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Review

Roles and regulation of phospholipid scramblases

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ABSTRACT

Phospholipid scramblase activity is involved in the collapse of phospholipid (PL) asymmetry at the plasma membrane leading to externalization of phosphatidylserine. This activity is crucial for initiation of the blood coagulation cascade and for recognition/elimination of apoptotic cells by macrophages. Efforts to identify gene products associated with this activity led to the characterization of PL scramblase (PLSCR) and XKR family members which contribute to phosphatidylserine exposure in response to apoptotic stimuli. Meanwhile, TMEM16 family members were identified to externalize phosphatidylserine in response to elevated calcium in Scott syndrome platelets, which is critical for activation of the coagulation cascade. Herein, we report their mechanisms of gene regulation, molecular functions independent of their scrambling activity, and their potential roles in pathogenic conditions.
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1. Introduction

Phospholipids are essential components of mammalian cell membranes [1]. Specifically, phospholipids are distributed asymmetrically between the inner and outer leaflets of the plasma membrane [1]. Phosphatidylserine (PS), one of the four major phospholipids in the plasma membrane, is located in the inner leaflet along with phosphatidylethanolamine (PE) and phosphatidylinositol (PI), whereas sphingomyelin (SM) and phosphatidylcholine (PC) are enriched in the outer leaflet [1]. This asymmetrical distribution of phospholipids is maintained by specific proteins that regulate the translocation of these phospholipids [1,2]. However, this distribution collapses in response to two major cellular events: blood coagulation and apoptosis. These events involve phospholipid "scrambling" between the inner and outer leaflets of the plasma membrane [1,2]. Transbilayer lipid motion seems to be mediated by distinct molecules [3], including flippases and floppases, which maintain membrane asymmetry via translocation of selective lipids in an ATP-dependent manner, as well as scramblases. Whereas flippases and floppases move phospholipids to the inner and outer leaflet, respectively, scramblases mediate transbilayer movement in a non-selective and energy-independent mechanism.

Injuries that lead to activation of the blood coagulation pathway involve PS externalization in platelets, allowing for interactions of this phospholipid with coagulation factors, and thereby stimulating production of thrombin and, consequently, blood clotting [4]. In apoptotic cells, scramblase activity disrupts phospholipid asymmetry resulting in PS externalization [4]. PS exposure provides a signal for recruitment of macrophages to bind to and engulf apoptotic cells

Abbreviations: AML, acute myelogenous leukemia; ANO, anoctamin; APL, acute promyelocytic leukemia; ATRA, all-*trans*-retinoic acid; BAC, bacterial artificial chromosome; BACE, β-secretase APP cleaving enzyme; ECM1, extracellular matrix protein 1; EGCG, epigallocatechin gallate; EGF, epidermal growth factor; EMCV, encephalomyocarditis virus; EMT, epithelial–mesenchymal transition; ERM, ezrin–radixin–moesin; G-CSF, granulocyte colony stimulating factor; GFP, green fluorescent protein; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HTLV-1, human T cell leukemia virus; IFN, interferon; IL, interleukin; ISGs, interferon-stimulated genes; IP_3R, inositol-1,4,5-triphosphate receptor 1; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LTR, long terminal repeat; MBP, maltose-binding protein; mRFP, monomeric red fluorescent protein; NLS, nuclear localization signal; PKC, protein kinase C; PL, phospholipid; PLSCR, phospholipid scramblase; PS, phosphatidylserine; RBC, red blood cell; RELT, receptors expressed in lymphoid tissues; SCF, stem cell factor; SLE, systemic lupus erythematosus; SLPI, secretory leukocyte protease inhibitor; STAT, signal transducer and activator of transcription; TCGA, The Cancer Genome Atlas; TLR, toll-like receptor; TMEM, transmembrane protein 16; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; VSV, vesicular stomatitis; XKR, XK-related protein

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[4]. Conditions that inhibit flippase and floppase activities activate phospholipid scramblase activity [2,5,6]. Thus, the regulation of the activities of these molecules is essential for regulating plasma membrane phospholipid asymmetry. As described in this review, the specific proteins that are capable of PS externalization at the plasma membrane include PLSCR, TMEM16 (also referred to as anoctamin (ANO)), and the XKR family members.

In red blood cells (RBC), PS externalization is not only important for "eryptosis", a form of programmed cell death activated by calcium (and required for RBC clearance) [7], but is also critical for the process of blood coagulation [2]. For example, the common environmental toxicants, lead and mercury, elevate scramblase and reduce flippase activities to promote PS externalization in RBCs and initiate coagulation events [8].

Interestingly, G-protein-coupled receptors (such as Rhodopsin, β_2-adrenergic, Adenosine A2A receptors) are associated with increased phospholipid scramblase activity, specifically at the ER membrane [9]. For the purpose of this review, we focus on the plasma membrane-described phospholipid scramblases. A detailed discussion of the regulation and molecular functions of these phospholipid scramblases, as well as their potential roles in disease are henceforth described below (See Figs. 1 and 2).

2. Phospholipid scramblase gene products

2.1. Initial identification of human phospholipid scramblase genes

The molecules that elicit scramblase activity had remained unidentified until 1996, when Basse and colleagues reported the purification of a type II transmembrane protein from erythrocytes [10]; when reconstituted into liposomes, this 37 kDa protein could mediate calcium-dependent redistribution of membrane phospholipids leading to PS externalization [10]. The cDNA encoding this protein was isolated via a plaque hybridization technique, and the gene was named *PLSCR1* [11]. PLSCR1 is expressed ubiquitously in multiple cell lines including erythrocytes and blood platelets [11]; these cells were associated with increased phospholipid scramblase activity in the presence of Ca^{+2} [11]. In 2000, Wiedmer and colleagues reported the cloning of the human *PLSCR1* gene from a human genomic bacterial artificial chromosome (BAC) library and estimated the size of its gene to be 29.7 Kb, encompassing 9 exonic regions [12]. Three additional cDNAs with high homology to *PLSCR1* (which encodes for a 318 aa protein) were cloned by a PCR-based approach utilizing a cDNA library, namely *PLSCR2* (which encodes for a 297 aa protein), *PLSCR3* (which encodes for a 295 aa protein) and *PLSCR4* (which encodes for a 329 aa protein) [12]. Another PLSCR family member, named *PLSCR5* (encoding for a 271 aa protein), was identified as a fifth member of the PLSCR family (http://www.genecards.org).

However, following this initial identification of PLSCR proteins as the molecules mediating phospholipid scramblase activity, members of the TMEM16 (i.e. TMEM16F and others) and XKR (i.e. XKR8 and others) family were also identified and reported to be involved in mediating scramblase activity [13,14].

