancer.

The physiological roles of PS in its normal location on the cytoplasmic leaflet of the plasma membrane are now beginning to be uncovered. New methods of creating long-lived synthetic asymmetric lipid vesicles into which specific membrane proteins can be reconstituted should provide valuable new information on the requirement of lipid asymmetry for many integral membrane proteins. To the authors it seems that comparisons between the roles of PS in both the cytoplasmic and extracellular leaflets could provide valuable insights into processes in which the lipid asymmetry changes, i.e. blood coagulation and apoptosis. In particular, the presence of polybasic PS-binding domains on the cytoplasmic-facing surface of many peripheral and integral membrane proteins has important consequences for trafficking and function of the proteins in healthy cells, but the same domains could act as "death sensors" to allow changes in protein function when PS is transferred from the cytoplasmic to the extracellular leaflet during apoptosis.

Although we have concentrated in this review in the role of PS, it is important not to forget other phospholipids such as PC. For example, the exposure of PS on the extracellular surface of the plasma membrane during blood coagulation and apoptosis causes the surface charge density of the membrane and its surface potential to become significantly more negative. However, such a change is only possible if the extracellular membrane initially has a net charge density close to zero. Thus, the maintenance of the high level of net neutrally charged PC in

the extracellular leaflet by the action of floppases is crucial to the signalling function of PS. Compensating fluxes of lipids between membrane leaflets are also necessary in order to maintain similar total numbers of lipids in each leaflet and avoid excessive lateral stress [168]. The physiological roles of lipid asymmetry hence involve intertwined activities of flippases, floppases and scramblases, as well as passive fluxes, and the asymmetric effect of one phospholipid cannot be disconnected from the asymmetric effect of another phospholipid. Thus, there is still much to be learnt regarding the roles of transverse lipid asymmetry in animal cells, but, based on what is already known, the rewards of further research will certainly be worth the effort.

Acknowledgement

The authors thank Prof. Philip Kuchel, Dr. Charles Cranfield and Prof. Toby Allen for helpful discussions and critically reading the manuscript. R.J.C. acknowledges financial support from the Australian Research Council (Discovery Grants DP121003548, DP150101112 and DP170101732).

References

[1] S. J. Singer, G. L. Nicolson, The fluid mosaic model of the structure of cell membranes, Science 175 (1972) 720-731.

[2] M. S. Bretscher, Asymmetrical lipid bilayer structure for biological membranes, Nature New Biol. 236 (1972) 11-12.

[3] R. D. Kornberg, H. M. McConnell, Inside-outside transitions of phospholipids in vesicle membranes, Biochemistry 10 (1971) 1111-1120.

[4] M. S. Bretscher, Phosphatidyl-ethanolamine: Differential labelling in intact cells and cell ghosts of human erythrocytes by a membrane impermeable reagent, J. Mol. Biol. 71 (1972) 523-528.

[5] S. E. Gordesky, G. B. Segel, G. V. Marinetti, Differences in reactivity of phospholipids with FDNB in normal, RBC, sickle cells and RBC ghosts, Biochem. Biophys. Res. Commun. 47 (1972) 1004-1009.

[6] S. E. Gordesky, G. V. Marinetti, R. Love, The reaction of chemical probes with the erythrocyte membrane, J. Membr. Biol. 20 (1975) 111-132.

[7] A. J. Verkleij, R. F. A. Zwaal, B. Roelofson, P. Comfurius, D. Kastelijn, L. L. M. van Deenen, The asymmetric distribution of phospholipids in the human red cell membrane: A combined study using phospholipases and freeze-etch electron microscopy, Biochim. Biophys. Acta 323 (1973) 178-193.

[8] G. van Meer, C. G. Gahmberg, J. A. F. Op den Kamp, L. L. M. Van Deenen, Phospholipid distribution in human En(a-) red cell membranes which lack the major sialoglycoprotein, glycophorin A, FEBS Lett. 135 (1981) 53-55.

[9] P. Bütikofer, Z. W. Lin, D. T.-Y. Chiu, B. Lubin, F. A. Kuypers, Transbilayer distribution of phosphatidylinositol in human red blood cells, J. Biol. Chem. 265 (1990) 16035-16038.

[10] P. Gascard, D. Tran, M. Sauvage, J.-C. Sulpice, T. Fukami, M. Claret, F. Giraud, Asymmetric distribution of phosphoinositides and phosphatidic acid in the human erythrocyte membrane, Biochim. Biophys. Acta 1069 (1991) 27-36.

[11] A. Zachowski, Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement, Biochem. J. 294 (1993) 1-14.

[12] J. A. F. Op den Kamp, Lipid asymmetry in membranes, Annu. Rev. Biochem. 48 (1979) 47-71.

[13] P. F. Devaux, Protein involvement in transmembrane lipid asymmetry, Annu. Rev. Biophys. Biomol. Struct. 21 (1992) 417-439.

[14] A. Zachowski, P. F. Devaux, Transmembrane movements of lipids, Experientia 46 (1990) 644-656.

[15] M. S. Bretscher, Some general principles of membrane structure, in: A. A. Moscona (Ed.), The Cell Surface in Development, Wiley, New York, 1974, pp. 17-27.

[16] M. S. Bretscher, Some aspects of membrane structure, in: S. Estrada-O. and C. Gitler (Eds.), Perspectives in Membrane Biology, Academic Press, New York, 1974, pp. 3-24.

