

RESEARCH ARTICLE

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

Masashi Maekawa¹ and Gregory D. Fairn^{1,2,3,4,5,*}

ABSTRACT

Cholesterol is an essential component of metazoan cellular membranes and it 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 in 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-stearoyl-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 relocate 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.

KEY WORDS: Biosensor, Cholesterol, Phosphatidylserine, Plasma membrane

INTRODUCTION

Despite the presence of a variety of phospholipids and sphingolipids within the cell, cholesterol is unique. Owing to its biophysical properties dictated by a rigid hydrophobic structure and small headgroup, cholesterol can readily flip-flop between the leaflets of membrane bilayers and can 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 sphingolipid–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). However, 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 whether this observation holds true for cholesterol.

The dynamics and functional roles of cholesterol in the cytosolic leaflet of the plasma membrane are currently unclear. Additionally, it is unknown whether inner leaflet membrane nanodomains exist or whether 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 (Börnig 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; Zaremborg et al., 2005; Jin et al., 2008). An alternative to filipin that has been developed more recently is to use cholesterol molecules directly conjugated to a fluorophore, such as BODIPY–cholesterol (Börnig and Geyer, 1974; Li et al., 2006; Hölttä-Vuori et al., 2008). However, exogenously added fluorophore-labeled 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). Although 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). The 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 analyzed 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 an mCherry-labeled D4 domain mutant with higher

¹Keenan Research Centre for Biomedical Science, St. Michael's Hospital, 209 Victoria Street, Toronto, ON, M5S 1T8, Canada. ²Department of Biochemistry, University of Toronto, Toronto, ON M5S 1A8, Canada. ³Department of Surgery, University of Toronto, Toronto, ON M5T 1P5, Canada. ⁴Institute of Medical Science, Faculty of Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada. ⁵Institute for Biomedical Engineering and Science Technology (iBEST) at Ryerson University and St. Michael's Hospital, Toronto, ON M5B 2K3, Canada.

*Author for correspondence (fairng@smh.ca)

affinity for cholesterol in the cytosol of mammalian cells. This probe, termed D4H, allows 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 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 reveal 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 cytolysins are known to bind to exofacial leaflet cholesterol, leading to pore formation (Dunstone and Tweten, 2012). Importantly, the fourth 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 by using flow cytometry and light microscopy (Fig. 1A; supplementary material Fig. S1A). The cholesterol dependence of GFP–D4 binding to the exofacial leaflet of the plasma membrane was confirmed by the use of methyl β -cyclodextrin (M β CD) to extract the cholesterol or by treating the cells with U18666A, a Niemann-Pick type C inhibitor, to deplete cholesterol from the plasma membrane (Fig. 1A; supplementary material Fig. S1A; 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 M β CD followed by incubation with recombinant GFP–D4, and analyzed the cells by using flow cytometry (Fig. 1B; supplementary material Fig. S1A). 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–M β CD complex led 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 material Fig. S2), whereas the protein bound to only the exofacial leaflets of the plasma membrane when incubated on ice or at room temperature (supplementary material Fig. S1A; Fig. S2). Taken together,

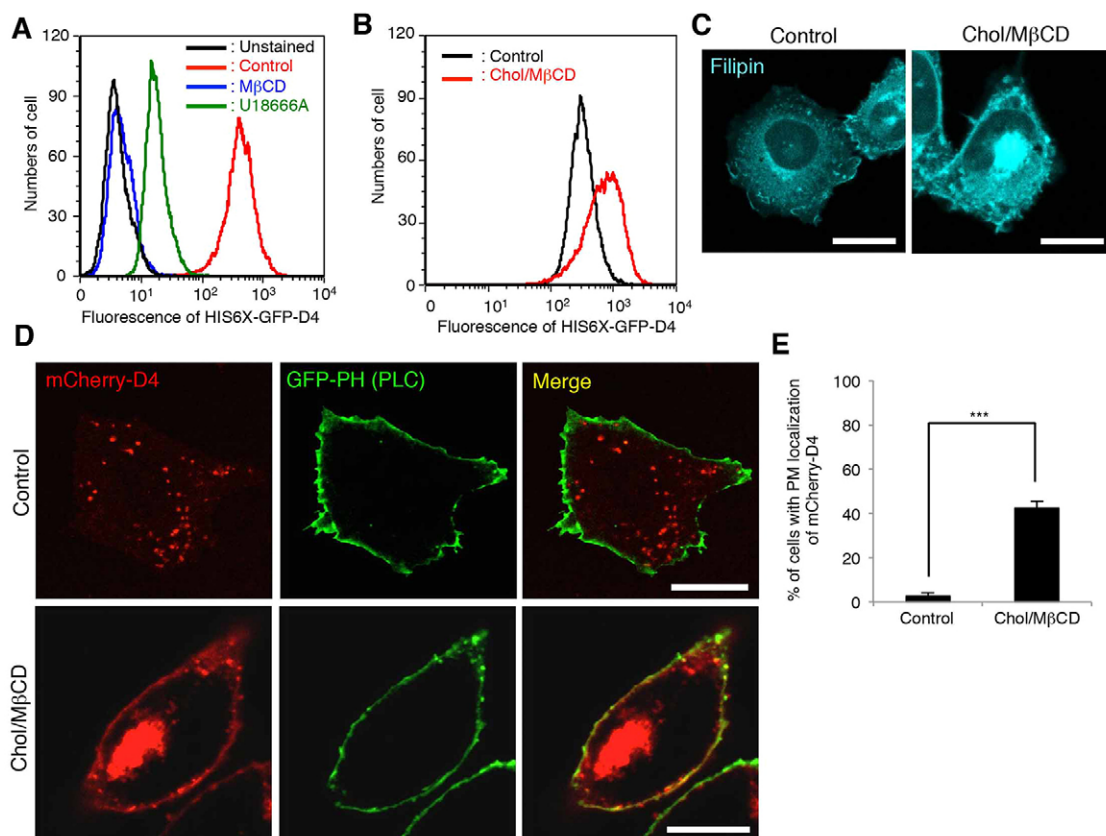


Fig. 1. D4 can detect cholesterol not in the cytosolic leaflets but in the exofacial leaflets of the plasma membrane. (A) Results of flow cytometric analysis of control CHO cells and cells treated with M β CD or U18666A incubated with recombinant HIS6X–GFP–D4 protein. (B) Results of flow cytometric analysis of cholesterol–M β CD complex (chol/M β CD)-loaded CHO cells treated with HIS6X–GFP–D4 protein. (C) Confocal images of chol/M β CD-loaded CHO cells stained with filipin. (D) Confocal images of chol/M β CD-loaded CHO cells expressing mCherry–D4 and GFP–PH–PLC δ . Scale bars: 10 μ m. (E) Quantification of the data shown in Fig. 1D. A total of 100 cells from three independent experiments were analyzed. PM, plasma membrane. Data show the mean $\pm$ s.e.m.; *** P < 0.001.

these results confirm that the D4 domain can act as a sensor of exofacial leaflet cholesterol by labeling at room temperature.