2.2. Gene location and protein structure of human phospholipid scramblases

2.2.1. PLSCR family members

With the exception of PLSCR3, all PLSCR family genes are clustered on the q arm of chromosome 3, specifically at the 3q23/3q24 loci (https://genome.ucsc.edu/, December 2013). Table 1A summarizes the genetic locations and gene sizes of all the PLSCR family members. The *PLSCR1* gene is composed of 9 exons with the coding region beginning at exon 2 and ending at exon 9. Interestingly, an ortholog of human PLSCR1 exists in *Caenorhabditis elegans*, namely *scrm1*, and is involved in PS externalization in apoptotic germ cells [15,16]. In addition, additional orthologs were identified in *Drosophila melanogaster*, *Mus musculus*, *Xenopus laevis*, and *Danio rerio* [17].

Although various alternate splice forms of PLSCR1 have been predicted (http://www.genecards.org), only three alternative forms have been identified experimentally [18], and their functional roles remain to be investigated. All PLSCR family members, with the exception of PLSCR2, possess a proline-rich N-terminal region containing PxxP and PPxY domains, a cysteine-rich region, a conserved calcium ion binding domain (EF-hand-like), and a putative transmembrane region enriched in hydrophobic amino acids. In addition, PLSCR1 contains a nuclear localization signal (NLS) and a DNA binding domain that are essential for its nuclear localization and associated nuclear function [19–24]. Although the NLS domain seems to be conserved in the other family members, it has only thus far been tested in PLSCR4 (minimal NLS domain) [25]. Further, the DNA-binding activity in the other isoforms has yet to be assessed.

PLSCR1 has been proposed to be a type II transmembrane protein that is tail-anchored to the plasma membrane at its C-terminal end [26]. Although recent homology modelling studies for PLSCR1 predicted that its transmembrane domain may not be inserted into membranes [27], fluorescence quenching studies and membrane binding assay studies suggest that the transmembrane domain is inserted into the lipid bilayer and plays an important anchoring

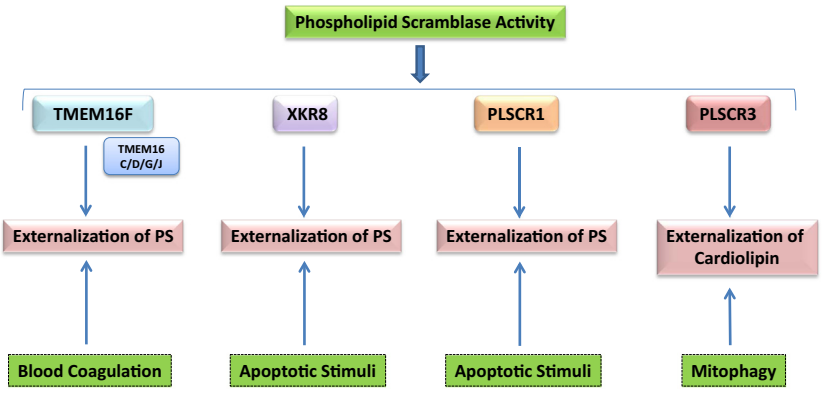

Fig. 1. Identity of Phospholipid Scramblases: a schematic showing the responsible gene products that contribute to scramblase activity including (1) TMEM16F involved in externalizing PS in response to cellular injury/activation (i.e. blood coagulation), (2) XKR8 involved in externalizing PS in response to apoptotic stimuli, (3) PLSCR1 involved in externalizing PS in response to apoptotic stimuli, and (4) PLSCR3 involved in movement of cardiolipin in the mitochondrial compartment important in mitophagy.

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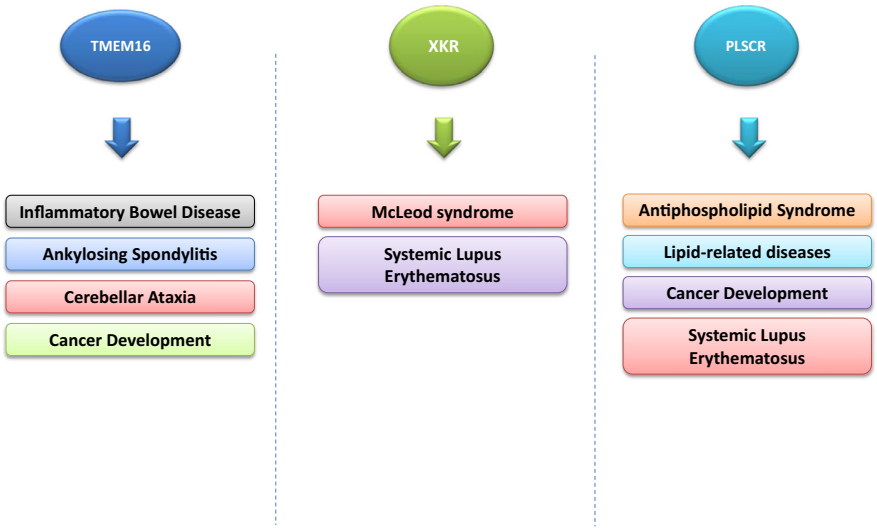

Fig. 2. Phospholipid scramblases in disease: a schematic showing the diseases associated with the phospholipid scramblase members of the (1) TMEM16 family, (2) XKR family and (3) PLSCR family.

Table 1
Chromosomal location of phospholipid scramblases.

Gene	Chromosomal location	Size
A		
PLSCR1	chr3:146,515,180-146,544,841	29,662 bp
PLSCR2	chr3:146,433,288-146,469,301	36,014 bp
PLSCR3	chr17:7,389,728-7,394,843	5116 bp
PLSCR4	chr3:146,192,336-146,251,179	58,844 bp
PLSCR5	chr3:146,576,608-146,606,216	20,379 bp
B		
TMEM16A	chr11:70,085,414-70,163,577	78,164 bp
TMEM16B	chr12:5,562,651-5,946,232	383,582 bp
TMEM16C	chr11:26,332,131-26,663,289	331,159 bp
TMEM16D	chr12:100,794,596-101,128,641	334,046 bp
TMEM16E	chr11:22,193,176-22,283,367	90,192 bp
TMEM16F	chr12:45,215,987-45,432,351	216,365 bp
TMEM16G	chr2:241,188,509-241,196,165	7657 bp
TMEM16H	chr19:17,323,223-17,334,829	11,607 bp
TMEM16J	chr11:417,930-442,011	24,082 bp
TMEM16K	chr3:43,366,326-43,622,068	255,743 bp
C		
XKR1	chrX:37,685,880-37,732,130	46,251 bp
XKR2	chrX:100,913,442-100,928,909	15,468 bp
XKR3	chr22:16,783,416-16,821,694	38,279 bp
XKR4	chr8:55,102,457-55,526,151	423,695 bp
XKR5	chr8:6,808,520-6,835,644	27,125 bp
XKR6	chr8:10,896,146-11,201,366	305,221 bp
XKR7	chr20:31,968,002-31,998,453	30,452 bp
XKR8	chr1: 27,959,993-27,968,093	8101 bp
XKR9	chr8:70,669,365-70,735,942	66,578 bp

role [28]. Moreover, a novel cholesterol recognition motif in the C-terminus of PLSCR1 is predicted to exist within the transmembrane and extracellular domain of PLSCR1 [29]. The cytoplasmic domain of PLSCR1 may also associate with the lipid bilayer when the transmembrane region is deleted; this finding implicates a role for the cytosolic region in membrane anchoring [30].