[17] F. Hirata, J. Axelrod, Enyzmatic synthesis and rapid translocation of phosphatidylcholine by two methyltransferases in erythrocyte membranes, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 2348-2352.

[18] B. de Kruijff, E. J. J. van Zoelen, L. L. M. van Deenen, Glycophorin facilitates the transbilayer movement of phosphatidylcholine in vesicles, Biochim. Biophys. Acta 509 (1978) 537-542.

[19] M. H. L. Nguyen, M. DiPasquale, B. W. Rickeard, M. Doktorova, F. A. Heberle, H. L. Scott, F. N. Barrera, G. Taylor, C. P. Collier, C. B. Stanley, J. Katseras, D.

Marquardt, Peptide-induced lipid flip-flop in asymmetric liposomes measured by small angle neutron scattering, Langmuir 35 (2019) 11735-11744.
[20] G. Taylor, M.-A. Nguyen, S. Koner, E. Freeman, C. P. Collier, S. A. Sarles, Electrophysiological interrogation of asymmetric droplet interface bilayers reveals surface-bound alamethicin induces flip-flop, Biochim. Biophys. Acta – Biomembr. 1861 (2019) 335-343.
[21] D. Marquardt, F. A. Heberle, T. Miti, B. Eicher, E. London, J. Katsaras, G. Pabst, ^{1}H NMR shows slow phospholipid flip-flop in gel and fluid bilayers, Langmuir 33 (2017) 3731-3741.
[22] B. Wah, J. M. Breidigan, J. Adams, P. Horbal, S. Garg, L. Porcar, U. Perez-Salas, Reconciling differences between lipid transfer in free-standing and solid supported membranes: A time-resolved small-angle neutron scattering study, Langmuir 33 (2017) 3384-3394.
[23] W. F. Drew Bennett, N. Sapay, D. P. Tieleman, Atomistic simulations of pore formation and closure in lipid bilayers, Biophys. J. 106 (2014) 210-219.
[24] R. J. Clarke, X. Fan, Pumping ions, Clin. Exp. Pharmacol. Physiol. 38 (2011) 726-733.
[25] J. C. Skou, The influence of some cations on an adenosine triphosphatase from peripheral nerves, Biochim. Biophys. Acta 23 (1957) 394-401.
[26] M. Ikeda, A. Kihara, Y. Igarishi, Lipid asymmetry of the eukaryotic plasma membrane: Functions and related enzymes, Biol. Pharm. Bull. 29 (2006) 1542-1546.
[27] T. Kobayashi, A. K. Menon, Transbilayer lipid asymmetry, Curr. Biol. 28 (2018) R367-R420.
[28] W. R. Bishop, R. M. Bell, Assembly of the endoplasmic reticulum phospholipid bilayer: The phosphatidylcholine transporter, Cell 42 (1985) 51-60.
[29] W. R. Bishop, R. M. Bell, Assembly of phospholipids into cellular membranes, Ann. Rev. Cell Biol. 4 (1988) 579-610.
[30] M. Seigneuret, P. F. Devaux, ATP-dependent asymmetric distribution of spin-labelled phospholipids in the erythrocyte membrane: relation to shape change, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 3751-3755.
[31] C. J. Féo, P. F. Leblond, The discocyte-echinocyte transformation: Comparison of normal and ATP-enriched human erythrocytes, Blood 44 (1974) 639-647.
[32] G. Pages, D. Szekely, P. W. Kuchel, Erythrocyte-shape evolition recorded with fast-measurement NMR diffusion-diffraction, J. Magn. Reson. Imaging 28 (2008) 1409-1416.
[33] G. Morrot, A. Zachowski, P. F. Devaux, Partial purification and characterization of the human erythrocyte Mg^{2+}-ATPase. A candidate aminophospholipidtranslocase, FEBS Lett. 266 (1990) 29-32.
[34] X. Tang, M. S. Halleck, R. A. Schlegel, P. Williamson, A subfamily of P-type ATPases with aminophospholipid transporting activity, Science 272 (1996) 1495-1497.
[35] M. Timcenko, J. A. Lyons, D. Januliene, J. J. Ulstrup, T. Dieudonné, C. Montigny, M.-R. Ash, J. Lykkegaard Karlsen, T. Boesen, W. Kühlbrandt, G. Lenoir, A. Moeller, P. Nissen. Structure and autoregulation of a P4-ATPase lipid flippase, Nature 571 (2019) 366-370.
[36] M. Hiraizumi, K. Yamashita, T. Nishizawa, O. Nureki, Cryo-EM structures capture the transport cycle of the P4-ATPase flippase, Science 365 (2019) 1149-1155.
[37] G. Lenoir, P. Williamson, C. F. Puts, J. C. M. Holthuis, Cdc50p plays a vital role in the ATPase reaction cycle of the putative aminophospholipid transporter Drs2p, J. Biol. Chem. 27 (2009) 17956-17967.