Based on the effectiveness of using 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 M β CD–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,E). 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 a higher affinity for cholesterol as a biosensor to monitor endogenous levels of cholesterol. To this end, we expressed an mCherry–D4^{D434S} mutant – which we have named D4H – that was previously shown to have a lower threshold for binding to 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 M β CD, 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,F). 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 assays (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 that of 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 material Fig. S3). These observations confirm 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 labeling of cholesterol by HIS6X–GFP–D4 or mCherry–D4H did not affect the intracellular distribution of cholesterol (supplementary material Fig. S4A). This approach to visualize cholesterol overcomes a major limitation of the canonical cholesterol probes such as filipin and fluorophore-labeled cholesterol, which 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.

Phosphatidylserine is required to retain cholesterol in the inner leaflet of the plasma membrane

The umbrella and condensed complex models are two related hypotheses to explain the interactions between cholesterol and other lipids in the plane of the membrane. Essentially, owing 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 microscopy results, we postulated that 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% of wild-type levels) resulting in ~80% decrease in 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 those of the parental CHO cells (Fig. 3A,B). As reported previously, the filipin-positive intracellular compartments in both cell types colocalized with the 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; supplementary material Fig. S1B). 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, whereas, in parallel, supplementing the medium with phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) had no effect (Fig. 4A; supplementary material Fig. S1B). Next, we examined both the localization of the remaining 20% of the PtdSer – using the biosensor Lact-C2 (Yeung et al., 2008) – and the localization of cholesterol, using mCherry–D4H, in PSB-2 cells. As illustrated in Fig. 4B,C, most of the remaining PtdSer in the PSB-2 cells was found in intracellular compartments in contrast to the distribution observed in 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, supplementing the cells with PtdSer caused both the PtdSer and cholesterol biosensors to relocalize to the plasma membrane (Fig. 4B,C). These results are consistent with PtdSer being important for the maintenance of cholesterol in the inner leaflet of the plasma membrane, and they indicate that PtdCho and PtdEtn cannot functionally replace PtdSer. To ensure that the binding of D4H to cholesterol was not influenced by PtdSer, we conducted both liposomal sedimentation and fluorescence resonance energy transfer (FRET)-based assays (Fig. 5A,B). In both assays, the presence of PtdSer had no significant impact on D4H binding to cholesterol. Taken 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 in the cytosolic leaflet. Additionally, the remaining cytosolic leaflet pools of cholesterol coincide with the presence of PtdSer.