2.2.2. TMEM16 family members

There are 10 TMEM16 family members and their genetic locations and gene sizes are summarized in Table 1B. Splice variants for TMEM16A and TMEM16B, as well as for TMEM16F have been identified [31]. Thus far, two *C. elegans* orthologs for TMEM16 have been identified: *ANOH-1* and *ANOH-2* which may be involved in sensory signaling [32]. Additionally, orthologs have been identified in *D. melanogaster* [33], *M. musculus* [34], *Aspergillus fumigatus* [35], and *Nectria haematococca* [36].

TMEM16F, initially identified to be associated with phospholipid scramblase activity [14], consists of 20 exons and encodes a protein of 910 aa that contains 8 transmembrane domains [37]. Its N-terminal region (1–95 aa) and its C-terminal region (842–910 aa) are required for its plasma membrane localization [38]. Mutational analyses of the putative pore-loop region between the 5th and 6th transmembrane helices of TMEM16 family members indicate that this region is essential for the chloride channel (TMEM16A) and phospholipid scramblase (TMEM16F) activities [38]. Interestingly, TMEM16A can form homo- as well as hetero-oligomers with other TMEM16 family members [39]. Recently, the X-ray crystal structure of nhTMEM16 (*N. haematococca*), reveals a hydrophilic cavity that traverses the membrane and contains a conserved calcium binding site embedded in the hydrophobic membrane core [36].

2.2.3. XKR family members

There are 9 human XKR family members and 8 XKR murine family members. The genomic locations and gene sizes of the human XKR family members are summarized in Table 1C. The orthologs of XKR8 in *C. elegans (CED-8)* and *M. musculus (mXKR8)* also promote PS externalization [13,40].

XKR8, initially identified to be associated with phospholipid scramblase activity [13], contains 3 exons, with the translation initiation site located within exon 1 (http://useast.ensembl.org/). The three exons of *XKR8* encode a 395 aa protein that contains 6 transmembrane segments. Both the N and C-termini are cytoplasmic in orientation [13]. XKR8 contains a caspase-3 binding site that, when mutated, abolishes its PS externalization ability [13,40,41].

3. Expression, regulation, and localization of phospholipid scramblases

3.1. Expression and transcriptional regulation

3.1.1. PLSCR family

Northern blot analyses of human tissues show ubiquitous expression of PLSCR1, PLSCR3, and PLSCR4 isoforms in multiple tissues tested, whereas PLSCR2 expression seems to be restricted to testis [12]. On the basis of their expression pattern, PLSCR family members may play a role in development of embryos/neonates, as

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PLSCR3 and PLSCR4, but not PLSCR1 and PLSCR2, are expressed in the endometrium and muscle layers of near-term rat uterine tissues [42]. Further, treatment of murine uterine tissue with lipopolysaccharide (LPS) induced mRNA and protein expression of all PLSCR family members with the exception of PLSCR4, which was reduced [43]. PLSCR4 expression correlates with total number of newborns, as well as number of pups born alive in a porcine bead-chip investigation, and this suggests a role for PLSCR4 in uterine functions [44]. Moreover, expression of PLSCR4 (and genes involved in lipid/cholesterol metabolism) is down-regulated in high-fat diet-receiving mice following treatment with Korean red ginseng extract, implicating this PLSCR family member in obesity [45]. PLSCR levels were also altered following induction of the acute innate immune response (induced by LPS, zymosan, and turpentine) in mice [46]; in cultured HepB3 hepatocytes (as well as 3T3-L1 adipocytes), PLSCR1 mRNA levels were increased following treatment with inflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukins (IL-1β and IL-6), as well as interferon (IFN)-γ) [46]. PLSCR1 expression is also upregulated in response to growth factors (i.e. epidermal growth factor (EGF) [47], stem cell factor (SCF) [48], and granulocyte colony stimulating factor (G-CSF) [48]) and by all-*trans*-retinoic acid (ATRA, a differentiation-inducing agent) which is mediated by PKC-δ activation in acute promyelocytic leukemia (NB4) and HL60 cells [49]. In MCF-7 breast cancer cells, PLSCR2 protein levels were down-regulated following γ-irradiation [50].

PLSCR1 was identified as one of the most potently induced interferon-stimulated genes (ISGs) by differential oligonucleotide array screening in the fibrosarcoma HT1080 cell line in response to Type I and II interferons (i.e. IFN-α, IFN-β, and IFN-γ) [51]. The PLSCR1 genomic sequence showed three possible IFN-regulated binding sites within the first 4 kb of 5′ flanking sequence, including a single consensus interferon-stimulated response element (ISRE) in its untranslated exon 1 that is essential for its induction [12,52]. Transcriptional activation of PLSCR1 by IFN-α required activation of Protein Kinase C (PKC)-δ, (Jun Kinase) JNK, and Signal Transducer and Activator of Transcription (STAT) 1 [53]. Recent studies implicate Snail, a transcriptional regulator of epithelial-mesenchymal transition (EMT), as a direct regulator of PLSCR1 transcription in IMR-32 and HEK-293 cell lines [54].

3.1.2. TMEM16 family

In mice, TMEM16 family members display differential expression depending on the developmental stage [34,55,56]. Notably, TMEM16A and TMEM16F-TMEM16J have epithelial expression, whereas TMEM16B-TMEM16E have neuronal-, skeletal-, and muscle-specific expression [34,55,56]. Additionally, TMEM16A is also expressed in Cajal interstitial cells in the smooth muscle of gut; indeed, TMEM16A null mice lack movement of their gastrointestinal muscles [57]. Decreased expression of TMEM16A in these cells has also been described in diabetic patients who have gastroparesis [58]. TMEM16A is also expressed in airway smooth muscle cells implicating this protein in tracheal development and may also be implicated in asthma development [59]. TMEM16B may play roles in the sensory signalling as it is localized to olfactory sensory neurons [60] and in photoreceptor synaptic terminals [61]. TMEM16F is expressed in a number of cells, including hematopoietic cells (involved in blood coagulation) [62] and osteoblasts (involved in bone matrix production) [63]). Mechanisms underlying the regulation of TMEM16 gene expression have yet to be investigated.

