Complementary probes reveal that phosphatidylserine is required for the proper transbilayer distribution of cholesterol.

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Abstract

Cholesterol is an essential component of metazoan cellular membranes and helps to maintain the structural integrity and fluidity of the plasma membrane. Here, we developed a cholesterol biosensor, termed D4H, based on the fourth domain of *Clostridium perfringens* theta-toxin, which recognizes cholesterol in the cytosolic leaflet of the plasma membrane and organelles. The D4H probe disassociates from the plasma membrane upon cholesterol extraction and after perturbations to cellular cholesterol trafficking. When used in combination with a recombinant version of the biosensor, we show that plasmalemmal phosphatidylserine is essential for retaining cholesterol in the cytosolic leaflet of the plasma membrane. In vitro experiments reveal that 1-stearoy-2-oleoyl phosphatidylserine can induce phase separation in cholesterol containing lipid bilayers and shield cholesterol from cholesterol oxidase. Finally, the altered transbilayer distribution of cholesterol causes flotillin-1 to relocalize to endocytic organelles. This probe should be useful in the future to study pools of cholesterol in the cytosolic leaflet of the plasma membrane and organelles.
Introduction

Despite the presence of a variety of phospholipids and sphingolipids within the cell, cholesterol is unique. Due to its biophysical properties dictated by a rigid hydrophobic structure and small headgroup, cholesterol can readily flip-flop between leaflets of membrane bilayers and be easily inserted and extracted by protein carriers (Ikonen, 2008). Additionally, the small headgroup is insufficient to shield the hydrophobic ring from water and thus cholesterol is thought to associate tightly with saturated phospholipids and sphingolipids. This concept along with biochemical experiments and microscopic observations has led to the development of the lipid raft hypothesis. This hypothesis suggests that cholesterol and sphingolipids form membrane nanodomains in the exofacial leaflet of the plasma membrane (Lingwood and Simons, 2010). However, due in part to technical limitations, the presence of sphingolipids-cholesterol nanodomains in the exofacial leaflet of the plasma membrane remains controversial. For example, a recent study using high-resolution secondary ion mass spectrometry has shown that sphingolipid-rich domains in the plasma membrane are not enriched in cholesterol (Frisz et al., 2013). Although this interpretation is complicated as the transbilayer distribution of cholesterol between the cytosolic and exofacial leaflet of the plasma membrane remains unclear. Indeed, through the use of intrinsically fluorescent sterols (dehydroergosterol, cholestatrienol) and their exposure to extracellular quenchers, it has been demonstrated that 60-70% of these cholesterol analogs reside in the cytosolic leaflet of the plasma membrane (Mondal et al., 2009). To date it remains unclear if this observation holds true for cholesterol.

The dynamics and functional roles for cholesterol in the cytosolic leaflet of the plasma membrane are currently unclear. Additionally, it is unknown if inner leaflet membrane nanodomains exist or if cholesterol is inhomogeneously distributed. The dearth of knowledge regarding cholesterol in the inner leaflet of the plasma membrane is due to the lack of a cholesterol biosensor (Maekawa and Fairn, 2014). Filipin, a fluorescent polyene macrolide, can bind to cholesterol directly and has been used extensively to visualize cellular cholesterol (Bornig and Geyer, 1974). However, filipin staining can be influenced by the accessibility of the sterols and cannot distinguish between cholesterol residing in cytosolic or exofacial/luminal leaflets (Miller, 1984; Zaremberg et al., 2005; Jin et al., 2008). An alternative to filipin that has been developed more recently is to use cholesterol molecules directly conjugated with a
fluorophore such as BODIPY-cholesterol (Bornig and Geyer, 1974; Li et al., 2006; Holttava et al., 2008). However, exogenously added fluorophore labelled cholesterol is also located in both leaflets of membrane bilayers (Milles et al., 2013). Furthermore, the addition of the fluorophore alters the properties and dynamics of cholesterol (Milles et al., 2013; Solanko et al., 2013). While these probes remain useful for certain types of experimentation the development of alternative probes is warranted.

In this study, we sought to develop an alternative approach to visualize cellular cholesterol. To this end, we made use of domain four (D4) of the theta-toxin produced by Clostridium perfringens as a basis for a genetically encoded cholesterol biosensor (Tweten, 1988; Shatursky et al., 1999). Addition of a recombinant GFP-tagged version of D4 to the extracellular medium results in cholesterol dependent binding to the cell surface that can be analysed microscopically or by using flow cytometry consistent with previous findings (Mizuno et al., 2011). We have expanded the use of this probe through the expression of a mCherry-D4 domain mutant with higher affinity for cholesterol in the cytosol of mammalian cells. This probe, termed D4H, allowed us to monitor cholesterol in the cytosolic leaflet of the plasma membrane and other organelles. To validate the D4H probe we used a number of treatments to decrease the plasmalemmal cholesterol content and/or increase cholesterol in endomembranes. Next, using these complementary probes we found that phosphatidylserine (PtdSer) is essential for retaining of cholesterol in the inner leaflet of the plasma membrane. Furthermore, we showed that the membrane nanodomain enriched 1-stearoyl-2-oleoyl phosphatidylserine can interact with cholesterol using model membranes (Pike et al., 2005). Our data revealed that this mutant D4 can serve as a cholesterol biosensor to monitor the distribution and appearance of cholesterol in the cytosolic leaflet of organelles.
Results

Complementary probes to visualize exofacial and cytosolic cholesterol.