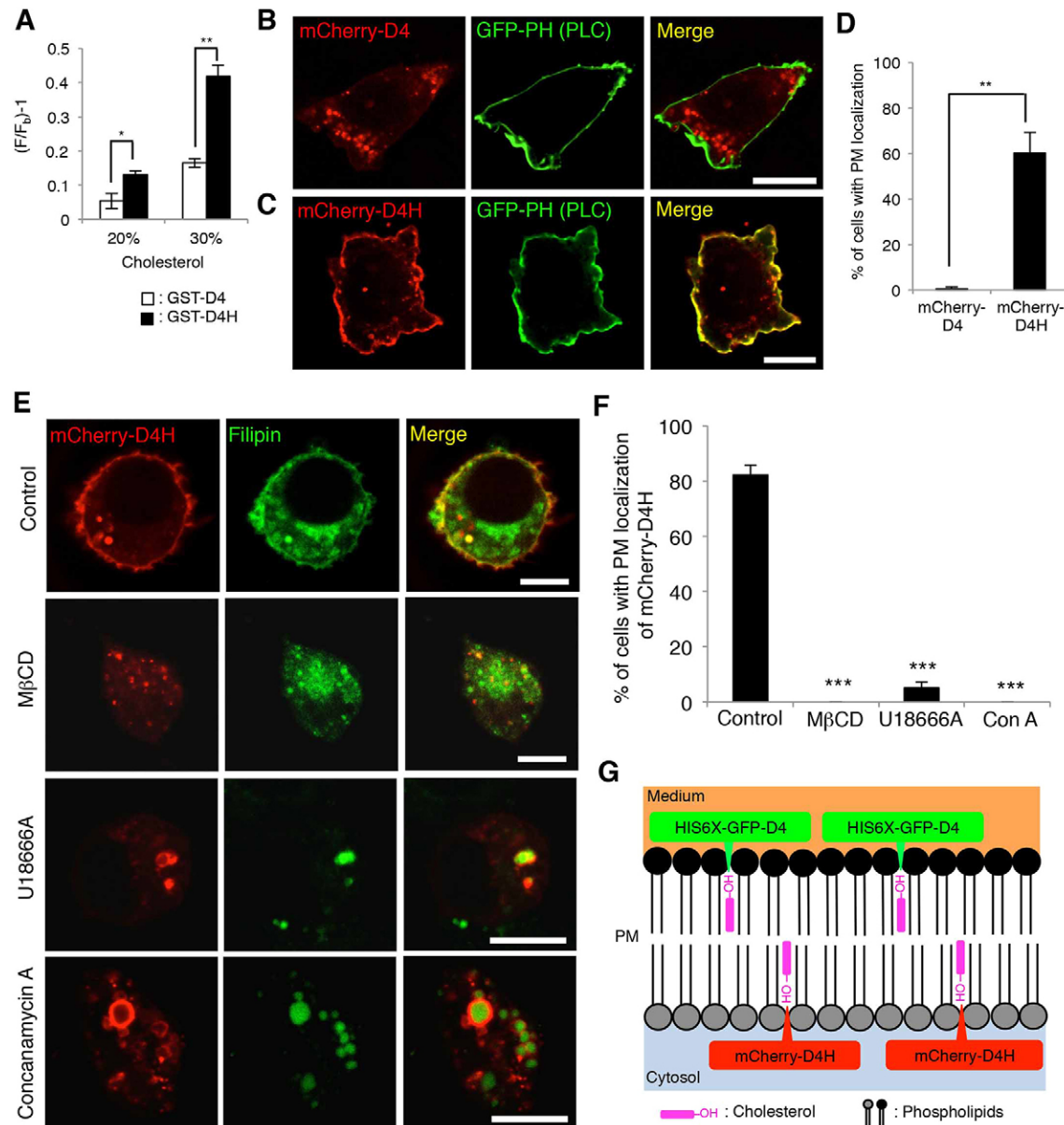


Fig. 2. Expressed mCherry-D4H allows the visualization of cholesterol in the cytosolic leaflets of the plasma membrane. (A) Binding of GST-D4 or GST-D4H to liposomes mimicking the cytosolic leaflet of the plasma membrane monitored by FRET. The lipid composition (mol %) of the liposomes was SOPS:liver PI:brain PI(4,5) P_2 :POPE:dansyl-PtdEtn:DOPC:cholesterol, 20:4:1:7.5:2.5:45:20 or SOPS:liver PI:brain PI(4,5) P_2 :POPE:dansyl-PtdEtn:DOPC:cholesterol, 20:4:1:7.5:2.5:35:30. The data show the mean $\pm$ s.e.m. ($n=3$); * $P < 0.05$; ** $P < 0.01$. (B) Confocal images of CHO cells expressing mCherry-D4 and GFP-PH-PLC δ . (C) Confocal images of CHO cells expressing mCherry-D4H and GFP-PH-PLC δ . (D) Quantification of Fig. 2B,C. A total of 100 cells from three independent experiments were analyzed. PM, plasma membrane. The data show the mean $\pm$ s.e.m.; ** $P < 0.01$. (E) Confocal images of filipin staining of Raw264.7 cells expressing mCherry-D4H. Scale bars: 10 μ m. (F) Quantification of Fig. 2E. A total of 100 cells from three independent experiments were analyzed. The data show the mean $\pm$ s.e.m.; *** $P < 0.001$. (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.

Acute alterations in PtdSer 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 led 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 mCherry-D4H relocalized from the plasma membrane to Lact-C2-positive endocytic structures (Fig. 6A,B). 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

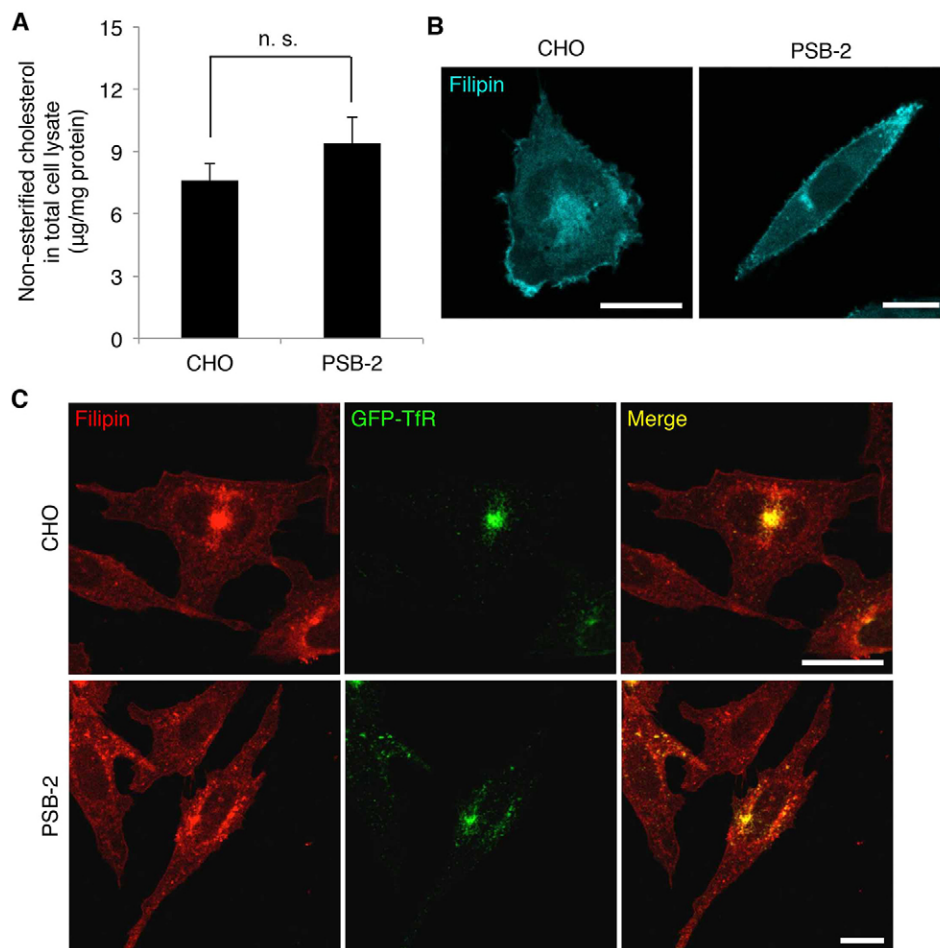


Fig. 3. Content and intracellular distribution of cholesterol in PSB-2 cells. (A) Cells were collected from one 10-cm dish and the cholesterol content of the cells was then measured using a cholesterol-oxidase-based Amplex Red kit. The data show the mean $\pm$ s.e.m. ($n=3$); n.s., not significant. (B) Confocal images of CHO cells and PSB-2 cells stained with filipin. (C) Confocal images of GFP-TfR-expressing CHO cells and PSB-2 cells stained with filipin are shown. Scale bars: 10 μ m.

