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Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol

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Abstract

Controlled distribution of lipids across various cell membranes is crucial for cell homeostasis and regulation. We developed an imaging method that allows simultaneous *in situ* quantification of cholesterol in two leaflets of the plasma membrane (PM) using tunable orthogonal cholesterol sensors. Our imaging revealed marked transbilayer asymmetry of PM cholesterol (TAPMC) in various mammalian cells, with the concentration in the inner leaflet (IPM) being ~12-fold lower than that in the outer leaflet (OPM). The asymmetry was maintained by active transport of cholesterol from IPM to OPM and its chemical retention at OPM. Furthermore, the increase in the IPM cholesterol level was triggered in a stimulus-specific manner, allowing cholesterol to serve as a signaling lipid. We found excellent correlation between the IPM cholesterol level and cellular Wnt signaling activity, suggesting that TAPMC and stimulus-induced PM cholesterol redistribution are crucial for tight regulation of cellular processes under physiological conditions.

Cell membranes contain a wide variety of lipid molecules with different structural and functional properties. Many lipids are asymmetrically distributed across various cell

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Author Contributions

R.S. contributed to sensor development and other biochemical studies and S.-L.L. performed all imaging work. L.W., S.S., R.K.B., R.A.W. and I.L. contributed to cell studies. J.H.J., K.B. and K.-P.K. performed MS analysis and K.U. contributed to lipid transporter studies. M.J.O.C. and D.L. prepared fluorophores. E.S. participated in sensor preparation. W.C. conceived the work and wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the [online version of the paper](#).

membranes, most notably in the PM of mammalian cells^{1–3}. Transbilayer asymmetry of membrane lipids is maintained by complex mechanisms involving lipid transporters and is crucial for cell homeostasis and regulation². However, conventional methods for determining transbilayer distribution of lipids fail to provide spatiotemporally resolved quantitative information because they depend on lipid extraction, use of labeled lipids or indirect estimation^{1–3}. The unavailability of direct real-time quantification methods makes it difficult to accurately determine the degree of dynamic transbilayer asymmetry of lipids and to elucidate when and how changes in transbilayer distribution of lipids modulate cellular processes.

Cholesterol is a major lipid in the PM of mammalian cells⁴ and has diverse structural and functional roles^{5,6}. Cellular unesterified cholesterol, which is either derived from low-density lipoprotein-receptor-mediated endocytosis or synthesized *de novo* in the endoplasmic reticulum^{5–7}, is primarily (up to 90%) localized in the PM⁸, where it constitutes 10–45 molar percentage (mol%) of total PM lipids^{8–10}. This high level of cholesterol is essential for the physical integrity of the PM. Cholesterol has been implicated in structural and functional modulation of integral membrane proteins¹¹ and in the formation of cholesterol-rich membrane domains, such as membrane (lipid) rafts¹². Recently, an increasing number of cellular proteins have been reported to bind cholesterol with varying affinity^{13–15}. In particular, cholesterol in the IPM might regulate various cell signaling pathways by specifically interacting with cytosolic scaffold proteins in a stimulus-dependent manner^{14–16}. In addition, IPM cholesterol levels have been reported to modulate neurotransmitter receptor trafficking¹⁷, social behaviors of cells¹⁸, and cell cytoskeleton and motility¹⁹. Collectively, these studies point to a potential link between the IPM cholesterol level ([Chol]_i) and diverse cellular processes and suggest the possibility of TAPMC.

Cholesterol levels in cell membranes are tightly regulated by sterol-sensing mechanisms that control cholesterol biosynthesis, uptake and transport^{7,20}. However, the actual cholesterol concentrations at the two leaflets of PM and other intracellular cell membranes have not been accurately determined because currently available methods^{21–24} depend on indirect and qualitative (or semiquantitative) analyses that primarily employ membrane-disrupting probes, such as filipin, or non-natural cholesterol derivatives. To overcome these technical difficulties, we developed tunable orthogonal cholesterol sensors that allow simultaneous *in situ* quantification of cholesterol in two PM leaflets and other cell membranes with high sensitivity and accuracy. Quantitative live-cell imaging using these sensors provided new information about the spatiotemporal distribution of cholesterol across PM in mammalian cells, which is crucial for cell homeostasis and regulation.