Our goal for this study was to develop and validate a pair of probes to monitor the distribution of endogenous cholesterol in the cytosolic leaflet of the plasma membrane in intact cells. Perfringolysin O (PFO) theta-toxin and other bacterial cytolsins are known to bind to exofacial leaflet cholesterol leading to pore formation (Dunstone and Tweten, 2012). Importantly, the 4th domain of PFO theta toxin in recombinant form has been used to detect exofacial cholesterol using light and super-resolution microscopy (Mizuno et al., 2011). We confirmed the ability of recombinant GFP-D4 proteins to bind to the exofacial leaflet of the plasma membrane in living Chinese hamster ovary (CHO) cells using flow cytometry and light microscopy (Fig. 1A and Supplementary Figure 1A). The cholesterol dependence of GFP-D4 binding to the exofacial leaflet of the plasma membrane was confirmed by the use of methyl β-cyclodextrin (MβCD) to extract the cholesterol or by treating the cells with U18666A, a Niemann-Pick type C inhibitor to deplete cholesterol from the plasma membrane (Roff et al., 1991)(Roff et al., 1991)(Roff et al., 1991)(Roff et al., 1991)(Roff et al., 1991)(Roff et al., 1991)(Fig. 1A and Supplementary Figure 1A; Roff et al., 1991). We also demonstrated that the capacity for GFP-D4 binding could be enhanced if cells were loaded with cholesterol. To this end, we treated cells with cholesterol-loaded MβCD followed by incubation with recombinant GFP-D4 and measured by flow cytometry (Fig. 1B and Supplementary Figure 1A). As a control for this experiment, cellular cholesterol was visualized using filipin and imaged by confocal microscopy. Consistent with previous findings, addition of the cholesterol-MβCD complex lead to increased filipin signal intensity and the enhancement of signal in a perinuclear region (Hao et al., 2002). In addition, we found that recombinant HIS6X-GFP-D4 proteins could be internalized by incubation at 37 °C (Supplementary Figure 2), whereas the protein bound to only the exofacial leaflets of the plasma membrane when incubated on ice or at room temperature (Supplementary Figure 1A and Supplementary Figure 2). Taken together these results confirm that the D4 domain can act as a sensor of exofacial leaflet cholesterol by labelling at room temperature.

Based on the effectiveness of recombinant GFP-D4 to monitor exofacial cholesterol by microscopy and flow cytometry, we hypothesized that a plasmid based fluorescently-tagged D4
could be used as a biosensor for cholesterol in the cytosolic leaflet of the plasma membrane and other organelles. However, expressed mCherry-D4 did not localize to the plasma membrane in CHO cells, suggesting that there is either not enough available cholesterol to recruit the probe to the plasma membrane and/or that the probe has insufficient affinity when expressed in the cytosol (Fig. 1D). To investigate these possibilities we incubated the CHO cells with a MβCD-cholesterol complex as before to increase plasmalemmal and total cellular cholesterol. Under this condition the genetically encoded mCherry-D4 relocalized to the plasma membrane and to a perinuclear region (Fig. 1D and 1E). This suggests that the D4 works sufficiently well in the cytosol when there is an increased abundance of cholesterol. We next sought to assess the effectiveness of a D4 molecule with higher affinity for cholesterol as a biosensor to monitor endogenous levels of cholesterol. To this end, we expressed a mCherry-D4D434S mutant - that we have named D4H - that was previously shown to have a lower threshold of binding for cholesterol in vitro (Johnson et al., 2012). Consistent with the previous findings, we found that recombinant GST-D4H had higher affinity for cholesterol than GST-D4 in vitro and when expressed in the cytosol the mCherry-D4H reporter localized to the cytosolic leaflet of the plasma membrane (Fig. 2A - D). Importantly, localization of mCherry-D4H to the cytosolic leaflet of the plasma membrane was also cholesterol dependent as treatment with MβCD, U18666A and concanamycin A (a V-ATPase inhibitor that inhibits cholesterol recycling) (Kozik et al., 2013) all caused disassociation from the plasma membrane (Fig. 2E and 2F). As a control in these experiments, we stained the cells with filipin to confirm the depletion of plasmalemmal cholesterol in the RAW264.7 murine monocyte-macrophage cells. In these cells, mCherry-D4H disappeared from the cytosolic leaflet of the plasma membrane, and localized to filipin positive intracellular vesicles (Fig. 2E). Together, the recombinant D4 and genetically encoded D4H constitute complementary probes to monitor the topological distribution of plasmalemmal cholesterol in mammalian cells (Fig. 2G). To confirm that the individual probes are detecting the proper leaflets of the plasma membrane, we performed fluorescence protease protection assay (Lorenz et al., 2006). The addition of protease K to cells expressing mCherry-D4H and incubated with HIS6X-GFP-D4 led to the abolishment of GFP signal but not mCherry as expected. In parallel experiments both HIS6X-GFP-D4 and mCherry-D4H were degraded by the protease after the plasma membrane was permeabilized by digitonin (Supplementary Figure 3). These observations confirm our new method that HIS6X-GFP-D4 and mCherry-D4H label cholesterol
in the exofacial and cytosolic leaflets of the plasma membrane, separately (Fig. 2G). We also confirmed that labelling of cholesterol by HIS6X-GFP-D4 or mCherry-D4H did not affect the intracellular distribution of cholesterol (Supplementary Figure 4A). This approach to visualize cholesterol overcomes a major limitation of the canonical cholesterol probes such as filipin and fluorophore labelled cholesterol that cannot distinguish cholesterol in the exofacial and cytosolic leaflets of the plasma membrane. This probe is also suitable for live cell or time lapse imaging that is not possible with filipin.