[38] S. Bryde, H. Hennrich, P. M. Verhulst, P. F. Devaux, G. Lenoir, J. C. M. Holthuis, CDC50 proteins are critical components of the human class-1 P_4-ATPase transport machinery, J. Biol. Chem. 285 (2010) 40562-40572.
[39] J. A. Coleman, R. S. Molday, Critical role of the β-subunit CDC50A in the stable expression, assembly, subcellular localization, and lipid transport of the P_4-ATPase ATP8A2, J. Biol. Chem. 286 (2011) 17205-17216.
[40] F. L. Theodoulou, I. D. Kerr, ABC transporter research: going strong 40 years on, Biochem. Soc. Trans. 43 (2015) 1033-1040.
[41] R. W. Robey, K. M. Pluchino, M. D. Hall, A. T. Fojo, S. E. Bates, M. M. Gottesman, Revisting the role of ABC transporters in multi-drug resistant cancer, Nature Rev. Cancer 18 (2018) 452-464.
[42] H. Glavinas, P. Krajcsi, J. Cserepes, B. Sarkadi, The role of ABC transporters in drug resistance, metabolism and toxicity, Curr. Drug Deliv. 1 (2004) 27-42.
[43] N. P. Greene, E. Kaplan, A Crow, V. Koronakis, Antibiotic resistance mediated by the MacB ABC transporter family: A structural and functional perspective, Front. Microbiol. 9 (2018) 950.
[44] J. Neumann, D. Rose-Sperling, U. A. Hellmich, Diverse relations between ABC transporters and lipids: An overview, Biochim. Biophys. Acta – Biomembr. 1859 (2017) 605-618.
[45] E. J. Tarling, T. Q. de Aguir Vallim, P. A. Edwards, Role of ABC transporters in lipid transport and human disease, Trends Endorinol. Metab. 24 (2013) 342-350.
[46] F. Quazi, R. S. Molday, Differential phospholipid substrates and directional transport by ATP-binding cassette proteins ABCA1, ABCA7, and ABCA4 and disease-causing mutants, J. Biol. Chem. 288 (2013) 34414-34426.
[47] C. Vedhachalam, P. T. Duong, M. Nickel, D. Nguyen, P. Dhanasekaran, H. Saito, G. H. Rothblat, S. Lund-Katz, M. C. Phillips, Mechanism of ATP-binding cassette transporter A1-mediated cellular lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles, J. Biol. Chem. 282 (2007) 25123-25130.
[48] G. R. Beilharz, C. R. Middlehurst, P.W. Kuchel, G.E. Hunt, G.F. S. Johnson, An experimental study and computer simulation of the turnover of choline in erythrocytes of patients treated with lithium carbonate, Aust. J. Exp. Biol. Med. Sci. 64 (1986) 271-289.
[49] C. F. Reed, Phospholipid exchange between plasma and erythrocytes in man and the dog, J. Clin Invest. 47 (1968) 749-760.
[50] H. Selle, B. R. Chapman, P. W. Kuchel, Release of choline by phospholipase D and a related phosphoric diester hydrolase in human erythrocytes, Biochem. J. 284 (1992) 61-65.
[51] T. G. Pomorski, A. K. Menon, Lipid somersaults: Uncovering the mechanisms of protein-mediated lipid flipping, Prog. Lipid Res. 64 (2016) 69-84.
[52] R. Watanabe, T. Sakuragi, H. Noji, S. Nagata, Single-molecule analysis of phospholipid scrambling by TMEM16F, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) 3066-3071.
[53] P. F.Devaux, A. Herrmann, N. Ohlwein, M. M. Kozlov, How flippases can modulate membrane structure, Biochim. Biophys. Acta 1778 (2008) 1591-1600.
[54] M. P. Sheetz, S. J.Singer, Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions, Proc. Natl. Acad. Sci. U. S. A. 71 (1974) 4457-4461.
[55] J. Zimmerberg, M. M. Kozlov, How proteins produce cellular membrane curvature, Nature Rev. Mol. Cell Biol. 7 (2006) 9-19.