3.1.3. XKR family

Murine *Xkr8* mRNA is ubiquitously expressed (with high expression in testis), whereas murine XKR4 and XKR9 are tissue specific [13,41]. In detail, murine XKR4 has the strongest expression in brain (with weak expression in spleen, thymus, uterus, blood vessels, and fetus), whereas XKR9 has the strongest expression in the small intestine (with weak expression in pancreas, liver, stomach and colon) [41]. Interestingly, methylation of CpG islands in the XKR8 promoter region in cancer cell lines hindered mRNA transcription of XKR8 and thus contributed to diminished phospholipid scramblase activity [13]. Mechanisms underlying transcriptional regulation of XKR expression remain unclear.

3.2. Post-translational modifications

Post-translational regulation of scramblases is, thus far, mainly reported to occur via phosphorylation. These phosphorylation events are regulated by several tyrosine kinases. EGF treatment rapidly promotes PLSCR1 tyrosine phosphorylation (Y69/Y74) mediated by c-Src [64]. c-Abl is another tyrosine kinase that can contribute to tyrosine phosphorylation of PLSCR1 [65]. By contrast, c-Src and c-Abl were both unable to tyrosine phosphorylate PLSCR2, PLSCR3, or PLSCR4 [64,65]. Additionally, activation of the IgE receptor leads to tyrosine phosphorylation of PLSCR1 in rat mast cells [66,67]; this PLSCR1 phosphorylation event is mediated in a cooperative fashion by src family tyrosine kinase members, Lyn and Fyn, as well as the FcRγ chain [68]. In addition, PLSCR3 is threonine phosphorylated (T21) by PKC-δ [69], and this phosphorylation event is antagonized by heat shock protein 27 (HSP27), which disrupts the physical interaction of PLSCR3 with PKC-δ [70]). PLSCR3 phosphorylation by PKC-δ promotes cardiolipin translocation across the mitochondrial membrane, thereby modulating apoptosis [71]. Although Frasch and colleagues reported that PLSCR1 could be phosphorylated by PKC-δ on threonine residues (Thr-161) [72], phosphorylation of purified PLSCR1 from erythrocytes by PKC-δ was undetectable in an *in vitro* phosphorylation assay [49]. In addition to phosphorylation, PLSCR family members also undergo palmitoylation (described in detail in Section 3.3).

Based on *in silico* analyses, TMEM16F might be ubiquitinated at Lys876/Lys888, whereas Asn329, Asn361, Asn493, Asn790 and Asn802 have been predicted as potential glycosylation sites [73]. A potential sumoylation site has been identified in TMEM16A and TMEM16F; however, its contribution to protein stability has yet to be assessed [38]. Although putative phosphorylation sites have been identified for TMEM16A and TMEM16B including PKC sites [74], experimental validation is needed. With respect to post-translational modifications of XKR8, its proteolytic processing by caspase-3 leads to its activation [13,40]. Other XKR family members (such as XKR4 and XKR9) also contain a caspase-cleavage site at their C-termini [41]. Cleavage of these proteins is needed for them to elicit functionality [13,41].

Collectively, further investigations into the post-translational modifications of all of the phospholipid scramblase family members would provide additional information with respect to their mechanisms of regulation.

3.3. Subcellular localization

3.3.1. PLSCR family

PLSCR1 contains structural domains that might regulate its subcellular localization: DNA binding domain (86–118 aa) [23], cysteine-rich palmitoylation site (^{184}CCCPCC^{189}) [75,76], and an atypical nuclear localization signal (NLS, 257–266 aa) [19,77].

Similar to other palmitoylated proteins, PLSCR1 localizes to the plasma membrane specifically to the low density membrane microdomain fraction (lipid rafts/caveolae). In support of this observation, increased PLSCR1 and phospholipid scramblase activity during the maturation process of chondrocytes results in PS enrichment in the outer leaflet of lipid-rich microdomains [78]. In addition, PLSCR colocalizes with EGFR in KB cells [47]. Interestingly, stimulation with EGF leads to internalization of

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plasma-membrane localized PLSCR1 into endocytic vesicles [47]; in contrast to EGF receptor (EGFR) which is degraded in lysosomes, PLSCR1 recycles back to the plasma membrane [47]. Moreover, PLSCR1 co-localizes with the tyrosine kinases Lyn and Syk (downstream components of the IgE receptor signaling cascade) at the lipid rafts of non-activated mast cells [79], and has been also found in the lipid rafts of fMLP (formylated Met-Leu-Phe)-activated neutrophils [80]. Interestingly, lipid rafts seem to be required for the secretion of PLSCR1 into the extracellular matrix (ECM) in HaCaT human keratinocyte cells, and this event is essential for the interaction of PLSCR1 with the extracellular matrix protein 1 (ECM1) [81]. PLSCR1 is also found in other sites including the Golgi and endosomal compartments [82,83].

The plasma membrane localization of PLSCR1 can be disrupted by cellular treatment with 2-bromopalmitate (a general inhibitor of palmitoylation) or via mutagenesis of the palmitoylation sites in specific cell types [75] leading to its nuclear localization. Nuclear localization of PLSCR1 can also be induced by IFN treatment of certain cell lines (i.e. HEY1B and SVT2 fibroblasts) [75]. Nuclear import of PLSCR1 requires its atypical NLS (^{257}GKISKHWTGI^{266}, 257–266 aa), and the activity of the importin α/β nuclear receptor pathway [19,20]. In the nuclear compartment, PLSCR1 regulates the transcription of IP_3R [23] and may contribute to DNA replication via its physical interaction with topoisomerase [22]. Nuclear PLSCR1 is involved in the production of mature neutrophils from myeloid progenitors following exposure to G-CSF [21], although the underlying mechanism is unclear. In addition, PLSCR1 regulates angiogenin-enhanced rRNA transcription in the nucleus [24].

PLSCR4 is also localized at the plasma membrane [84], whereas PLSCR3 is enriched in the mitochondrial membrane [85]. PLSCR3 has been experimentally shown to be palmitoylated using ^{3}H-palmitate labelling [85]. For example, mutagenesis of the cysteine residues of PLSCR3 critical for its palmitoylation led to its redistribution to the nuclear compartment [85]. Furthermore, secretion of PLSCR3 in exosomes was inhibited by 2-bromopalmitate [86]. NLS signals are also found in PLSCR2, PLSCR3, and PLSCR4. PLSCR2 is predominantly nuclear localized when stably expressed in Chinese hamster ovary K1 cell line [87], although its nuclear function remains to be investigated. Mutation of the PLSCR4 minimal nuclear localization sequence (^{273}GSIIRKWN^{280}) disrupts its localization to the nuclear compartment [25].