binding and led to the relocation 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 induced the formation of liquid-ordered and disordered domains (as determined by the absence and presence of Rhodamine–PtdEtn, respectively) in cholesterol-containing GUVs, whereas 18:0-18:1 PtdCho (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 with 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 the variability that exists with regards to the strength of interactions between cholesterol and the different acyl chain species of PtdSer, with the interaction between cholesterol and SOPS being the strongest. Our findings are consistent with 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 whether 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 catalyzes the conversion of the hydroxyl group of cholesterol to generate chole-4-en-3-one 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 sphingomyelin-containing liposomes and, owing to its small headgroup, high in PtdEtn-containing liposomes. As predicted, the oxidase accessibility to cholesterol in sphingomyelin or SOPS-containing liposomes was lower, whereas that in SOPE-containing liposomes was higher than that of SOPC-containing liposomes (Fig. 7B). Next, to examine the fatty acyl chain 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

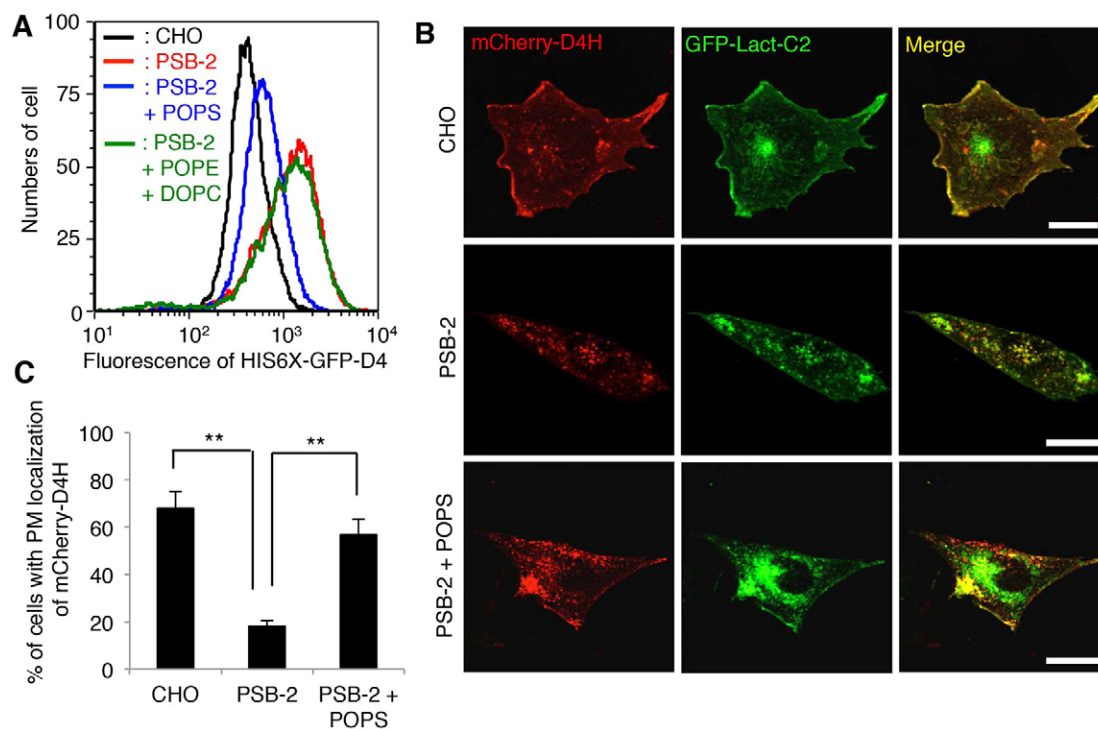


Fig. 4. The transbilayer distribution of cholesterol in the plasma membrane is defective in PSB-2 cells. (A) The results of flow cytometric analysis of CHO cells and PSB-2 cells treated with HIS6X-GFP-D4 protein. For rescue experiments, PSB-2 cells were incubated with lipids for 24 h. (B) Confocal images of CHO cells and PSB-2 cells expressing mCherry-D4H and GFP-Lact-C2. Scale bars: 10 μ m. (C) Quantification of the data shown in Fig. 4B. PM, plasma membrane. A total of 100 cells from three independent experiments were analyzed. The data show the mean $\pm$ s.e.m.; ** $P < 0.01$.

liposomes containing phosphatidic acid or in brain-derived PtdIns(4,5) P_2 -containing liposomes to SOPS and found a minimal impact (Fig. 7D). Taken together, these data indicate 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 phosphatidylserine-depleted and mislocalized cells

We examined the consequences of PtdSer depletion or altered transbilayer distribution of cholesterol in the plasma membrane. We predicted that, under conditions with increased amounts of GFP-D4, more cholesterol should be extractable with short

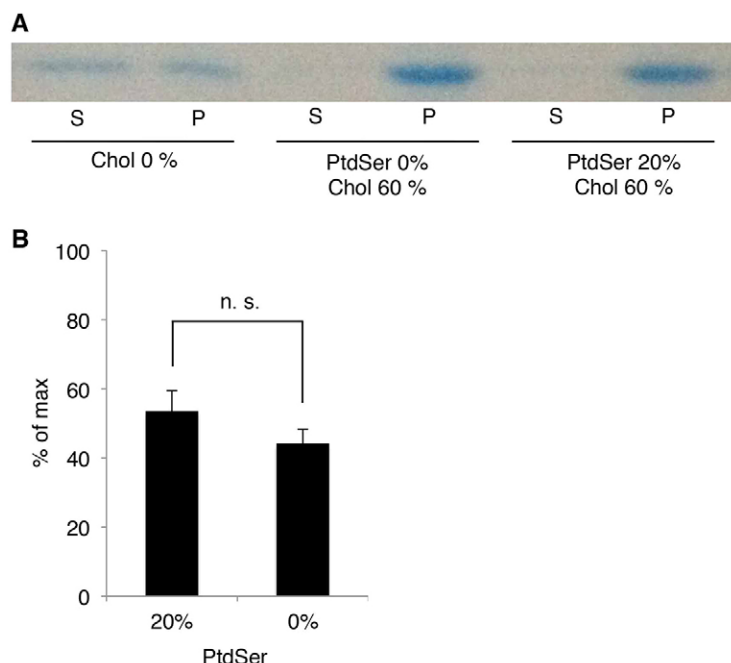


Fig. 5. PtdSer does not affect the binding of GST-D4H to cholesterol *in vitro*. (A) A representative image of a G250-stained gel from the *in vitro* cosedimentation assay. GST-D4H was mixed with liposomes for 15 min, then the mixture was spun at 100,000 g for 1 h. The resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The lipid compositions (mol %) of LUVs used in this experiment were as follows: 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) The 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) P_2 :POPE:dansyl-PtdEtn:DOPC:cholesterol, 20:4:1:7.5:2.5:35:30 or SOPS:liver PI:brain PI(4,5) P_2 :POPE:dansyl-PtdEtn:DOPC:cholesterol, 0:4:1:7.5:2.5:55:30. The data show the mean $\pm$ s.e.m. ($n=3$). n.s., not significant.