**Phosphatidylycerine is required to retain cholesterol in the inner leaflet of the plasma membrane.**

The umbrella and condensed complex models are two related hypothesis to explain the interactions between cholesterol and other lipids in the plane of the membrane. Essentially, due to its small headgroup, cholesterol associates closely with lipids with large headgroups and primarily saturated acyl chains to maintain itself in a low energy state (Ikonen, 2008). Based on a variety of biochemical and microscopic results we postulated that phosphatidylycerine (PtdSer) would be the most likely candidate to interact with cholesterol in the cytosolic leaflet of the plasma membrane (Leventis and Silvius, 2001; Niu and Litman, 2002; Kay et al., 2012) and that a reduction in PtdSer would potentially lead to less cholesterol in the cytosolic leaflet of the plasma membrane. To test this hypothesis, we made use of the PSB-2 cell line, derived from CHO cells that have very low PtdSer synthase activities (~11%) resulting in approximately 80% decrease of PtdSer content (Saito et al., 1998; Stone and Vance, 2000). We determined that the total cholesterol content and its localization by filipin staining were normal in PSB-2 cells compared to the parental CHO cells (Fig. 3A and 3B). As reported previously, the filipin positive intracellular compartments in both cell types co-localized with the an transiently expressed GFP-transferrin receptor (TfR), a marker of recycling endosomes, indicating that cholesterol is enriched not only in the plasma membrane but also in the recycling endosomes (Fig 3C; Gagescu et al., 2000; Mondal et al., 2009). What about the transbilayer distribution of cholesterol in the plasma membrane? We found that in PSB-2 cells the binding of the recombinant GFP-D4 to the exofacial leaflet of the plasma membrane was increased (Fig. 4A and Supplementary Figure 1B). As a control we restored the levels of PtdSer in our PSB-2 cells by supplementing the medium with PtdSer (Saito et al., 1998) and found that GFP-D4 binding was largely restored to normal
while in parallel supplementing the medium with phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) had no effect (Fig. 4A and Supplementary Figure 1B). Next, we examined the localization of the remaining 20% of the PtdSer using the biosensor Lact-C2 (Yeung et al., 2008) and cholesterol using the mCherry-D4H in the PSB-2 cells. As illustrated in Fig. 4B and 4C, most of the remaining PtdSer in the PSB-2 cells is found in intracellular compartments compared to the parental CHO cells. Likewise the mCherry-D4H probe was found to be displaced from the plasma membrane and now found primarily on internal structures. Importantly, by supplementing the cells with PtdSer, both the PtdSer and cholesterol biosensors relocalize to the plasma membrane (Fig. 4B and 4C). These results are consistent with PtdSer being important to maintain cholesterol in the inner leaflet of the plasma membrane and that PtdCho and PtdEtn cannot functionally replace PtdSer. To ensure that binding of the D4H to cholesterol was not influenced by PtdSer, we conducted both liposomal sedimentation and FRET-based assays, respectively (Fig. 5A and 5B). In both assays the presence of PtdSer had no significant impact on D4H binding to cholesterol. Together these results suggest that in the absence of PtdSer, more cholesterol is retained in the exofacial leaflet of the plasma membrane, likely at a lower energetic state, than the cytosolic leaflet. Additionally, the remaining cytosolic leaflet pools of cholesterol coincide with the presence of PtdSer.

**Acute alterations in phosphatidylserine distribution alter cholesterol distribution.**

To complement the experiments using the PSB-2 cells that have a chronic diminution of PtdSer we sought to examine the impact of PtdSer redistribution on cholesterol localization. Treating MDCK cells with staurosporine (STS) at low sub-apoptotic concentrations causes the relocalization of PtdSer from the plasma membrane to endosomes through an uncharacterized mechanism (Cho et al., 2012). Consistent with this finding we found that treatment of CHO cells with STS lead to the relocalization of the PtdSer biosensor, GFP-Lact-C2, to endosomal compartments. In parallel, the binding of the recombinant GFP-D4 to the exofacial leaflets of the plasma membrane was increased and the mCherry-D4H relocalized from the plasma membrane to Lact-C2 positive endocytic structures (Fig. 6A and 6B). Consistent with the findings using the PSB-2 cells, these results also demonstrate that in the absence of PtdSer, the cytosolic leaflet of the plasma membrane has a reduced capacity to retain cholesterol. Next as a control, we demonstrate that replenishment of the plasmalemmal PtdSer restored GFP-D4 binding and led to
the relocalization of the D4H and Lact-C2 probes back to the cytosolic leaflet of the plasma membrane (Fig. 6A-C). From these experiments we concluded that in the absence of PtdSer, cholesterol is retained in the exofacial leaflet of the plasma membrane.

**Phosphatidylserine and cholesterol coalesce in model membranes.**

To investigate the possible interactions between PtdSer and cholesterol we examined the ability of PtdSer and cholesterol to form liquid ordered domains. Giant unilamellar liposomes (GUVs) have been used extensively as a model to visualize the phase separation of lipids. As reported previously (Baumgart et al., 2007; van Meer et al., 2008), sphingomyelin (SM) induced the formation of liquid ordered and disorders domains (as monitored with absence and presence of rhodamine-PtdEtn, respectively) in cholesterol containing GUVs while SOPC did not (Fig. 7A). We next examined GUVs and observed phase separation with a composition of cholesterol/PtdSer/PtdCho/rhodamine-PtdEtn (33:33:33:1) with 18:0-18:1 PtdSer (SOPS) but not the following PtdSer species: 16:0/18:1 (POPS), 18:1/18:1 (DOPS), or 16:0/18:2 (PLPS) (Fig. 7A). This highlights that variability exists with regards to the strength of interactions between cholesterol and the different acyl chain species of PtdSer with SOPS being the strongest. Consistent with our findings is previous results demonstrating that cholesterol and SOPS are enriched in detergent resistant membrane fractions and caveolae from CHO cells (Pike et al., 2005).

Our preceding results demonstrate that PtdSer but not PtdCho or PtdEtn is important for the cellular distribution of cholesterol possibly through direct interactions. We wanted to further examine if the headgroup of PtdSer could shield the hydroxyl group of cholesterol from the environment thereby supporting the umbrella model for cholesterol-lipid interactions. To do this we used accessibility of the hydroxyl group to cholesterol oxidase in an *in vitro* liposome based assay (Patzer and Wagner, 1978). Cholesterol oxidase catalyses the conversion of the hydroxyl group of cholesterol to generate chole-4-en-3-on and hydrogen peroxide, which can be measured by a coupled chemical reaction. We speculated that the cholesterol oxidase accessibility to cholesterol would be low in SM containing liposomes and due to its small headgroup high in PtdEtn containing liposomes. As predicted, the oxidase accessibility to cholesterol in SM or SOPS containing liposomes was lower while SOPE containing liposomes was higher than that of SOPC containing liposomes (Fig. 7B). Next, to examine the fatty acyl chains specificity of
PtdSer species, we monitored the oxidase accessibility to cholesterol in various species of PtdSer containing liposomes, and found that cholesterol was well shielded from the oxidase only in SOPS containing liposomes (Fig. 7C). To ensure that the presence of anionic phospholipids was not acting as an inhibitor of the cholesterol oxidase we next compared the accessibility of cholesterol in liposomes contain phosphatidic acid or brain PtdIns(4,5)P2 containing liposomes to SOPS and found a minimal impact (Fig. 7D). Together these data indicated that both the headgroup of PtdSer and fatty acyl chain composition are important to shield cholesterol from cholesterol oxidase and to support phase separation in model membranes.