3.3.2. TMEM16 family

Murine TMEM16F was found to localize to the plasma membrane, when expressed as a monomeric red fluorescent protein (mRFP) conjugate in 293T cells [38]. However, another independent report presented evidence for a cytoplasmic localization of TMEM16F [88]. Following tagging with C-terminal GFP, several other TMEM16 family members were reported not to reach the plasma membrane, but rather were retained intracellularly in HEK293, CHO, and COS-7 cells [88]. In detail, TMEM16A has been found to localize to the plasma membrane, whereas GFP-conjugated TMEM16C-TMEM16G and TMEM16J localized in the endoplasmic reticulum; this differential localization pattern may associate with the differential ability of these proteins to generate calcium-activated Cl^- currents [88]. As protein overexpression (and tagging proteins with fluorescent proteins) might result in artefacts, investigation of the endogenous expression of these family members is still needed. Thus far, the endogenous localization of TMEM16G was assessed in human prostate tissue, where TMEM16G was found to be enriched in its epithelium, and localized mainly to intracellular regions [88].

3.3.3. XKR family

According to Genecards (http://www.genecards.org), XKR8 has been suggested to be predominantly localized at the plasma membrane, and this would correspond to its role as a phospholipid scramblase mediating PS externalization in apoptotic cells [41]. Indeed, when expressed as GFP-fusion proteins, murine XKR-family members (with the exception of XKR2) were found to localize to the plasma membrane [41]. However, only XKR4, XKR8, and XKR9 promote PS externalization in response to apoptotic stimuli [41]. Truncated GFP-fusion proteins of XKR8 and XKR9 (which lack the C-terminal caspase-cleavage site) were mislocalized to the endoplasmic reticulum. Thus, the dibasic/diaromatic amino acids that are located at the C-terminal of the caspase cleavage site may contain an ER export signal [41]. By contrast, cleaved XKR4 was not mistargeted suggesting that the C-terminal segment (after the caspase cleavage site) is not essential for its appropriate subcellular localization [41].

3.4. Molecular functions of phospholipid scramblases

3.4.1. PLSCR family

With regards to the protein interactome for PLSCR family members, several targets have thus far been identified which may implicate PLSCR in regulating their function. For example, PLSCR1 interacts with toll-like receptor 9 (TLR9) in endosomal compartments to regulate TLR9-mediated production of IFN-α [83]. TLRs contribute to innate immune system cellular responses by functioning as receptors recognizing foreign and pathogenic nucleic acids/proteins that enter cells [89–92]. Although the detailed mechanism by which PLSCR1 modulates the TLR signaling and innate immune responses is presently unclear, these observations provide a link between PLSCR1 regulation and innate immune responses. PLSCR1 has also been shown to interact with EGFR and Shc-A, an adaptor molecule in EGF stimulated A431 epidermoid carcinoma cells [64]. PLSCR1 interaction with Shc-A requires Src-mediated phosphorylation on tyrosines 69 and 74 in the N-terminal region of PLSCR1 [64]. In T cells, PLSCR1 and PLSCR4 interact with plasma membrane localized CD4 receptor which is regulated by the presence of the secretory leukocyte protease inhibitor (SLPI), which has anti-viral activity against human immunodeficiency virus 1 (HIV-1) [84]. These results suggest a potential role of PLSCR1 in receptor-mediated signaling pathways and receptor internalization. In neutrophils, PLSCR1 was shown to interact with Proteinase 3 (PR3), which is a target of autoantibodies in Wegener granulomatosis [93]. In SH-SY5Y human neuroblastoma cells, PLSCR1 interacts with β-secretase APP cleaving enzyme (BACE), a membrane proteinase enzyme that regulates the production of amyloid β-peptide molecules (a characteristic feature of Alzheimer's disease) in the Golgi and endosomal compartments [82]. Thus, PLSCR1 may regulate the intracellular distribution of BACE and potentially implicates a role for PLSCR1 in Alzheimer's disease pathophysiology. In myeloid cells, PLSCR1 binds to onzin, a negative transcriptional regulatory target of c-Myc which regulates cell proliferation [94]. This finding potentially implicates PLSCR1 in cancer cell survival and proliferation. Furthermore, PLSCR1 interacts with receptors expressed in lymphoid tissues (RELT), and this interaction leads to phosphorylation of PLSCR1 by OSR1, an oxidative stress response kinase protein [95]; this finding may implicate PLSCR1 in oxidative stress response pathways. As described earlier, PLSCR1 is secreted into the ECM via a lipid raft-dependent pathway thus promoting its interaction with ECM1; this suggests a potential role for PLSCR1 in epidermal differentiation mediated by ECM1 [81]. In addition, PLSCR1 is an angiogenin-interacting protein in the nuclear compartment to modulate rRNA transcription [24].

Since PLSCR1 is highly inducible by IFN-α, IFN-β, and IFN-γ [51], the role of PLSCR1 in mediating antiviral activities has recently come under much investigation. Cells with reduced expression of PLSCR1 (via siRNA or fibroblasts from PLSCR1$^{-/-}$ knockout mice)

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contained increased viral titers of vesicular stomatitis (VSV) and encephalomyocarditis (EMCV) viruses [96]. In HepG2 and Huh7 cell lines (as well as in mice), expression of PLSCR1 affects Hepatitis B virus (HBV) replication (reduction of viral proteins, RNAs, and DNA replicative intermediates) [97]. Although PLSCR1 was upregulated via IFN-α and IFN-γ to oppose hepatitis C virus (HCV) replication [98], PLSCR1 was also reported to promote viral entry of HCV [99]. In HEK293T and Huh7.5.1 cells, PLSCR1 interacts with the E1 and E1 HCV envelope proteins as well as other entry factors (i.e. OCLN) to coordinate HCV infection [99]. Further studies of PLSCR1 expression and its effects on viral infection, however, continue to support the role of PLSCR1 in mediating antiviral activity. For instance, PLSCR1 interacts with and regulates the transactivation of HTLV-1 tax, a protein associated with the transcription of the Human T-cell Leukemia Virus type-1 provirus [100]. In a similar study, PLSCR1 was found to interact with HIV-1 Tat, a protein essential to the transcription of the Human Immunodeficiency Virus type-1 long terminal repeat (LTR) [101]. Collectively, these studies implicate PLSCR1 in viral responses in which it can both assist in the viral infection and inhibit viral replication and propagation. Interestingly, IFN-α stimulates expression of PLSCR1 in order to protect cells from staphylococcal α-toxin produced during *S. aureus* infection [102,103]. Knockdown of PLSCR1 by shRNA supports a protective role for PLSCR1 following α-toxin exposure [102,103].