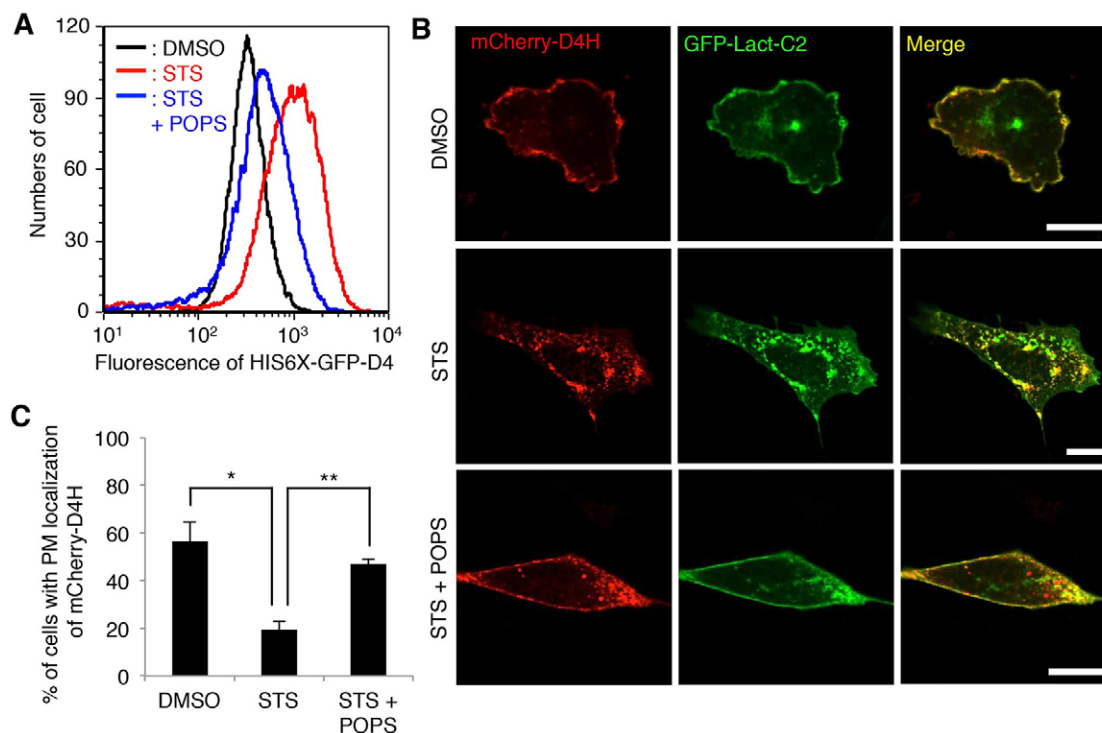


Fig. 6. Transbilayer distribution of cholesterol in the plasma membrane is defective in staurosporine-treated cells. (A) The results of flow cytometric analysis of STS-treated CHO cells incubated with HIS6X-GFP-D4 protein. Cells were incubated with STS (50 nM) for 24 h. For rescue experiments, STS-treated CHO cells were incubated with lipids for 2 h. (B) Confocal images of STS-treated CHO cells expressing mCherry-D4H and GFP-Lact-C2. Scale bars: 10 μ m. (C) Quantification of the data shown in Fig. 6B. PM, plasma membrane. A total of 100 cells from three independent experiments were analyzed. The data show the mean $\pm$ s.e.m.; * P < 0.05; ** P < 0.01.

(5 min) incubations with M β CD. For these experiments, cells were equilibrated with [³H]cholesterol and subjected to a 5-min exposure with 10 mM M β CD. In these experiments, 65–70% of the cholesterol was extracted by M β CD 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 show that it is easily extractable by extracellular M β CD when the plasmalemmal PtdSer is depleted.

Finally, we considered whether membrane proteins normally associated with membrane nanodomains would be impacted upon by the alterations in cholesterol transbilayer distribution. For these experiments, we chose 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), flotillin-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 palmitoylation on cysteine 34 (Liu et al., 2005). When flotillin-1-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 results observed in control cells (Fig. 8B). Importantly, this defect could be rescued by supplementation with PtdSer and restoration of the cholesterol distribution (Fig. 8B,C). Taken together, these results suggest that the altered cholesterol transbilayer distribution of the plasma membrane impacts upon the localization of cholesterol-dependent or nanodomain-associated proteins and allows for cholesterol to be more readily extracted from the cell.

DISCUSSION

In this study, we developed a genetically encoded cholesterol biosensor, mCherry-D4H, which allows us to 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 the analysis of the dynamics and organization of cholesterol in the cytosolic leaflet of the plasma membrane using advanced microscopy techniques (e.g. fluorescence recovery after photobleaching or super-resolution microscopy). Despite the advantages of this probe, it does have the same limitation that applies to all of these types of biosensors, in that they only have access to available lipid. Recent evidence suggests that cholesterol might 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 is able to detect cholesterol over a range from 20–60 mol% in liposomes that mimic the inner leaflet of the plasma membrane (supplementary material Fig. S4B). These results suggest that there is likely to be a minimal threshold of cholesterol required for the recruitment of the mCherry-D4H *in vivo*. Previous studies have shown that binding affinity of the full-length 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