Alteration to the plasma membrane in phosphatidylycerine depleted and mislocalized cells

We examined the consequences of PtdSer depletion/ altered transbilayer distribution of cholesterol in the plasma membrane. We predicted that, under conditions with increased GFP-D4, more cholesterol should be extractable with short (5 min) incubations with MβCD. For these experiments, cells were equilibrated with [3H]-cholesterol and subjected to a 5 min exposure with 10 mM MβCD. In these experiments, 65-70% of the cholesterol was extracted by MβCD in the PSB-2 and STS-CHO cells whereas ~50% of the cholesterol was extracted from the control CHO cells (Fig. 8A). These results are consistent with more cholesterol in the exofacial leaflet of the plasma membrane and that it is easily extractable by extracellular MβCD in conditions when the plasmalemmal PtdSer is depleted.

Finally, we considered if membrane proteins normally associated with membrane nanodomains would be impacted by the alterations in cholesterol transbilayer distribution. For these experiments we choose to examine flotillin-1. Flotillin-1 is a membrane nanodomain associated protein that has been implicated in endocytosis and signaling. Unlike proteins such as the HIV gag protein (Dick et al., 2012) and caveolin-1 (Wanaski et al., 2003), flotilin-1 has not been documented to bind to PtdSer. Flotillin-1 localizes to the plasma membrane in a cholesterol dependent manner through a combination of two hydrophobic stretches and a palmitolyation on cysteine 34 (Liu et al., 2005). When flotillin1-GFP was expressed in PSB-2 and STS-CHO cells, it did not localize to the cytosolic leaflet of the plasma membrane in contrast to control cells (Fig. 8B). Importantly this defect could be rescued by supplementation of PtdSer and restoration of cholesterol distribution (Fig. 8B and 8C). Together these results suggest that the altered
cholesterol transbilayer distribution of the plasma membrane impacts the localization of cholesterol dependent or nanodomain associated proteins and allow for cholesterol to be more readily extracted from the cell.
Discussion

In this study, we developed a genetically encoded cholesterol biosensor, mCherry-D4H, which can visualize cholesterol in the cytosolic leaflet of the plasma membrane and organelles. This probe offers advantages over the canonical cholesterol stain, filipin, which is typically used with fixed cells to stain cholesterol in cellular membranes regardless of its transbilayer distribution. Additionally, the plasmid-borne mCherry-D4H is also suitable for live-cell imaging. In this regard, expressed mCherry-D4H should prove useful for analysis of cholesterol dynamics and organization in the cytosolic leaflet of the plasma membrane using advanced microscopic techniques (e.g. fluorescence recovery after photo-bleaching or super-resolution microscopy). Despite the advantages of this probe it does have the limitation that all of these types of biosensors have in that they only have access to available lipid. Recent evidence suggests that cholesterol may act as an allosteric regulator for many membrane proteins such as ion channels and scaffolding proteins which will likely influence the amount of freely accessible cholesterol (Sheng et al., 2012; Levitan et al., 2014; Sheng et al., 2014).

The in vitro binding assay revealed that recombinant D4H has is able to detect cholesterol over a range from 20 to 60 mol% in liposomes that mimic the inner leaflet of the plasma membrane (Supplementary Figure 4B). These results suggest that there is likely a minimal threshold of cholesterol required for the recruitment of the mCherry-D4H in vivo. Previous studies have shown that binding affinity of full length of the Perfringolysin O (PFO) theta-toxin to cholesterol is influenced by the local environment (e.g. neighboring phospholipids) of cholesterol in model membranes (Flanagan et al., 2009; Sokolov and Radhakrishnan, 2010; Das et al., 2013). This is likely due to alterations in the chemical potential of the cholesterol (Bennett et al., 2009). These findings led us to consider an alternative hypothesis that in the absence of PtdSer, the chemical activity of cholesterol in the inner leaflet of the PM is altered and as a result no longer accessible to the D4H probe. However, the experiment evidence is more consistent with there being a redistribution of cholesterol from the inner leaflet of the PM to the exofacial leaflet. First, the in vitro data suggests that removal of PtdSer should increase the chemical activity of cholesterol and make it more accessible to the probe not less. Second, altering the inner leaflet of the PM should not impact the binding of recombinant D4 to the exofacial leaflet unless there is more cholesterol present. Third, the replacement of PtdSer with PtdCho and
PtdEtn, two lipids that shield cholesterol less effectively than PtdSer and SM, did not restore binding of the D4H to the plasma membrane. Taken together, we believe the evidence indicates in the absence of PtdSer, the inner leaflet of the plasma membrane has less ability to retain cholesterol and that the levels dip below the minimal threshold required for detection by the D4H probe.

Cholesterol has various physiological functions in cell signaling and vesicular trafficking as well as pathophysiological states such as atherosclerosis and Alzheimer’s disease (Simons and Toomre, 2000; Simons and Ehehalt, 2002; Chadda et al., 2007; Kozik et al., 2013). However, it is currently unclear to what extent PtdSer-dependent cytosolic leaflet cholesterol plays in these processes. Clearly, understanding the organization of the plasma membrane is required to understand proteins and signaling hubs that reside here. The existence of lipid rafts or nanodomains in the exofacial leaflet of the plasma membrane remains controversial, although electron and super-resolution microscopy has visualized lipid clusters (Mizuno et al., 2011; Zhou et al., 2013). Our results suggest that a number of the same organizing principles exist for the cytosolic leaflet resident lipids, especially for the anionic lipid PtdSer and cholesterol.