[56] P. Müller, T. Pomorski, A. Herrmann, Incorporation of phospholipid analogues into the plasma membrane affects ATP-induced vesiculation of human erthrocyte ghosts, Biochem. Biophys. Res. Commun. 199 (1994) 881-887.
[57] T. W. Yau, R. P. Kuchel, J. M. S. Koh, D. Szekely, P. J. Mirtschin, P. W. Kuchel, Cytoskeletal rearrangements in human red blood cells induced by snake venoms: light microscopy of shapes and NMR studies of membrane function, Cell Biol. Int. 36 (2012) 87-97.
[58] P. F. Devaux, Is lipid translocation involved during endo- and exocytosis? Biochimie 82 (2000) 497-509.
[59] M. Siggel, R. M. Bhaskara, G. Hummel, Phospholipid scramblases remodel the shape of asymmetric membranes, J. Phys. Chem. Lett. 10 (2019) 6351-6354.
[60] D. Blockmans, H. Deckmyn, J. Vermylen, Platelet activation, Blood Reviews 9 (1995) 143-156.
[61] J. W. M. Heemskerk, E. M. Bevers, T. Lindhout, Platelet activation and blood coagulation, Thromb. Haemost. 88 (2002) 186-193.
[62] B. Nieswandt, S. P. Watson, Platelet-collagen interaction: is GPVI the central receptor? Blood 102 (2003) 449-461.
[63] P. Blair, R. Flaumenhaft, Platelet α-granules: Basic biology and clinical correlates, Blood Reviews 23 (2009) 177-189.
[64] E. W. Davie, K. Fujikawa, W. Kisiel, The coagulation cascade: Initiation, maintenance, and regulation, Biochemistry 30 (1991) 10363-10370.
[65] R. F. A. Zwaal, P. Comfurius, E. M. Bevers, Lipid-protein interactions in blood coagulation, Biochim. Biophys. Acta 1376 (1998) 433-453.
[66] R. F. A. Zwaal, P. Comfurius, E. M. Bevers, Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids, Biochim. Biophys. Acta 1636 (2004) 119-128.
[67] E. M. Bevers, P. Comfurius, J. L. M. L. van Rijn, H. C. Hemker, R. F. A. Zwaal, Generation of prothrombin-converting activity and exposure of phosphatidylserine at the outer surface of platelets, Eur. J. Biochem. 122 (1982) 429-436.
[68] M. Hoffman, D. M. Monroe, A cell-based model of hemostasis, Thromb. Haemost. 85 (2001) 958-965.
[69] R. F. A. Zwaal, P. Comfurius, E. M. Bevers, Surface exposure of phosphatidylserine in pathological cells, Cell. Mol. Life Sci. 62 (2005) 971-988.
[70] B. Fadeel, D. Xue, The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease, Crit. Rev. Biochem. Mol. Biol. 44 (2009) 264-277.
[71] J. Suzuki, M. Umeda, P. J. Sims, S. Nagata, Calcium-dependent phospholipid scrambling by TMEM16F, Nature 468 (2010) 834-838.
[72] H. M. Hankins, R. D. Baldridge, P. Xu, T. R. Graham, Role of flippases, scramblases and transfer proteins in phosphatidylserine subcellular distribution, Traffic 16 (2014) 35-47.
[73] R. B. Birge, S. Boeltz, S. Kumar, J. Carlson, J. Wanderley, D. Calianese, M. Barcinski, R. A. Brekken, X. Huang, J. T. Hutchins, B. Freimark, C. Empig, J. Mercer, A. J. Schroit, G. Schett, M. Herrmann, Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer, Cell Death Differ. 23 (2016) 962-978.
[74] J. D. Brunner, N. K. Lim, S. Schenck, A. Duerst, R. Dutzler, X-ray structure of a calcium-activated TMEM16 lipid scramblase, Nature 516 (2014) 207-212.
[75] M. E. Falzone, J. Rheinberger, B.-C. Lee, T. Peyear, L. Sasset, A. M. Raczkowski, E. T. Eng, A. Di Lorenzo, O. S. Andersen, C. M. Nimigean, A. Accardi, Structural basis

of Ca^{2+}-dependent activation and lipid transport by a TMEM16 scramblase, eLife 8 (2019) e43229.
[76] N. P. Bethel, M. Grabe, Atomistic insight into lipid translocation by a TMEM16 scramblase, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 14049-14054.
[77] B.-C. Lee, G. Khelashvili, M. Falzone, A. K. Menon, H. Weinstein, A. Accardi, Gating mechanism of the extracellular entry to the lipid pathway in a TMEM16 scramblase, Nature Commun. 9 (2018) 3251.
[78] D. Varga-Szabo, A. Braun, B. Nieswandt, Calcium signaling in platelets, J. Thromb. Haemostat. 7 (2009) 1057-1066.
[79] M. P. Mahaut-Smith, The unique contribution of ion channels to platelet and megakaryocyte function, J. Thromb. Haemostat. 10 (2012) 1722-1732.
[80] Z. Ilkan, J. R. Wright, A. H. Goodall, J. M. Gibbins, C. I. Jones, M. P. Mahaut-Smith, Evidence for shear-mediated Ca^{2+} entry through mechanosensitive cation channels in human platelets and a megakaryocytic cell line, J. Biol. Chem. 292 (2017) 9204-9217.
[81] E. M. Bevers, P. Comfurius, D. W. C. Dekkers, M. Harmsma, R. F. A. Zwaal, Regulatory mechanisms of transmembrane phospholipid distributions and pathophysiological implications of transbilayer lipid scrambling, Lupus 7 (1998) S126-S131.
[82] J. Rosing, J. L. M. L. van Rijn, E. M. Bevers, G. van Dieijen, P. Comfurius, R. F. A. Zwaal, The role of activated platelets in prothrombin and factor X activation, Blood 65 (1985) 319-332.
[83] E. M. Bevers, P. Comfurius, R. F. A. Zwaal, Changes in membrane distribution during platelet activation, Biochim. Biophys. Acta 736 (1983) 57-66.
[84] O. Morel, L. Jesel, J.-M. Freyssinet, F. Toti, Cellular mechanisms underlying the formation of circulating microparticles, Arterioscl. Throm. Vasc. 31 (2011) 15-26.
[85] R. F. A. Zwaal, P. Comfurius, E. M. Bevers, Platelet procoagulant activity and microvesicle formation. Its putative role in hemostasis and thrombosis, Biochim. Biophys. Acta 1180 (1992) 1-8.
[86] H. J. Weiss, W. J. Vivic, B. A. Lages, J. Rogers, Isolated deficiency of platelet procoagulant activity, Am. J. Med. 67 (1979) 206-213.
[87] N. Pedemonte, L. J. V. Galietta, Structure and function of TMEM16 proteins (anoctamins), Physiol. Rev. 94 (2014) 419-459.
[88] S. Nagata, J. Suzuki, K. Segawa, T. Fujii, Exposure of phosphatidylserine on the cell surface, Cell Death Differ. 23 (2016) 952-961.
[89] H. W. A. Ehlen, M. Chinenkova, M. Moser, H.-M. Munter, Y. Krause, S. Gross, B. Brachvogel, M. Wuelling, U. Kornak, A. Vortkamp, Inactivation of anoctamin-6/Tmem16F, a regulator of phosphatidylserine scrambling in osteoblasts, leads to decreased mineral deposition in skeletal tissues, J. Bone Mineral Research 28 (2013) 246-259.
[90] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495-516.
[91] M. A. Savitskaya, G. E. Onishchenko, Mechanisms of apoptosis, Biochem.-Moscow 80 (2015) 1393-1405.
[92] S. Nagata, Apoptosis and clearance of apoptotic cells, Annu. Rev. Immunol. 36 (2018) 489-517.
[93] V. A. Fadok, D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, P. M. Henson, Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages, J. Immunol. 148 (1992) 2207-2216.