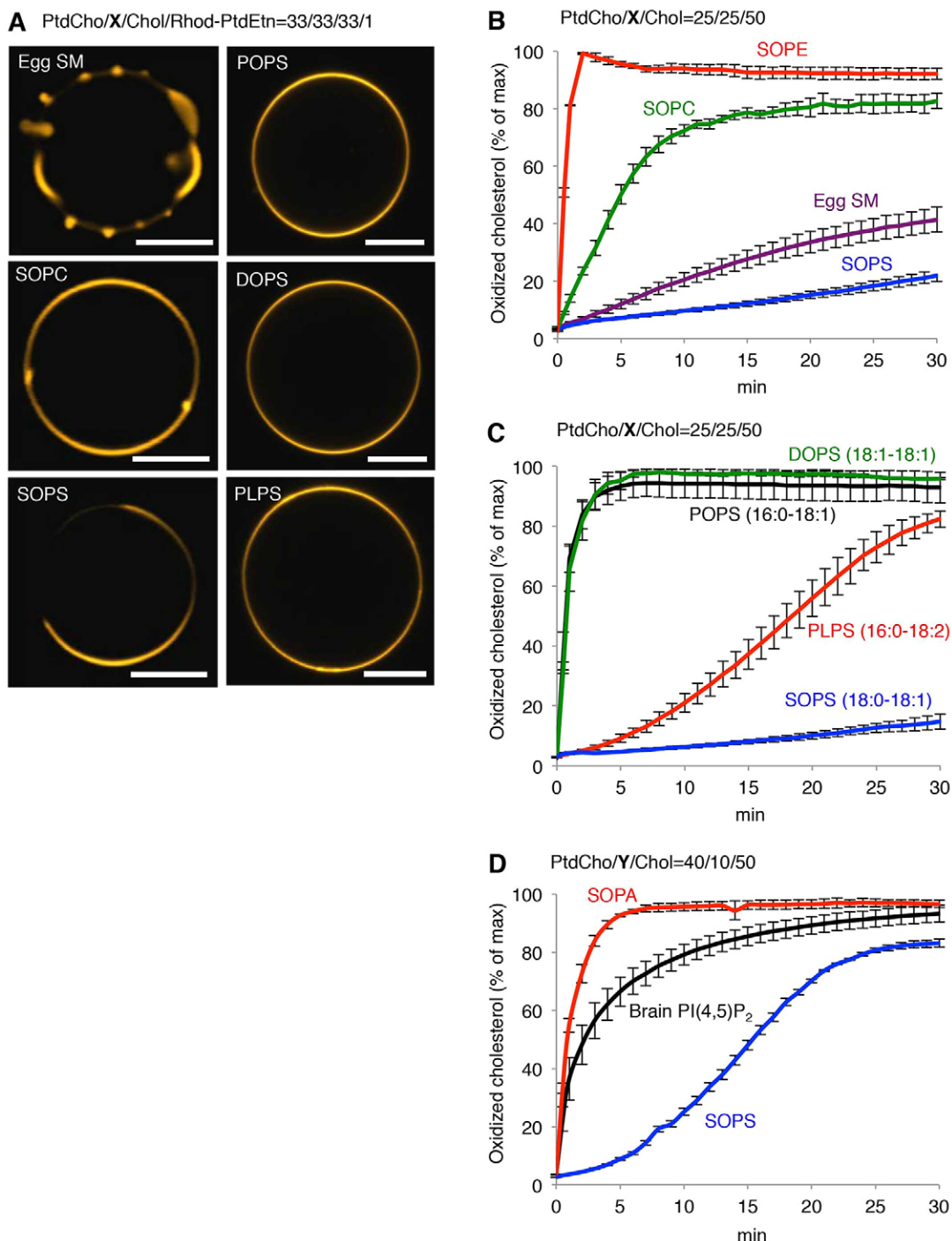


Fig. 7. SOPS can interact with cholesterol in model membranes. (A) Confocal images of giant unilamellar liposomes (GUVs). The lipid composition (mol %) of the liposomes was egg PtdCho:X:cholesterol (chol):Rhodamine–PtdEtn, 33:33:33:1. X was one of the following: egg SM, SOPC, SOPS, POPS, DOPS or PLPS. Scale bars: 5 μ m. (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, 25:25:50 (B,C). X was one of the following: SOPE, SOPC, egg SM, SOPS, DOPS, POPS or PLPS. The lipid composition (mol %) of the liposomes was egg PtdCho:Y:cholesterol, 40:10:50 (D). Y was one of the following: SOPA, brain PI(4,5)P₂ or SOPS. The data show the mean $\pm$ s.e.m. ($n=3$).

et al., 2013). This is likely due to alterations in the chemical potential of 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 plasma membrane is altered and, as a result, no longer accessible to the D4H probe. However, the experimental evidence is more consistent with there being a redistribution of cholesterol

from the inner leaflet of the plasma membrane to the exofacial leaflet. First, the *in vitro* data suggest that the 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 plasma membrane should not impact on the binding of recombinant D4 to the exofacial leaflet unless there is more cholesterol present. Third, the replacement of PtdSer with PtdCho