Although there is controversy surrounding the role of PLSCR family members in mediating PS externalization, there is evidence demonstrating PLSCR contribution to this function. For example, *scrm-1* (*C. elegans* ortholog of human PLSCR1) mediates PS externalization in apoptotic cells [16]. In addition to PLSCR1-mediated apoptotic events, PLSCR3 is another PLSCR family member that contributes to apoptosis induced by UV and tBid [104]. The PLSCR3 isoform markedly increases TNF-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial apoptotic pathway [105]. During PKC-δ -induced apoptosis, PLSCR3 is phosphorylated at Thr-161 [69,106]. The phosphorylation of PLSCR3 by PKC-δ is also an early event in apoptotic cells treated with N-benzyladriamycin-14-valerate (AD198) [107].

Recent studies on hybrid mapping of yeast and human interaction networks identified PLSCR1 as one of the interaction partners of ATG12, an ubiquitin-like protein that helps in the elongation step of autophagosome formation [108]. These findings suggest a potentially important role for PLSCR1 in the process of autophagosome formation. In addition, LC3-II (via western analysis) and GFP-LC3 punctae (via immunofluorescence) levels were altered following PLSCR1 knockdown in ovarian cancer cells treated with arsenic trioxide [109]. However, the exact mechanism by which PLSCR1 contributes to autophagy requires further investigation. PLSCR3 is also implicated in regulating pro-death autophagy; in particular, PLSCR3 is involved in externalizing cardiolipin, an event important in targeting the damaged mitochondria to autophagosomes/lysosomes during the process of mitophagy (a type of autophagy that specifically targets the damaged mitochondria for degradation) [71].

3.4.2. TMEM16 family

Ist2, a yeast homolog of TMEM16 ion channels, has been described as an endoplasmic reticulum-plasma membrane (ER-PM) tethering proteins and is localized at these junctional sites in yeast cells [110]. Deletion of Ist2 (as well as other tethering proteins such as tricalbins and Scs2/Scs22) promotes detachment of the ER from the PM [110]. The ER-PM attachment sites are important in calcium and PI signaling at the plasma membrane [110]. In addition to its suggested phospholipid scramblase activity, TMEM16F is a critical factor in the outwardly rectifying Cl^- channel (ORCC) [111], and may play a role in promoting chloride currents in response to FasL-activation in Jurkat cells [112] and staurospaurine mediated ORCC in epithelial cells [111]. Indeed, this activity is absent in TMEM16F$^{-/-}$ cells [113]. There also are conflicting reports of TMEM16F as a Cl^- channel [31,113,114]. Nonetheless, mutation of Q559 to a lysine in TMEM16F increased its Cl^- channel activity [113]. Mutation at E670Q in TMEM16F downregulated its plasma membrane expression while mutation at E667Q markedly decreased the calcium-sensitivity of its channel activity in the absence of any changes in plasma membrane localization [113,115].

With respect to TMEM16 family members, TMEM16A forms both homo- and heterodimers with TMEM16B but not TMEM16F [74,116]. The cellular role for formation of these oligomers remains unclear. A proteomics approach was implemented to identify TMEM16A binding proteins; 73 proteins were increased in their screen by >3-fold including SNARE proteins as well as the ezrin–radixin–moesin (ERM) scaffolding complex [74,116]. Downregulation of these targets via knockdown methodology could modulate Cl^- channel activity of TMEM16A [74,116]. In addition, the IP_3R1 (inositol 1,4,5-triphosphate receptor 1) was identified to bind to TMEM16A [74,117].

3.4.3. XKR family

Cleaved XKR4 (with highest expression in brain) may be involved in functions of the synapse, axons, and dendrites by externalization of PS which may lead to remodelling of the "neural network" [41]. In small intestinal epithelial cells, cleaved XKR9 may promote migration via the process of "shedding" in which PS externalization may be involved [41].

4. PLSCR, TMEM16, and XKR-associated scramblase activities

When erythrocyte-purified PLSCR1 was reconstituted into liposomes, rapid bidirectional movement occurred in a similar manner to that in plasma membranes following Ca^{+2} mobilization [10]. Similar results were obtained with purified recombinant PLSCR1 protein fused to maltose-binding protein (MBP) reconstituted in liposomes [11] as well as with PLSCR1 fused to green fluorescent protein (GFP) expressed in Burkitt's lymphoma Raji cells treated with A23187, a calcium ionophore [118]. The calcium binding site in PLSCR1 (273–284 aa) is required for mediating calcium-induced phospholipid scrambling activity [119].

With respect to other PLSCR family members, mutations in the calcium-binding site of PLSCR4 markedly reduces its Ca^{+2} and Mg^{+2}-induced scramblase activity [120]. PLSCR3, which is localized in mitochondrial membranes, externalizes cardiolipin from the inner to the outer mitochondrial membrane [121]. By contrast, PLSCR2 lacks scramblase activity; this was attributed to the absence of the proline-rich N-terminal region that is present in the other PLSCR family members [122]. Indeed, when fused to the proline-rich domain of PLSCR1, PLSCR2 is capable of calcium-dependent scrambling activity [122]. Although the above studies implicate PLSCRs in the redistribution of phospholipids between membrane leaflets, there may be other factors contributing to PS externalization in response to apoptotic stimuli [123]. PLSCR1 expression does not always correlate with PS exposure during apoptosis [123]. Indeed, PLSCR1$^{-/-}$ mice were fully proficient in mediating scramblase activity (with no apparent defect in coagulation) suggesting that PLSCR1 may not be essential for mediating PS exposure [48]. As PLSCR3 and PLSCR4 also seem to contribute to scrambling activity [120,121], these isoforms may overcome the loss of PLSCR1 by eliciting redundant scramblase activity that enables PS externalization in PLSCR1$^{-/-}$ mice. However, PLSCR1$^{-/-}$ and PLSCR3$^{-/-}$ double knockout mice still retain scramblase activity [124]. In addition, PLSCR1 protein was normal in patients with Scott syndrome, a severe bleeding disorder that is characterized by

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a lack of blood coagulant activity [125], PS exposure in platelets, red cells, and lymphocytes resulting in a defective blood coagulation process [126] that is now attributed to TMEM16F loss of function mutations [14]. Another factor that results in PS exposure is *wah-1* (apoptosis inducing factor) which interacts with *scrm-1* to promote PS exposure [15]; phospholipid scramblase activity is elevated when these two proteins interact [15]. Interestingly, treatment with an anti-phospholipid scramblase 1 antibody (NP1) increased apoptosis in colorectal cancer cells [127].