PtdSer and cholesterol are synthesized in the endoplasmic reticulum and enriched in the plasma membrane and recycling endosomes (Gagescu et al., 2000; Ikonen, 2008; Uchida et al., 2011). Thus, it is possible that they influence the others trafficking or retention in a given organelle. Segregation of PtdSer and cholesterol in the cytosolic leaflet of organelles could generate nanoscale enrichments of anionic charge that in turn could be recognized by sorting or vesiculating machinery. To examine the presence of the nanodomains of PtdSer and cholesterol in the cytosolic leaflets of organelles, cluster analysis will be required using high resolution electron microscopy or super-resolution light microscopy with the Lact-C2 and D4H probes (Saka et al., 2014; Zhou et al., 2014). Additionally, the presence of soluble carriers of cholesterol and PtdSer, such as the steroidogenic acute regulatory-related lipid-transfer (START) proteins and Oxysterol-binding protein (OSBP) related protein (ORP) family suggest that the concerted actions of these types of proteins may help regulate the co-segregation of these two lipids (Mesmin et al., 2011; Maeda et al., 2013; Olkkonen and Li, 2013).
Materials and Methods

Plasmids

pET28b vector with GFP-D4 was a kind gift from Dr. Y. Ohno-Iwashita (Iwaki Meisei University, Japan). D4 was amplified using this vector as a template for PCR using the following pairs of primers: 5’-GCGCTCGAGCCAAGGAAAAATAAAACTTAGA-3’ (D4 sense primer) and 5’-GCGGAATTCTTAATTGTAAGTAATACCTAG-3’ (D4 antisense primer). The PCR product was introduced into pmCherry-C1 vector at XhoI/EcoRI site. D4 (D434S), named as a D4H, was generated with the following pairs of primers: 5’-CTCAGATTCAACAGTAATACCTCT-3’ (D434S sense primer) and 5’-CTGTTTTAGATTGATAATTTCCATC-3’ (D434S antisense primer) from pmCherry-C1 vector with D4 using Phusion Site-Directed Mutagenesis Kit (Thermo). Human flotillin-1 was amplified with the vector (HsCD00003177) obtained from the Harvard plasmid repository using the following pairs of primer: 5’-GCGCTCGAGCCATGTTTTTCACTTCCTAGGGCCC-3’ (flotillin-1 sense primer) and 5’-CGCGAATTCTGGCTGTTCTCAAAAGGCTTG-3’ (flotillin-1 antisense primer). The product was introduced into pEGFP-N1 vector at XhoI/EcoRI site. To subclone D4 and D4H into the pGEX-6P1 vector, D4 and D4H were PCR amplified with mCherry-D4 and mCherry-D4H, respectively, using the following pairs of primers: 5’-GCGGGATCCAAGGAAAAATAAAACTTAGA-3’ (D4 sense primer 2) and 5’-CGCGAATTCTTAATTGTAAGTAATACCTAG-3’ (D4 antisense primer 2). The products were introduced into pGEX-6P1 vector at BamHI/EcoRI site. The GFP-Lact-C2, GFP-PH-PLCδ, RFP-PH-PLCδ and GFP-TfR plasmids were kind gifts from Dr. S. Grinstein (The Hospital for Sick Children, Toronto, Canada).

Purification of GST-D4 and GST-D4H

*E. Coli.* strain BL21 (Rosetta) was used for the overexpression of GST-tagged D4 or D4H fusion proteins. *E. coli* transformed with pGEX-6P1-D4 or D4H were cultured in LB media at 37°C with constant shaking until the OD600 reached 0.8. Cultures were induced with 1 mM IPTG for 5 hours at 25 °C. Next, cells were collected by centrifugation and lysed using B-PER (Pierce Biotechnology) according to the manufacturer’s instructions. Cell lysate supernatants were bound to Pierce Glutathione Agarose (Thermo). The resin was washed with PBS (pH 7.4) and
the protein was eluted with 25 mM glutathione in Tris-EDTA buffer (10 mM Tris, 5 mM EDTA, pH 8.0). The fractions were analysed for GST-D4 and GST-D4H by G-250 staining (BioRad) of SDS-PAGE gels.

**Purification of His-GFP-D4**

*E. Coli.* strain BL21 (Rosetta) was used for the overexpression of the hexahistidine-tagged HIS6-GFP-D4 fusion proteins (HIS6X-GFP-D4). *E. Coli.* transformed with pET28b-GFP-D4 were cultured in LB media at 37 °C with constant shaking until the OD600 reached 0.4. Cultures were induced with 0.5 mM IPTG for 4 hours at 30 °C, then *E. coli* cells were harvested by centrifugation and lysed in B-PER (Pierce Biotechnology) according to the manufacturer’s instructions. Cell lysate supernatants were bound to TALON Metal Affinity Resin (Clontech). The slurry was washed by PBS (pH 7.4) and the protein was eluted with Tris-EDTA buffer (100 mM Tris, 50 mM EDTA, pH 7.5). The fractions were analysed for HIS6X-GFP-D4 by G-250 staining (BioRad) of SDS-PAGE gels.

**Lipids**

Cholesterol and [3H]-cholesterol were from Sigma and PerkinElmer, respectively. Synthetic 1-palmitoyl-2-oleoyl (PO) phosphatidylserine (POPS), 1-stearoyl-2-oleoyl (SO) PS (SOPS), 1,2-dioleoyl (DO) PS (DOPS), 1-palmitoyl-2-linoleoyl (PL) PS (PLPS), PO phosphatidylethanolamine (POPE), SOPE, dansyl-PE, rhodamine-PE, DO phosphatidylcholine (DOPC), SOPC, SO phosphatidic acid (SOPA), egg yolk L-α-PC, (egg PC) egg yolk sphingomyelin (egg SM), bovine liver L-α-phosphatidylinositol (liver PI) and porcine brain L-α-phosphatidylinositol-4, 5-bisphosphate (brain PI(4,5)P2) were from Avanti Polar Lipids.

**Liposome preparation**

Liposomes with various lipid compositions were prepared by adding the required amount of stock lipids in chloroform (total 1 μmol) into glass vials, and drying chloroform using nitrogen gas. Large multilamellar vesicles (MLVs) were prepared by gently vortexing with 1 ml of PBS until all lipids was suspended. For large unilamellar vesicles (LUVs) formation, 1 mM of MLVs was incubated at 37 °C for 1 hr. During this 1 hr incubation, MLVs were vortexed once every 10
min. Then, after sonication in a bath sonicator for 30 min, LUVs were prepared by extruding MLV suspensions 15 times through two stacked polycarbonate membranes with 100 nm diameter pores (Avestin) in a LiposoFast (Avestin), according to the manufacturer’s instructions. Giant unilamellar liposomes (GUVs) were prepared using 1 % agarose with ultralow melting temperature (Type IX-A) as described previously (Horger et al., 2009). Briefly, 300 μl of 1 % agarose with ultralow melting temperature was laid on a slide glass, spread evenly and put on the heater at 40 °C for 3 hr to dry the gel. Then, 50 μl of 6.2 mM lipid in chloroform was laid on the agarose-coated slide glass, spread evenly and dried with nitrogen gas. The slide glass with thin lipid films on the agarose gel was soaked in PBS in a petri dish at 37 °C for 3 hr, and then GUVs in PBS. Vesicles were used fresh. Storage at 4 °C before usage did not exceed 24 hours.