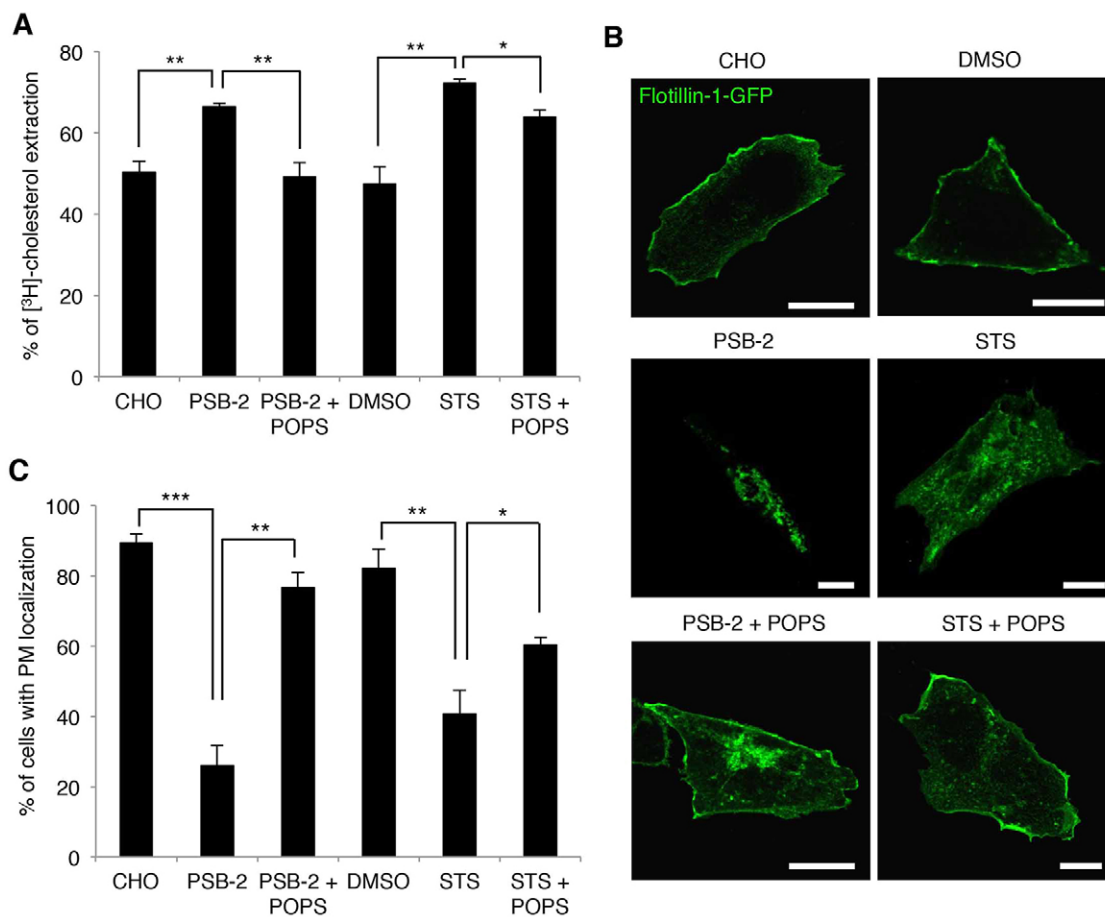


Fig. 8. Cytosolic leaflet cholesterol is required for the localization of flotillin-1. (A) Extraction of [^3H]cholesterol from the plasma membrane in PtdSer-manipulated cells. Cells were treated with 10 mM MbCD for 5 min at 37°C. The data show the mean $\pm$ s.e.m. ($n=3$); * $P<0.05$; ** $P<0.01$. (B) Confocal images of cells expressing human flotillin-1-GFP. Scale bars: 10 μm . (C) Quantification of the data shown in Fig. 8B. A total of 100 cells from three independent experiments were analyzed. PM, plasma membrane. The data show the mean $\pm$ s.e.m.; * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Cells were incubated with STS (50 nM) for 24 h. For rescue experiments, PSB cells and STS-treated CHO cells were incubated with PtdSer for 24 h and 2 h, respectively (A,B).

and PtdEtn, two lipids that shield cholesterol less effectively than PtdSer and sphingomyelin, did not restore the binding of D4H to the plasma membrane. Taken together, we believe the evidence indicates that, 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 in 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 is involved 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 revealed lipid clusters (Mizuno et al., 2011; Zhou et al., 2014). 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 trafficking of other lipids or their 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 the plasma membrane, 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 might help to regulate the co-segregation of these two lipids (Mesmin et al., 2011; Maeda et al., 2013; Olkkonen and Li, 2013).

MATERIALS AND METHODS

Plasmids

The pET28b vector with GFP-D4 was a kind gift from Dr Yoshiko Ohno-Iwashita (Iwaki Meisei University, Japan). D4 was amplified using this vector as a template for PCR using the following pairs of primers:

5'-GCGCTCGAGCCAAGGGAAAAATAAACTTAGA-3' (D4 sense primer) and 5'-GCGGAATTCTTAATTGTAAGTAATACTAG-3' (D4 antisense primer). The PCR product was introduced into pmCherry-C1 vector at the *XhoI/EcoRI* site. D4 (D434S), named D4H, was generated with the following pairs of primers: 5'-CTCACTATTCAACAGT-AATACCTCT-3' (D434S sense primer) and 5'-CTGTTTGTAGATTGTAATTTCCATC-3' (D434S antisense primer) from pmCherry-C1 vector with D4 using the 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 primers: 5'-GCGCTCGAGCCATGTTTTTC-ACTGTGGCCC-3' (flotillin-1 sense primer) and 5'-CGCGAATTCTGGCTGTTCTCAAAGGCTTGT-3' (flotillin-1 antisense primer). The product was introduced into the pEGFP-N1 vector at the *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'-GCGGGATCCAAGGGAAAA-ATAAACTTAGA-3' (D4 sense primer 2) and 5'-CGCGAATTCTTAATTGTAAGTAATACTAG-3' (D4 antisense primer 2). The products were introduced into pGEX-6P1 vector at the *BamHI/EcoRI* site. The GFP-Lact-C2, GFP-PH-PLC δ , RFP-PH-PLC δ and GFP-TfR plasmids were kind gifts from Dr Sergio Grinstein (The Hospital for Sick Children, Toronto, Canada).

Purification of GST-D4 and GST-D4H

Escherichia 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 medium at 37°C with constant shaking until the OD₆₀₀ reached 0.8. Cultures were induced with 1 mM IPTG for 5 h 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 analyzed 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 medium at 37°C with constant shaking until the OD₆₀₀ reached 0.4. Cultures were induced with 0.5 mM IPTG for 4 h 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 with 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 analyzed 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) PtdSer (POPS), 1-stearoyl-2-oleoyl (SO) PtdSer (SOPS), 1,2-dioleoyl (DO) PtdSer (DOPS), 1-palmitoyl-2-linoleoyl (PL) PtdSer (PLPS), PO PtdEtn (POPE), SOPE, dansyl-PtdEtn, Rhodamine-PtdEtn, DO PtdCho (DOPC), SOPC, SO phosphatidic acid (SOPA), egg yolk L- α -PtdCho (egg PtdCho), 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 μ mol) 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 lipid was suspended. For large unilamellar vesicle (LUV) formation, 1 mM of MLVs was incubated at 37°C for 1 h. During this 1-h 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 (Hoger et al., 2009). Briefly, 300 μ l of 1% agarose with ultralow melting temperature was laid on a slide glass, spread evenly and placed on a heater at 40°C for 3 h to dry the gel. Then, 50 μ l 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 h, and then GUVs in PBS. Vesicles were used fresh; storage at 4°C before usage did not exceed 24 h.