Recently, TMEM16F, a calcium-dependent chloride channel was identified as the factor mutated in Scott syndrome platelets that leads to scramblase deficiency [14]. Further, TMEM16F knockout mice (TMEM16F$^{-/-}$) displayed excessive bleeding defects and defective lipid scrambling activity in platelets [113], whereas another TMEM16F-knockout model had severe bone abnormalities with defective phospholipid scrambling in their osteoblasts in the absence of excessive bleeding defects (i.e. which may be expected in females after giving birth) [63]. Even with this family of proteins, there is controversy regarding whether TMEM16F elicits scramblase activity or is a membrane channel regulating scramblase activity. In some studies TMEM16F overexpression failed to promote calcium-dependent PS externalization, whereas other reports support a role for TMEM16F as a scramblase [14,62,113,128]. Therefore, it has been suggested that TMEM16F may function as a channel which regulates a scramblase protein [14,62,113,128]. Recently, this was addressed using a biochemically pure protein from *A. fumigatus* (afTMEM16, a calcium-gate channel and a calcium-dependent scramblase) whose scramblase activity was assessed directly in a liposomal reconstitution system [35]. Compared to TMEM16A and Ist2p (a *Saccharomyces cerevisiae* protein ortholog involved in salt tolerance [129]), only afTMEM16 directly exerted scramblase activity [35]. Some other TMEM family members including TMEM16C, TMEM16D, TMEM16F, TMEM16G, and TMEM16J are capable of scrambling lipids with some differences in their specificity [62]. However, TMEM16A and TMEM16B are Cl^- anion channels and are not associated with scramblase activity [62]. Mutational study of the pore region in both TMEM16A and TMEM16F (between the 5th and 6th transmembrane region) is critical for these proteins to elicit Cl^- channel and phospholipid scramblase activity, respectively [38]. Domain swapping studies were performed in which specific regions between TMEM16A and TMEM16F were exchanged leading to the elimination of Cl^- channel and scramblase activity, respectively [38]. The active sites therefore are likely to be quite different; this conclusion was also obtained by inhibitor studies (drugs targeting TMEM16A but not TMEM16F and vice versa) [38]. For example, tannic acid and epigallocatechin gallate (EGCG) have antithrombotic activities and inhibit TMEM16F scramblase activity. Both molecules are dependent on calcium although no EF hand has been identified [38].

As TMEM16F$^{-/-}$ cells exhibit normal levels of PS externalization in response to apoptotic stimuli, other factors are probably responsible for PS externalization under apoptotic conditions. Interestingly, XKR8, when activated by caspase-3 (an important mediator in the apoptotic signalling cascade), was shown to be responsible for externalizing PS in response to apoptotic stimuli; this was demonstrated using XKR8$^{-/-}$ murine and human cancer cells treated with XKR8 siRNA [13,40]. XKR8-deficient immortalized fetal thymocytes (IFETs) and murine embryonic fibroblasts (MEFs) (both cell types have low to undetectable levels of XKR4 and XKR9) did not externalize PS in response to apoptotic stimuli [41]. Additionally, Jurkat and Namalwa cells, which can both externalize PS, have high levels of XKR8 and XKR9 (but lack XKR4), whereas PLB-985 and Raji do not externalize PS and completely lack detectable expression of any XKR family members [41]. When murine XKR8$^{-/-}$ IFETs were reconstituted with XKR8 and XKR9, they regained the ability to externalize PS in response to apoptotic stimuli [41]. In addition, UV-irradiated PLB-985 cells overexpressing XKR8 elicited a similar PS response [41]. Interestingly, although C-terminal deletion variants of XKR family members, specifically XKR8 (at Asp-354) and XKR9 (at Asp-357), prevented PS exposure in apoptotic cells, apoptotic cells expressing truncated XKR4 (at Asp-564) still externalized PS in response to staurospaurine, an apoptotic stimuli [41]. From extensive mutational analyses, E141 and S184 seem to be required for the ability of XKR8 and *CED-8* to externalize PS in response to apoptotic stimuli [41]. However, when *CED-8* was expressed in murine cells, they failed to promote PS externalization, suggesting that another factor mediates scramblase activity in *C. elegans* [41]. Thus, it is presently unclear whether XKR4, XKR8, and XKR9 are direct mediators of phospholipid scramblase activity or work collaboratively with another protein to regulate this activity [41]. An *in vitro* liposomal reconstitution assay has yet to be performed to directly assess phospholipid scramblase activity of XKR family members.

5. Phospholipid scramblases in human diseases

5.1. PLSCR

5.1.1. Autoimmunity and inflammation

Approximately 10% of patients with systemic lupus erythematosus (SLE) exhibit an unusual increase in blood coagulation [18]; in the monocytes of these patients, the expression level of the full-length PLSCR1, as well as of the three PLSCR1 splice variants was found to be increased, along with elevated PS externalization [18]. Although the significance of these findings remains to be investigated, this observation implicates PLSCR1 in the pathophysiology of SLE. It is interesting to note that all three splice variants had an intact calcium binding (EF-hand-like) domain, which contributes to PLSCR1 scramblase activity [18]. In addition, PLSCR1 expression was elevated in monocytes isolated from patients with anti-phospholipid syndrome [130]. In this autoimmune disease, the recognition of externalized PS by anti-phospholipid antibodies leads to damaged cell membranes and thus increased blood clots and uncontrolled thrombin production [130].

5.1.2. Cancer development

PLSCR1 mRNA expression was markedly increased upon treatment with ATRA, a chemotherapeutic agent used in treatment of acute promyelocytic leukemia (APL) [49]. Further, reduced PLSCR1 expression significantly suppressed the differentiation of APL cells implicating PLSCR1 as an anti-leukemic molecule [49,131]. Studies in acute myelogenous leukemia (AML) patients revealed that elevated PLSCR1 mRNA expression correlated with increased survival [132]. In addition, PLSCR1 expression and its localization were altered when AML cells were treated with wogonoside, a Chinese herbal medicine used to treat blood-related malignancies [133]. PLSCR1 expression could also inhibit U937 myeloid leukemia cell proliferation by modulating the expression of pro-survival and anti-apoptotic proteins [133]. These above-described observations implicate PLSCR1 as a tumor suppressor in leukemia development. A similar function is attributed to PLSCR1 in ovarian cancer cells wherein murine xenograft (HEY1B ovarian cancer cells expressing PLSCR1) growth suppressed tumor development, although there was no observed functional defect observed *in vitro* [134].

Nevertheless, accumulating evidence also supports PLSCR1 as an oncogenic molecule in ovarian [109], colorectal [135,136], and metastatic liver cancers [137]. PLSCR1 was not highly elevated in various colorectal cancer (CRC) patient tissues but antibody or siRNA mediated downregulation of PLSCR1 in colorectal and liver cancers dramatically reduced *in vivo* tumorigenesis [135–137]. Although PLSCR1 suppresses tumor development in a mouse

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xenograft model, PLSCR1 is highly expressed in ovarian carcinoma cells (and cancer specimens based on TCGA analyses [109]) in comparison to normal T80 ovarian cells [109]. As discussed earlier, Snail plays an important role in tumor progression and is upregulated in several cancers [138]. Since Snail modulates PLSCR1 expression [54], this may be one mechanism involved in the transcriptional regulation of PLSCR1 in cancer.