**in vitro liposome fluorescence resonance energy transfer (FRET) assay**

Binding of GST-D4 and GST-D4H to liposomes was measured by FRET between donor tryptophan residues to acceptor dansyl conjugated to the headgroup of PtdEtn as described previously (Gilbert et al., 1990). Briefly, binding of GST-D4 and GST-D4H to the liposome was reflected by an increase in the fluorescence of the acceptor (dansyl) at 510 nm following excitation at 280 nm of the tryptophan residues on D4 and D4H. The LUVs (10 μM) were incubated with purified proteins (800 nM) at 37 °C for 20 min, and then fluorescence intensity were measured in a 2 x 10 x 45 mm 18F-Q-10 quartz cuvette, 2 x 10 x 45 mm, (Starna Cells) using a SpectraMax M5e (Molecular Devices) at room temperature (RT). Excitation and emission wavelength were 280 and 510 nm, respectively, with 9 nm (ex) and 15 nm (em) slit width. Data were analyzed as described previously (Gilbert et al., 1990). The lipid compositions (mol %) of LUVs used in this experiment are following: SOPS/liver PI/brain PI(4,5)P2/POPE/Dansyl-PE/DOPC/cholesterol = 20/4/1/7.5/2.5/5-65/0-60, or SOPS/liver PI/brain PI(4,5)P2/POPE/Dansyl-PE/DOPC/cholesterol = 0-20/4/1/7.5/2.5/35-55/30. FRET efficiency was calculated using the formula:

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\text{FRET efficiency} = \left( \frac{F}{F_b} \right) - 1
\]

Where, F is the fluorescence intensity in the presence of proteins and F_b is the fluorescence intensity in the absence of proteins.
**in vitro liposome sedimentation assay**

The liposome co-sedimentation assay used in this study was basically the same as that previously described (Uchida et al., 2011). Briefly, 10 μg GST-D4H proteins were incubated with 50 nmol LUVs in PBS (100 μl) at 37 °C for 15 min and the mixture was centrifuged at 100,000 g for 1 hr at 20 °C. The resultant supernatant and pellet were subjected to SDS-PAGE and the gels were stained with G-250 (BioRad). The lipid compositions (mol %) of LUVs used in this experiment are following: SOPS/POPE/DOPC/cholesterol = 20/20/60/0, SOPS/POPE/DOPC/cholesterol = 20/20/0/60 or SOPS/POPE/DOPC/cholesterol = 0/20/20/60.

**in vitro cholesterol oxidase accessibility assay**

Cholesterol oxidase oxidizes 3-hydroxy group of cholesterol and yields H$_2$O$_2$ and the ketone product (chole-4-en-3-on). In this experiment, the H$_2$O$_2$ generated by cholesterol oxidase from cholesterol in LUVs are detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex). In the presence of horseradish peroxidase (HRP), Amplex dye reacts with H$_2$O$_2$ with a 1:1 ratio to produce a fluorescent resorufin (Zhou et al., 1997). The resorufin has absorption and fluorescence emission maxima of 571 nm and 585 nm, respectively. Using these enzymatic reactions, the accessibility of cholesterol oxidase to cholesterol in LUVs was measured. LUVs (0.5 mM) in 50 μl PBS were prepared in 96 well plates and 50 μl working solution containing 0.3 mM Amplex Red (Invitrogen), 2 U/ml cholesterol oxidase from *Streptomyces* (Sigma), 2 U/ml HRP (Sigma) in PBS, were added to each well. Then, the plate were immediately set in SPECTRA max PLUS (Molecular Devices) and abs 571 nm were monitored every 1 min for 30 min at 37 °C. For normalization of data, maximum oxidation of cholesterol by the cholesterol oxidase was determined by incubation of LUVs and working solution in the presence of 0.5 % Triton and abs 571 nm were monitored every 1 min for 30 min at 37 °C. The lipid compositions (mol %) of LUVs used in this experiment are following: egg PC/X/cholesterol = 25/25/50 or egg PC/Y/cholesterol = 10/40/50. X is one of the following: SOPE, SOPC, egg EM, SOPS, DOPS, POPS, PLPS. Y is one of the following: SOPA, brain PI(4,5)P$_2$, SOPS.

**Cell culture and transfection**

RAW264.7 cells were maintained at 37 °C with 5 % CO$_2$ in RPMI (Wisent, Burlington, ON) supplemented with 10 % FBS. Raw264.7 cells were treated with 3 μg/ml U18666A (Sigma) and
1 μM concanamycin A (Sigma) in RPMI with 10 % FBS at 37 °C for 24 hr. Wild-type CHO-K1 and PSB-2 cells were routinely maintained at 37 °C with 5 % CO2 in Ham’s F-12 medium (Wisent) supplemented with 5 % FBS or 5 % Lipoprotein deficient serum, respectively. For cholesterol extraction, cells were treated with 10 mM MβCD (Sigma) in serum free Ham’s F-12 medium for 30 min at 37 °C. For PtdSer relocalization studies CHO-K1 cells were treated with 50 nM staurosporine (BioShop, Burlington, Ontario) in Ham’s F-12 medium with 5 % FBS at 37 °C for 24 hr. For supplementation of lipids, cells were incubated with 30 μM MLVs in serum containing Ham’s F-12 medium for 24 hr or 2 hr at 37 °C. Raw264.7 cells and PSB-2 cells were transiently transfected with plasmids using Fugene HD (Promega) according to the manufacturer’s instructions. Wild-type CHO-K1 cells were transiently transfected with plasmids using Fugene 6 (Promega) according to the manufacturer’s instructions. Cells were fixed with 3.7 % formaldehyde (FA)-PBS for 30 min at RT at 24 hr post-transfection.