[94] M. Miyanishi, K. Tada, M. Koike, Y. Uchiyama, T. Kitamura, S. Nagata, Identification of Tim4 as a phosphatidylserine receptor, Nature 450 (2007) 435-439.
[95] D. Park, A. Hochreiter-Hufford, K. S. Ravichandran, The phosphatidylserine receptor TIM-4 does not mediate direct signalling, Curr. Biol. 19 (2009) 346-351.
[96] P. A. Leventis, S. Grinstein, The distribution and function of phosphatidylserine in cellular membranes, Annu. Rev. Biophysics 39 (2010) 407-427.
[97] J. Suzuki, D. P. Denning, E. Imanishi, H. R. Horvitz, S. Nagata, Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells, Science 341 (2013) 403-406.
[98] J. Suzuki, E. Imanishi, S. Nagata, Exposure of phosphatidylserine by Xk-related protein family during apoptosis, J. Biol. Chem. 289 (2014) 30257-30267.
[99] J. Suzuki, T. Fujii, T. Imao, K. Ishikara, H. Kuba, S. Nagata, Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members, J. Biol. Chem. 288 (2013) 13305-13316.
[100] T. Sakuragi, H. Kosako, S. Nagata, Phosphorylation-mediated activation of mouse Xkr8 scramblase for phosphatidylserine exposure, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 2907-2912.
[101] K. Segawa, S. Nagata, An apoptotic 'eat me' signal: phosphatidylserine exposure, Trends Cell Biol. 25 (2015) 639-650.
[102] K. Segawa, S. Kurata, Y. Yanagihashi, T. R. Brummelkamp, F. Matsuda, S. Nagata, Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure, Science 344 (2014) 1164-1168.
[103] K. Segawa, S. Kurata, S. Nagata, Human type IV P-type ATPases that work as plasma membrane phospholipid flippases and their regulation by caspase and calcium, J. Biol. Chem. 291 (2016) 762-772.
[104] V. A. Tyurin, K. Balasubramanian, D. Winnica, YY Tyurina, R. R. He, A. A. Kapralov, C. H. Macphee, V. E. Kagan, Oxidatively modified phosphaptidylserines on the surface of apoptotic cells are essential phagocytic 'eat-me' signals: cleavage and inhibition of phagocytosis by Lp-PLA$_2$, Cell Death Differ. 21 (2014) 825-835.
[105] G. Lemke, How macrophages deal with death, Nat. Rev. Immunol. 19 (2019) 539-549.
[106] V. E. Kagan, J. P. Fabisiak, A. A. Shvedova, Y. Y. Tyurina, V. A. Tyurin, N. F. Schor, K. Kawai, Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis, FEBS Lett. 477 (2000) 1-7.
[107] J. Jiang, V. Kini, N. Belikova, B. F. Serinkan, G. G. Borisenko, Y. Y. Tyurina, V. A. Tyurin, V. E. Kagan, Cytochrome c release is required for phosphatidylserine peroxidation during Fas-triggered apoptosis in lung epithelial A549 cells, Lipids 39 (2004) 1133-1142.
[108] H. Bayir, B. Fadeel, M. J. Palladino, E. Witasp, I. V. Kumikov, Y. Y. Tyurina, V. A. Tyurin, A. A. Amoscato, J. Jiang, P. M. Kochanek, S. T. DeKosky, J. S. Greenberger, A. A. Shvedova, V. E. Kagan, Apoptotic interactions of cytochrome c: Redox flirting with anionic phospholipids within and outside of mitochondria, Biochim. Biophys. Acta – Bioenerg. 1757 (2006) 648-659.
[109] R. E. Voll, M. Herrmann, E. A. Roth, C. Stach, J. K. Kalden, Immunosuppressive effects of apoptotic cells, Nature 390 (1997) 350-351.
[110] E. M. Bevers, P. L. Williamson, Phospholipid scramblase: An update, FEBS Lett. 584 (2010) 2724-2730.
[111] M. K. Callahan, P. M. Popernack, S. Tsutsui, L. Truong, R. A. Schlegel, A. J. Henderson, Phosphatidylserine on HIV envelope is a cofactor for infection of monocytic cells, J. Immunol. 170 (2003) 4840-4845.