5.1.3. Lipid-related diseases

Overexpression of PLSCR3 through mRNA upregulation leads to increased cardiolipin production, primarily via upregulation of cardiolipin synthase activity (as opposed to phosphatidylglycerolphosphate synthase) [121]. Furthermore, there was a notable increase in ER monolysocardiolipin acyltransferase activity (including its mRNA levels) in the absence of changes in the decarboxylation of PS to PE in the mitochondria [121]. Interestingly, PLSCR3 is expressed to a high level in fat and muscle [124]. Knockout PLSCR3$^{-/-}$ mice have a marked accumulation of abdominal fat; they also were intolerant for glucose and resistant to insulin with abnormal amounts of lipids in their blood (cholesterol, triacylglycerides, fatty acids, and leptin) [124]. Studies using isolated adipocytes and macrophages from the bone-marrow of PLSCR3$^{-/-}$ knockout mice indicated an abnormality in the levels of neutral lipids and resistance to insulin presentation [124]. Metabolic profiling in PLSCR3$^{-/-}$ (as well as PLSCR1$^{-/-}$/PLSCR3$^{-/-}$ double knockout) mice led to the identification of 19 metabolites that were regulated by these phospholipid scramblases, specifically the upregulation of pro-inflammatory lipids and genes involved in lipid metabolism in adipose tissues [124]. These observations suggest that the loss of PLSCR1 may lead to the development of lipid-related diseases.

5.2. TMEM16 family

Aside from mutations in Scott syndrome patients [14,37], polymorphisms within TMEM16F have been associated with inflammatory bowel disease, a complication of the autoimmune disease ankylosing spondylitis (observed in a Taiwanese population) [139]. The mechanism by which TMEM16F contributes to the development of this disease remains unclear and requires further investigation. Although TMEM16K mutations have been shown to lead to adult-onset cerebellar ataxia and coenzyme Q10 deficiency in muscle [140], TMEM16E missense mutations occur in patients with gnathodiaphyseal dysplasia (a disease characterized by skeletal defects) [141,142], and TMEM16C mutations are linked to autosomal dominant disease, craniocervical dystonia [143], the mechanisms by which these occur and their contribution to disease progression remains unclear.

Dysregulated expression of TMEM16 family members in cancer has also been described. For instance, TMEM16A (the original identified member of this large family of transmembrane proteins), is amplified at chromosome 11q13 locus [73,144] in a subset of cancers [88,145,146] while TMEM16G is dysregulated in prostate cancer [147]. It is presently unclear whether these changes are a cause or an effect of cancer initiation or evolution. Interestingly, from an exon-array analysis, the splicing pattern of TMEM16F is altered in metastatic breast cancers [148]. The functional consequences of the expressed altered splice variants of TMEM16F require further investigation. Interestingly, TMEM16F (and TMEM16A) have been shown to modulate cellular motility and thus, may be involved in promoting cancer aggressiveness [149].

5.3. XKR family

XKR mutations are responsible for the McLeod syndrome, an X-linked genetic disorder characterized by muscular dystrophy, cardiomyopathy, with multiple other defects including those of haematological origin [150]. However, the mechanism by which XKR leads to these functional defects remains unclear. Polymorphisms in the XKR4 gene seem to be associated with the effect of the dopamine iloperidone antagonist (used in schizophrenia treatment) [151]; this finding may provide important information with respect to patient response to this drug. Meanwhile, a polymorphism in XKR6 was correlated with lupus nephritis which is a defect in patients with SLE [152] which is characterized by the immune system attacking the cells and tissues of the host, thereby leading to inflammation in various organs of the body. However, the role of XKR6 in the development of this disease yet remains unclear.

With respect to cancer initiation and evolution, XKR members have thus far only been studied in cancer cell lines in terms of their ability to externalize PS under apoptotic conditions [13]. Whether XKR family members are dysregulated in specific cancers has yet to be investigated. Furthermore, it is unclear whether the ability of XKR to modulate PS externalization can alter cancer development or response to chemotherapeutic agents.

6. Conclusions and perspectives

PLSCR, TMEM16, and XKR family members have been implicated in the plasma membrane externalization of PS. The process of phospholipid scrambling between the two leaflets of plasma membrane is critical in cellular events such as blood coagulation and apoptosis. Earlier studies have implicated phospholipid scramblase (PLSCR) family members in this process. However, later studies in Scott Syndrome patients revealed that PLSCR members may not be responsible for the PS externalization during blood coagulation. This activity has now been ascribed to TMEM16F (and some additional family members). Furthermore, since scrambling activity remained unaltered in knockout mice with a deletion in PLSCR1 (and/or PLSCR3), there may be redundant function for various members of the PLSCR family. It remains to be investigated whether combinatorial deletion in mice of all known PLSCR members would alter scrambling activity. Nonetheless, XKR8 (and other XKR family members) has recently been implicated in PS externalization in response to apoptotic stimuli. In addition to externalization of PS, phospholipid scramblases elicit a number of other diverse and important cellular functions. Specifically, they play roles in autoimmunity, protection against viruses and bacteria, cell death (apoptosis and autophagy), lipid metabolism, genetic diseases, and cancer.

Although PKC-δ, JNK, and STAT1 signalling molecules as well as the ISRE element present in the untranslated exon 1 of PLSCR1 contribute to the transcriptional induction of PLSCR1, the complete mechanism of its transcriptional regulation remains to be investigated. Thus far, the only transcriptional regulatory molecule identified to regulate PLSCR1 is Snail [54]. Further investigations are required to identify transcriptional regulators of PLSCR1 and other phospholipid scramblase family members. Further, it is currently unknown how TMEM16 and XKR family members are transcriptionally regulated. Although there are multiple links between PLSCR1 and autophagy, the detailed underlying mechanism of how PLSCR1 contributes to this process remains unclear. As XKR family members can modulate the apoptotic pathway, it is possible that this molecule can alter autophagic flux; thus, studies can be conducted to investigate this possibility. PLSCR1 is described to elicit both tumour suppressive and oncogenic roles in cancer development, but further investigations are needed to clarify the role of this molecule (as well as TMEM16 and XKR family members) in the pathogenesis of cancer. From TCGA data, PLSCR1 is altered in ovarian cancer patients and these alterations are highly correlated with that of SnoN/SkiL, an oncogene that is overexpressed at 3q26.2 in ovarian and other epithelial cancers [153]. Another

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question to address is to assess the subcellular distribution of endogenous phospholipid scramblase in cell lines as well as in cancer specimens; as described earlier, the localization of PLSCR1 plays an important role in its functions. For example, although PLSCR1 is overexpressed in colorectal cancer tissue specimens [136], its subcellular localization was not assessed. Further studies are needed to further elucidate the role of PLSCR1 as well as other phospholipid scramblase family members (TMEM16 and XKR) in ovarian and other cancers.

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