**Binding assay of HIS6X-GFP-D4 to the exofacial leaflet of the plasma membrane in living cells**

Cells were incubated with HIS6X-GFP-D4 (15 μg/ml) in the serum free RPMI media for 15 min at RT, washed with PBS and observed live at RT. For quantitative analysis with a flow cytometry cells were detached from the plates with 0.05 % trypsin-EDTA for 5 min at 37 °C, collected and resuspended in 0.3 ml of ice-cold PBS. Cells were analyzed using FACS Calibur (BD Bioscience) with CellQuest software (BD Bioscience).

**Filipin staining**

Filipin specifically binds to non-esterified cholesterol (Bornig and Geyer, 1974). Cells were fixed with 3.7 % FA-PBS for 30 min at RT and then incubated with 0.5 mg/ml filipin (Polysciences, Warrington, PA) in PBS for 16 hr at 4 °C. Samples stained with filipin were visualized using an LSM700 using a 405 nm laser.

**Confocal microscopy**

At 24 hr post-transfection, cells were imaged live or fixed with 3.7 % FA-PBS for 30 min at RT and mounted for later examination. Images were acquired using a Zeiss LSM 700 inverted confocal microscopy (Zeiss) using a Plan-Apochromat 60x/ 1.4 NA oil objective and acquired
using Zen 2010 software (Zeiss). Analysis of images was performed with Zen 2010 or ImageJ software (NIH). Cells were classified as being positive for plasmalemmal D4H if there was a >2-fold enrichment of the mean fluorescence intensity of the probe compared to cytosol using the following equation: \((\text{MFI of PM} - \text{MFI of background})/\text{(MFI of cytosol – MFI of background)}\). MFI was measured for regions of interest using ImageJ software.

**Cholesterol supplementation**

Cholesterol was added to cells by incubating cells for 1 hr at 37 °C with 50 μg/ml cholesterol complexed with 1.5 mg/ml MβCD in serum free F12 medium (Blom et al., 2001).

**Measurement of cholesterol**

Cellular cholesterol determinations were performed using the Amplex Red Cholesterol Assay Kit (Invitrogen), according to the manufacturer’s instructions.

**Cholesterol extraction assay**

Cholesterol extraction assay were performed as described previously (Low et al., 2012). Briefly, cells were incubated with 0.5 μCi/well \(^{3}\text{H}\)-cholesterol for 24 hr at 37 °C in 12 well plates. After equilibration incubation with serum free medium for 24 hr at 37 °C, cells were incubated with 10 mM MβCD for 5 min at 37 °C. Radioactivity in both the medium and cell lysates were measured using a scintillation counter (Beckman Coulter LS6500, Beckman). For calculation of rates of cholesterol extraction, the following formula is used.

\[
\text{Cholesterol Extraction} = \frac{\text{(media counts x dilution factor)}}{\text{[(media counts x dilution factor)+(cell counts x dilution factor)]}}
\]

The specific extraction is calculated as a difference between the rate in the presence or absence of MβCD (blank). Final Extraction = Cholesterol Extraction - Blank Extraction

**FPP (fluorescence protease protection) assay**

FPP assay was performed as described previously with minor changes (Lorenz et al., 2006). Briefly, after treatment of mCherry-D4H expressing cells with HIS6X-GFP-D4 recombinant proteins for 15 min at RT, protease K (50 μg/ml) was added to cells in the serum free RPMI media. After incubation for 5 min at RT, cells were observed live at RT. To permeabilize the
plasma membrane, cells were treated with digitonin (20 μM) in the serum free RPMI media for 1 min at RT, then protease K (50 μg/ml) was added to cells following washing with PBS. After incubation for 5 min at RT, cells were observed live at RT.

**Statistical analysis**

Statistical analysis was carried out using Student’s two-tailed $t$-test.
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Author contributions

M.M. and G.D.F. designed research; M.M. performed research; M.M. analyzed data; and M.M. and G.D.F. wrote the paper.

Competing interests

The authors declare no competing interests.
References


Figure Legends

**Fig. 1.** D4 can detect cholesterol not in the cytosolic leaflets but in the exofacial leaflets of the plasma membrane. (A) A histogram of flow cytometric analysis of control CHO cells or treated with MβCD or U18666A incubated with recombinant HIS6X-GFP-D4 protein. (B) A histogram of FACS analysis of cholesterol-MβCD complex (chol/MβCD) loaded CHO cells treated with HIS6X-GFP-D4 protein. (C) Confocal images of chol/MβCD loaded CHO cells stained with filipin. Bar, 10 μm. (D) Confocal images of chol/MβCD loaded CHO cells expressing mCherry-D4 and GFP-PH-PLCδ. Bar, 10 μm. (E) Quantitation of Fig. 1D. Total 100 cells from three independent experiments were analysed. Data are means ± sem. ***, p<0.001.

**Fig. 2.** Expressed mCherry-D4H can visualize cholesterol in the cytosolic leaflets of the plasma membrane. (A) Binding of GST-D4 or GST-D4H to liposomes mimicking the cytosolic leaflet of the plasma membrane monitored by FRET. The lipid composition (mol %) of the liposomes was SOPS/liver PI/brain PI(4,5)P2/POPE/dansyl-PE/DOPC/cholesterol = 20/4/1/7.5/2.5/45/20 or SOPS/liver PI/brain PI(4,5)P2/POPE/dansyl-PE/DOPC/cholesterol = 20/4/1/7.5/2.5/35/30. Data are means ± sem (n = 3). *, p<0.05, **, p<0.01. (B) Confocal images of CHO cells expressing mCherry-D4 and GFP-PH-PLCδ. (C) Confocal images of CHO cells expressing mCherry-D4H and GFP-PH-PLCδ. (D) Quantitation of Fig. 2B and Fig. 2C. Total 100 cells from three independent experiments were analysed. Data are means ± sem. **, p<0.01. (E) Confocal images of filipin staining of Raw264.7 cells expressing mCherry-D4H. (F) Quantitation of Fig. 2E. Total 100 cells from three independent experiments were analysed. Data are means ± sem. ***, p<0.001. (G) Schematic representation of the probes used to monitor cholesterol in the exofacial and cytosolic leaflets of the plasma membrane using HIS6X-GFP-D4 and mCherry-D4H, respectively. Scale bar for all images = 10 μm.