[112] S. B. Willingham, J.-P. Volkmer, A. J. Gentles, D. Sahoo, P. Dalerba, S. S. Mitra, J. Wang, H. Conteras-Trujillo, R. Martin, J. D. Cohen, P. Lovelace, F. A. Scheeren, M. P. Chao, K. Weiskopf, C. Tang, A. K. Volkmer, T. J. Naik, T. A. Storm, A. R. Mosley, B. Edris, S. M. Schmid, C. K. Sun, M.-S. Chua, O. Murillo, P. Rajendran, A. C. Cha, R. K. Chin, D. Kim, M. Adorno, T. Raveh, D. Tseng, S. Jaiswal, P. Ø. Enger, G. K. Steinberg, G. Li, S. K. So, R. Majeti, G. R. Harsh, M. van den Rijn, N. N. H. Teng, J. B. Sunwoo, A. A. Alizadeh, M. F. Clarke, I. L. Weissman, The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 6662-6667.
[113] A. Rivera, X. Fu, L. Tao, X. Zhang, Expression of mouse CD47 on human cancer cells profoundly increases tumor metastasis in murine models, BMC Cancer 15 (2015) 964.
[114] H. Zhao, J. Wang, X. Kong, E. Li, Y. Liu, X. Du, Z. Kang, Y. Tang, Y. Kuang, Z. Yang, Y. Zhou, Q. Wang, CD47 promotes tumor invasion and metastasis in non-small cell lung cancer, Sci. Rep. 6 (2016) 29719.
[115] B. Sharma, S. S. Kanwar, Phosphatidylserine: A cancer cell targeting biomarker, Semin. Cancer Biol. 52 (2018) 17-25.
[116] O. Belzile, X. Huang, J. Gong, J. Carlson, A. J. Schroit, R. A. Brekken, B. D. Freimark, Antibody targeting of phosphatidylserine for the detection and immunotherapy of cancer, ImmunoTargets and Therapy 7 (2018) 1-14.
[117] R. A. Brekken, Antibody targeting of phosphatidylserine for the detection and immunotherapy of cancer. Reply, ImmunoTargets and Therapy 7 (2018) 53.
[118] K. F. N'Guessan, P. H. Patel, X. Qi, SapV-DOPS – a phosphatidylserine-targeted nanovesicle for selective cancer therapy, Cell Commun. Signal. 18 (2020) 6.
[119] C. Andrechak, L. J. Dooling, D. Discher, The macrophage checkpoint CD47:SIRPα for recognition of 'self' cells: from clinical trials of blocking antibodies to mechanobiological fundamentals, Phil. Trans. R. Soc. 374 (2019) 20180217.
[120] K. Segawa, Y. Yanagihashi, K. Yamada, C. Suzuki, Y. Uchiyama, S. Nagata, Phospholipid flippases enable precursor B cells to flee engulfment by macrophages, Proceedings of the National Academy of Sciences of the U. S. A. 115 (2018) 12212-12217.
[121] S. McLaughlin, A. Aderem, The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions, Trends Biochem. Sci. 20 (1995) 272-276.
[122] D. Murray, N. Ben-Tal, B. Honig, S. McLaughlin, Electrostatic interaction of myristoylated proteins with membranes: simple physics, complicated biology, Structure 5 (1997) 985-989.
[123] D. Murray, A. Arbuzova, B. Honig, S. McLaughlin, The role of electrostatic and nonpolar interactions in the association of peripheral proteins with membranes, Curr. Topics Membranes 52 (2002) 277-307.
[124] A. Mulgrew-Nesbitt, K. Diraviyam, J.Wang, S. Singh, P. Murray, Z. Li, L. Rogers, N. Mirkovic, D. Murray, The role of electrostatics in protein-membrane interactions, Biochim. Biophys. Acta 1761 (2006) 812-826.
[125] A. M. Whited, A. Johs, The interactions of peripheral membrane proteins with biological membranes, Chem. Phys. Lipids 192 (2015) 51-59.
[126] J. Kim, P. J. Blackshear, J. D. Johnson, S. McLaughlin, Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin, Biophys. J. 67 (1994) 227-237.
[127] J. Kim, T. Shishido, X. Jiang, A. Aderem, S. McLaughlin, Phosphorylation, high ionic strength, and calmodulin reverse the binding of MARCKS to phospholipid vesicles, J. Biol. Chem. 269 (1994) 28214-28219.