In vitro liposome fluorescence resonance energy transfer assay

Binding of GST-D4 and GST-D4H to liposomes was measured by fluorescence resonance energy transfer (FRET) between donor tryptophan residues and 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 μ M) were incubated with purified proteins (800 nM) at 37°C for 20 min, and then fluorescence intensity was measured in a 2×10×45-mm 18F-Q-10 quartz cuvette (Starna Cells) using a SpectraMax M5e (Molecular Devices) at room temperature. Excitation and emission wavelengths were 280 and 510 nm, respectively, with 9 nm (excitation) and 15 nm (emission) slit width. Data were analyzed as described previously (Gilbert et al., 1990). The lipid compositions (mol %) of LUVs used in this experiment were as follows: SOPS:liver PI:brain PI(4,5)P₂:POPE:dansyl-PtdEtn:DOPC:cholesterol, 20:4:1:7.5:2.5:5-65:0-60 or SOPS:liver PI:brain PI(4,5)P₂:POPE:dansyl-PtdEtn:DOPC:cholesterol, 0-20:4:1:7.5:2.5:35-55:30. FRET efficiency was calculated using the formula:

$$\text{FRET efficiency} = (F/F_b) - 1$$

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 cosedimentation assay used in this study was basically the same as that described previously (Uchida et al., 2011). Briefly, 10 μ g of GST-D4H protein was incubated with 50 nmol LUVs in PBS (100 μ l) at 37°C for 15 min, and the mixture was centrifuged at 100,000 g for 1 h 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 were as follows: SOPS:POPE:DOPC:cholesterol, 20:20:60:0; SOPS:POPE:DOPC:cholesterol, 20:20:0:60; SOPS:POPE:DOPC:cholesterol, 0:20:20:60.

In vitro cholesterol oxidase accessibility assay

Cholesterol oxidase oxidizes the 3-hydroxy group of cholesterol and yields H₂O₂ and the ketone product (chole-4-en-3-on). In this experiment, the H₂O₂ generated by cholesterol oxidase from cholesterol in LUVs was detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex). In the presence of horseradish peroxidase (HRP), Amplex dye reacts with H₂O₂ 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 μ l PBS were prepared in 96-well plates and 50 μ l of working solution containing 0.3 mM Amplex Red (Invitrogen), 2 U/ml cholesterol oxidase from *Streptomyces* (Sigma) and 2 U/ml HRP (Sigma) in PBS was added to each well. Then, the plate was immediately placed in

a SPECTRA max PLUS (Molecular Devices) and the absorbance at 571 nm was 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 X-100 and the absorbance at 571 nm was monitored every 1 min for 30 min at 37°C. The lipid compositions (mol %) of LUVs used in this experiment were as follows: egg PtdCho:X:cholesterol, 25:25:50 or egg PtdCho:Y:cholesterol, 10:40:50. X is one of the following: SOPE, SOPC, egg SM, SOPS, DOPS, POPS or PLPS. Y is one of the following: SOPA, brain PI(4,5) P_2 or SOPS.

Cell culture and transfection

RAW264.7 cells were maintained at 37°C with 5% CO₂ 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 h. 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 relocation 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 h. For supplementation of lipids, cells were incubated with 30 µM MLVs in serum-containing Ham's F-12 medium for 24 h or 2 h at 37°C. Raw264.7 cells and PSB-2 cells were transiently transfected with plasmids using Eugene HD (Promega) according to the manufacturer's instructions. Wild-type CHO-K1 cells were transiently transfected with plasmids using Eugene 6 (Promega) according to the manufacturer's instructions. Cells were fixed with 3.7% formaldehyde-PBS for 30 min at room temperature at 24 h 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 serum-free RPMI medium for 15 min at room temperature, washed with PBS and observed live at room temperature. For quantitative analysis by 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 a FACS Calibur (BD Bioscience) with CellQuest software (BD Bioscience).

Filipin staining

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

Confocal microscopy

At 24 h post-transfection, cells were imaged live or fixed with 3.7% formaldehyde-PBS for 30 min at room temperature and mounted for later examination. Images were acquired using a Zeiss LSM 700 inverted confocal microscope (Zeiss) with a Plan-Apochromat 60×/1.4 NA oil objective and 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 greater than twofold enrichment of the mean fluorescence intensity (MFI) of the probe compared with fluorescence in the cytosol using the following equation: (MFI of plasma membrane – MFI of background)/(MFI of cytosol – MFI of background). MFI was measured for regions of interest using ImageJ software.

Cholesterol supplementation and measurement of cholesterol

Cholesterol was added to cells by incubating cells for 1 h at 37°C with 50 µg/ml cholesterol complexed with 1.5 mg/ml MβCD in serum-free F12 medium (Blom et al., 2001). Cellular cholesterol measurements were performed using the Amplex Red Cholesterol Assay Kit (Invitrogen), according to the manufacturer's instructions.

Cholesterol extraction assay

Cholesterol extraction assays were performed as described previously (Low et al., 2012). Briefly, cells were incubated with 0.5 µCi/well [³H]cholesterol for 24 h at 37°C in 12-well plates. After equilibration incubation with serum-free medium for 24 h at 37°C, cells were incubated with 10 mM MβCD for 5 min at 37°C. Radioactivity in both the medium and cell lysates was measured using a scintillation counter (Beckman Coulter LS6500, Beckman). For calculation of the rates of cholesterol extraction, the following formula was used: cholesterol extraction = (medium counts × dilution factor) / [(medium counts × dilution factor) + (cell counts × dilution factor)]. The specific extraction is calculated as the difference between the rate in the presence or absence of MβCD (blank). Final extraction = cholesterol extraction – blank extraction.

FPP assay

The fluorescence protease protection (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 room temperature, protease K (50 µg/ml) was added to cells in the serum-free RPMI medium. After incubation for 5 min at room temperature, cells were observed live at room temperature. To permeabilize the plasma membrane, cells were treated with digitonin (20 µM) in the serum-free RPMI medium for 1 min at room temperature, and then protease K (50 µg/ml) was added to cells following washing with PBS. After incubation for 5 min at room temperature, cells were observed live at room temperature.

Statistical analysis

Statistical analysis was carried out using Student's two-tailed *t*-test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.164715/-/DC1>

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