**Fig. 3.** Content and intracellular distribution of cholesterol in PSB-2 cells. (A) Cells were collected from one 10 cm dish and the cholesterol content of the cells was then measured using a cholesterol oxidase-based Amplex Red kit. Data are means ± sem (n = 3). n. s, not significant. (B) Confocal images of CHO cells and PSB-2 cells stained with filipin. Bar, 10 μm. (C)
Confocal images of GFP-TfR expressing CHO cells and PSB-2 cells stained with filipin were shown. Bar, 10 μm.

**Fig. 4. Transbilayer distribution of cholesterol in the plasma membrane is defective in PSB-2 cells.** (A) A histogram of FACS analysis of CHO cells and PSB-2 cells treated with HIS6X-GFP-D4 protein. For rescue experiments, PSB-2 cells were incubated with lipids for 24 hr. (B) Confocal images of CHO cells and PSB-2 cells expressing mCherry-D4H and GFP-Lact-C2. Bar, 10 μm. (C) Quantitation of Fig. 4B. Total 100 cells from three independent experiments were analysed. Data are means ± sem. **, p<0.01.

**Fig. 5. PtdSer does not affect on binding of GST-D4H to cholesterol in vitro.** (A) A representative image of a G250 stained gel of in vitro cosedimentation assay. GST-D4H was mixed with liposomes for 15 min, then the mixture was spun at 100,000 g for 1 hr. The resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The lipid compositions (mol %) of LUVs used in this experiment are following: SOPS/POPE/DOPC/cholesterol (chol) = 20/20/60/0, SOPS/POPE/DOPC/cholesterol = 0/20/20/60 or SOPS/POPE/DOPC/cholesterol = 20/20/0/60. (B) Binding of GST-D4H to liposomes mimicking the cytosolic leaflet of the plasma membrane by FRET assay. The lipid composition (mol %) of the liposomes was SOPS/liver PI/brain PI(4,5)P2/POPE/dansyl-PE/DOPC/cholesterol = 20/4/1/7.5/2.5/35/30 or SOPS/liver PI/brain PI(4,5)P2/POPE/dansyl-PE/DOPC/cholesterol = 0/4/1/7.5/2.5/55/30. Data are means ± sem (n = 3). n. s, not significant.

**Fig. 6. Transbilayer distribution of cholesterol in the plasma membrane is defective in staurosporine (STS) treated cells.** (A) A histogram of FACS analysis of staurosporine treated CHO (STS-CHO) cells incubated with HIS6X-GFP-D4 protein. STS (50 nM) was incubated for 24 hr. For rescue experiments, STS-CHO cells were incubated with lipids for 2 hr. (B) Confocal images of STS-CHO cells expressing mCherry-D4H and GFP-Lact-C2. Bar, 10 μm. (C) Quantitation of Fig. 6B. Total 100 cells from three independent experiments are analysed. Data were means ± sem. *, p<0.05, **, p<0.01.
Fig. 7. SOPS can interact with cholesterol in model membranes. (A) Confocal images of giant unilamellar liposomes (GUVs). The lipid composition (mol %) of the liposomes was egg PtdCho/X/cholesterol (chol)/rhodamine (Rhod)-PtdEtn = 33/33/33/1. X was one of the following: egg SM, SOPC, SOPS, POPS, DOPS, PLPS. Bar, 5 μm. (B-D) The oxidation rate of cholesterol in liposomes at each time point. The lipid composition (mol %) of the liposomes was egg PtdCho/X/cholesterol (chol) = 25/25/50 (B, C). X was one of the following: SOPE, SOPC, egg SM, SOPS, DOPS, POPS, PLPS. The lipid composition (mol %) of the liposomes was egg PtdCho/Y/cholesterol (chol) = 40/10/50 (D). Y was one of the following: SOPA, brain PI(4,5)P2, SOPS. Data are means ± sem (n = 3).

Fig. 8. Cytosolic leaflet cholesterol is required for the localization of flotillin-1. (A) Extraction of [3H]-cholesterol from the plasma membrane in PtdSer manipulated cells. Cells were treated with 10 mM MbCD for 5 min at 37 °C. Data are means ± sem (n = 3). *, p<0.05, **, p<0.01. (B) Confocal images of cells expressing human Flotillin-1-GFP. Bar, 10 μm. (C) Quantitation of Fig. 8B. Total 100 cells from three independent experiments were analysed. Data are means ± sem. *, p<0.05, **, p<0.01, ***, p<0.001. STS (50 nM) was incubated for 24 hr. For rescue experiments, PSB-cells and STS-CHO cells were incubated with PtdSer for 24 hr and 2 hr, respectively (A, B).
Fig. 2.

A

\[
\frac{[F]}{[F_{\text{H}}]} \quad \text{Cholesterol}
\]

- 20%
- 30%

\[\text{GST-D4} \quad \text{GST-D4H}\]

B

mCherry-D4

GFP-PH (PLC)

Merge

C

mCherry-D4H

GFP-PH (PLC)

Merge

D

% of cells with PM localization

mCherry-D4

mCherry-D4H

E

mCherry-D4H

Filipin

Merge

Control

MβCD

U18666A

Con A

F

% of cells with PM localization of mCherry-D4H

Control

MβCD

U18666A

Con A

G

Medium

HIS6-GFP-D4

HIS6-GFP-D4

PM

mCherry-D4H

mCherry-D4H

Cytosol

Cholesterol

Phospholipids
**Fig. 5.**

**A**

<table>
<thead>
<tr>
<th>S</th>
<th>P</th>
<th>Chol 0 %</th>
<th>PtdSer 0% Chol 60 %</th>
<th>PtdSer 20% Chol 60 %</th>
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**B**

% of max

- 20% PtdSer
- 0% PtdSer

n.s.
Fig. 6.

(A) Numbers of DMSO, STS, and STS + POPS treated cells expressing HIS6X-GFP-D4.

(B) Immunofluorescence images of DMSO, STS, and STS + POPS treated cells expressing mCherry-D4H and GFP-Lact-C2.

(C) Bar graph showing the percentage of cells with PM localization of mCherry-D4H in DMSO, STS, and STS + POPS treatments.