[128] C. T. Sigal, W. Zhou, C. A. Buser, S. McLaughlin, M. D. Resh, Amino-terminal basic residues of Src mediate membrane binding through electrostatic interaction with acidic phospholipids, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 12253-12257.
[129] M. A. Noguera-Salvà, F. Guardiola-Serrano, M. L. Martin, A. Marcilla-Etxenike, M. O. Bergo, X. Busquets, P. V. Escribá, Role of the C-terminal basic amino acids and the lipid anchor of the $G\gamma_2$ protein in membrane interactions and cell localization, Biochim. Biophys. Acta – Biomembr. 1859 (2017) 1536-1547.
[130] T. J. Maures, H.-W. Su, L. S. Argetsinger, S. Grinstein, C. Carter-Su, Phosphorylation controls a dual-function polybasic nuclear localization sequence in the adapter protein SH2B1β to regulate its cellular function and distribution, J. Cell Sci. 124 (2011) 1542-1552.
[131] F. Lei, J. Jin, C. Herrmann, W. Mothes, Basic residues in the matrix domain and multimerization target murine leukemia virus Gag to the virological synapse, J. Virol. 87 (2013) 7113-7126.
[132] T. Yeung, G. E. Gilbert, J. Shi, J. Silvius, A. Kapus, S. Grinstein, Membrane phosphatidylserine regulates surface charge and protein localization, Science 11 (2008) 210-213.
[133] D. Diaz, R.J. Clarke, Evolutionary analysis of the lysine-rich N-terminal cytoplasmic domains of the gastric H^+,K^+-ATPase and the Na^+,K^+-ATPase, J. Membr. Biol. 251 (2018) 653–666.
[134] P. Beguin, A. T. Beggah, A. V. Chibalin, P. Burgener-Kairuz, F. Jaisser, P. M. Mathews, B. C. Rossier, S. Cotecchia, K. Geering, Phosphorylation of the Na,K-ATPase α-subunit by protein kinase A and C in vitro an in intact cells. Identification of a novel motif for PKC-mediated phosphorylation, J. Biol. Chem. 269 (1994) 24437-24445.
[135] M. S. Feschenko, K. J. Sweadner, Structural basis for species-specific differences in the phosphorylation of Na,K-ATPase by protein kinase C, J. Biol. Chem. 270 (1995) 14072-14077.
[136] N. S. Loginvenko, I. Dulubova, N. Fedosova, S. H. Larsson, A. C. Nairn, M. Esmann, P. Greegard, A. Aperia, Phosphorylation by protein kinase C of serine-23 of α-1 subunit of rat Na^+,K^+-ATPase affects its conformational equilibrium, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9132-9137.
[137] K. Togawa, S. Kaya, A. Shimada, T. Imagawa, S. Mårdh, J. Corbin, U. Kikkawa, K. Taniguchi, Ser-27, Tyr-10 and Tyr-7 in the α-chain as Ca^{2+}-dependent phosphorylation sites by intrinsic and extrinsic protein kinases, Biochem. Biophys. Res. Commun. 227 (1996) 810-815.
[138] F. Cornelius, Y. A. Mahmmoud, Direct activation of gastric H,K-ATPase by N-terminal protein kinase C phosphorylation. Comparison of the acute regulation mechanisms of H,K-ATPase and Na,K-ATPase, Biochemistry 84 (2003) 1690-1700.
[139] P. L. Jørgensen, Purification and characterization of (Na^+,K^+)-ATPase. 5. Conformational-changes in enzyme-transitions between Na-form and K-form studied with tryptic digestion as a tool, Biochim. Biophys. Acta 401 (1975) 399-415.
[140] P. L. Jørgensen, E. Skriver, H. Hebert, A. B. Maunsbach, Structure of the Na,K-pump: crystallization of pure membrane-bound Na,K-ATPase and identification of functional domains of the α-subunit, Ann. N. Y. Acad. Sci. 402 (1982) 207-225.
[141] P. L. Jørgensen, J. H. Collins, Tryptic and chymotryptic cleavage sites in sequence of α-subunit of ($Na^+ + K^+$)-ATPase from outer medulla of mammalian kidney, Biochim. Biophys. Acta 860 (1986) 570-576.
[142] P. L. Jørgensen, J. P. Andersen, Structural basis for E1-E2 conformational transitions in Na,K-pump and Ca-pump proteins, J. Membr. Biol, 103 (1988) 95-120.

[143] C. H. Wu, L. A. Vasilets, K. Takeda, M. Kawamura, W. Schwarz, Functional role of the N-terminus of Na^+,K^+-ATPase α-subunit as an inactivation gate of palytoxin-induced pump channel, Biochim. Biophys. Acta 1609 (2003) 55-62.
[144] F. Cornelius, Y. A. Mahmmoud, L. Meischke, G. Cramb, Functional significance of the shark Na,K-ATPase N-terminal domain. Is the strucurally variable N-terminus involved in tissue-specific regulation by FXYD proteins? Biochemistry 44 (2005) 13051-13062.
[145] R. Scanzano, L. Segall, R. Blostein, Specific sites in the cytoplasmic N terminus modulate conformational transitions of the Na,K-ATPase, J. Biol. Chem. 282 (2007) 33691-33697.
[146] C. Lüpfert, E. Grell, V. Pintschovius, H.-J. Apell, F. Cornelius, R. J. Clarke, Rate limitation of the Na^+,K^+-ATPase pump cycle, Biophys. J. 81 (2001) 2069-2081.
[147] P. A. Humphrey, C. Lüpfert, H.-J. Apell, F. Cornelius, R. J. Clarke, Mechanism of the rate-determining step of the Na^+,K^+-ATPase pump cycle, Biochemistry 41 (2002) 9496-9507.
[148] Q. Jiang, A. Garcia, M. Han, F. Cornelius, H.-J.Apell, H. Khandelia, R. J. Clarke, Electrostatic stabilization plays a central role in autoinhibitory regulation of the Na^+,K^+-ATPase, Biophys. J. 112 (2017) 288-299.
[149] K. Nguyen, A. Garcia, M.-A. Sani, D. Diaz, V. Dubey, D. Clayton, G. Dal Poggetto, F. Cornelius, R. J. Payne, F. Separovic, H. Khandelia, R. J. Clarke, Interaction of N-terminal peptide analogues of the Na^+,K^+-ATPase with membranes, Biochim. Biophys. Acta – Biomembr. 1860 (2018) 1282-1291.
[150] J. P. Morth, B. P. Pedersen, M. S. Toustrup-Jensen, T. L.-M. Sørensen, J. Petersen, J. P. Andersen, B. Vilsen, P. Nissen, Crystal structure of the sodium-potassium pump, Nature 450 (2007) 1043-1049.
[151] T. Shinoda, H. Ogawa, F. Cornelius, C. Toyoshima, Crystal structure of the sodium-potassium pump at 2.4 Å resolution, Nature 459 (2009) 446-450.
[152] R. Kanai, H. Ogawa, B. Vilsen, F. Cornelius, C. Toyoshima, Crystal structure of a Na^+-bound Na^+,K^+-ATPase preceding the E1P state, Nature 502 (2013) 201-206.
[153] M. Nyblom, H. Poulsen, P. Gourdon, L. Reinhard, M. Andersson, E. Lindahl, N. Fedosova, P. Nissen, Crystal structure of Na^+,K^+-ATPase in the Na^+-bound state, Science 342 (2013) 123-127.
[154] K. Abe, K. Irie, H. Nakanishi, H. Suzuki, Y. Fujiyoshi, Crystal structures of the gastric proton pump, Nature 556 (2018) 214-218.
[155] S. E. Daly, L. K. Lane, R. Blostein, Structure/function analysis of the amino-terminal region of the α1 and α2 subunits of Na,K-ATPase, J. Biol. Chem. 271 (1996) 23683-23689.
[156] S. Asano, K. Miwa, H. Yashiro, Y. Tabuchi, N. Takeguchi, Significance of lysine/glycine cluster structure in gastric H^+,K^+-ATPase, Jpn. J. Physiol. 50 (2000) 419-428.
[157] J. I. E. Bruce, Metabolic regulation of the PMCA: Role in cell death and survival, Cell Calcium 69 (2018) 28-36.
[158] Q. Wang, S.-B. Li, Y.-Y. Zhao, D.-N. Dai, H. Du, Y.-Z. Lin, J.-C. Ye, J. Zhao, W. Xiao, Y. Mei, Y.-T. Xiao, S. C. Liu, Y. Li, Y.-F. Xia, E.-W. Song, G.-H. Tang, W.-G. Zhang, Z.-G. Zhang, Z.-J. Li, X.-B. Zheng, D.-H. Cao, M.-Z. Li, Q. Zhong, Z.-P. Chen, C.-N. Qian, W. Fan, G.-K. Feng, M.-S. Zeng, Identification of a sodium pump Na^+.K^+ ATPase α1-targeted peptide for PET imaging of breast cancer, J. Controlled Release 281 (2018) 178-188.
[159] P. J. Hamilton, A. N. Belovich, G. Khelashvili, C. Saunders, K. Erreger, J. A. Javitch, H. H. Sitte, H. Weinstein, H. J. G. Matthews, A. Galli, PIP_2 regulates psychostimulant

behaviors through its interaction with a membrane protein, Nature Chem. Biol. 10 (2014) 582-589.
[160] C. P. Ptak, L. G. Cuello, E. Perozo, Electrostatic interaction of a K^+ channel RCK domain with charged membrane surfaces, Biochemistry 44 (2005) 62-71.
[161] F. A. Heberle, D. Marquardt, M. Doktorova, B. Geier, R. F. Standaert, P. Heftberger, B. Kollmitzer, J. D. Nickels, R. A. Dick, G. W. Feigenson, J. Katsaras, E. London, G. Pabst, Subnanometer structure of an asymmetric model membrane: Interleaflet coupling influences domain properties, Langmuir 32 (2016) 5195-5200.
[162] M. Doktorova, F. A. Heberle, B. Eicher, R. F. Standaert, J. Katsaras, E. London, G. Pabst, D. Marquardt, Preparation of asymmetric phospholipid vesicles for use as cell membrane models, Nat. Protoc. 13 (2018) 2086-2101.
[163] M. Markones, C. Drechsler, M. Kaiser, L. Kalie, H. Heerklotz, S. Fiedler, Engineering asymmetric lipid vesicles: Accurate and convenient control of the outer leaflet composition, Langmuir 34 (2018) 1999-2005.
[164] C. Drechsler, M. Markones, J.-Y. Choi, N. Frieling, S. Fiedler, D. R. Voelker, R. Schubert, H. Heerklotz, Preparation of asymmetric liposomes using phosphatidylserine decarboxylase, Biophys. J. 115 (2018) 1509-1517.
[165] R. Takaoka, H. Kurosaki, H. Nakao, K. Ikeda, M. Nakano, Formation of asymmetric vesicles via phospholipase D-mediated transphosphorylation, Biochim. Biophys. Acta – Biomembr. 1860 (2018) 245-249.
[166] M. H. L. Nguyen, M. DiPasquale, B. W. Rickeard, C. B. Stanley, E. G. Kelley, D. Marquardt, Methanol accelerates DMPC flip-flop and transfer: A SANS study on lipid dynamics, Biophys. J. 116 (2019) 755-759.
[167] M. Markones, A. Fippel, M. Kaiser, C. Drechsler, C. Hunte, H. Heerklotz, Stairway to asymmetry: five steps to lipid-asymmetric proteoliposomes, Biophys. J. 118 (2020) 294-302.
[168] S. Frickenhaus, A. Herrmann, R. Heinrich, Compensating lipid fluxes generated by the aminophospholipid translocase, Mol. Membrane Biol. 15 (1998) 213-220.