RESULTS

Development of tunable orthogonal cholesterol sensors

Subcellular localization of diverse cellular lipids has typically been monitored using fluorescence-protein-tagged lipid-binding proteins²⁵. Despite its popularity due to experimental convenience, this approach has major drawbacks, most notably its inability to provide robust quantitative information^{25,26}. We recently developed an *in situ* quantitative imaging analysis that allows quantification of lipids in live cells with minimal perturbation

of cellular processes²⁷. Our method employs lipid sensors that are prepared by labeling engineered lipid-binding domains with a solvatochromic fluorophore. Lipid binding of these sensors results in a large solvatochromic shift of a fluorophore, from which the lipid concentration can be accurately determined in a spatiotemporally resolved manner through ratiometric calibration and analysis (Fig. 1a). Given that local concentrations of a lipid in two leaflets of cell membranes may vary widely, application of this technology to quantitative determination of transbilayer distribution of a lipid would require tunable lipid sensors covering a broad range of lipid concentrations.

To achieve simultaneous *in situ* quantification of cholesterol at OPM and IPM, we first prepared cholesterol-specific molecular sensors from the D4 domain of perfringolysin O (PFO), which is known to have high specificity for cholesterol^{28–30}. Although PFO is a bacterial toxin that disrupts the PM of mammalian cells, the isolated D4 domain is not toxic²⁹. Despite high cholesterol specificity, the wild-type (WT) D4 domain is not well suited for quantitative determination of transbilayer distribution of cholesterol because its cholesterol binding requires a high threshold cholesterol concentration, has a narrow dynamic range and highly depends on the lipid environment in which cholesterol is embedded^{21,23,24,30}. We therefore developed a strategy of preparing tunable cholesterol sensors with incrementally higher cholesterol affinity by systematically introducing Trp, which enhances the membrane affinity of soluble proteins^{31,32}, to the membrane binding surface of the protein (Supplementary Results, Supplementary Fig. 1a). This approach successfully generated a panel of D4 domain mutants with partially overlapping cholesterol affinity. These mutants were then labeled on a single Cys (C459) with two solvatochromic fluorophores with minimal spectral overlap, for example, acrylodan (DAN)²⁷ and NR3 (ref. 33), respectively, to generate a collection of tunable orthogonal sensors (Fig. 1a) that seamlessly cover a wide dynamic range of cholesterol concentration down to <1 mol% (Fig. 1b,c). All these mutants retained high cholesterol specificity after labeling with DAN and NR3 (Supplementary Fig. 1b,c). In addition, DAN- and NR3-labeled D4 sensors had faster membrane binding kinetics than their unlabeled counterparts (Supplementary Fig. 1d). Furthermore, DAN- and NR3-labeled D4 sensors, presumably as a result of their improved membrane penetration activity conferred by amphiphilic fluorophores²⁷, showed little to no dependence on the lipid environment of cholesterol-containing vesicles (Supplementary Fig. 1e–k and Supplementary Table 1). For example, DAN-D434A (Supplementary Fig. 1e–g) and DAN-WT (Supplementary Fig. 1h,i), which were employed for quantification of the cholesterol concentration in the OPM ($[Chol]_o$), exhibited no dependence on either the content of sphingomyelin (SM) and ceramide or the nature of *sn*-2 acyl group of phosphatidylcholine (PC) in the OPM-mimetic giant unilamellar vesicles (GUVs) during calibration. Similarly, NR3-YDA (Supplementary Fig. 1j) and NR3-QYDA (Supplementary Fig. 1k), which were used for $[Chol]_i$ quantification, were insensitive to the relative composition of phosphatidylethanolamine (PE) and phosphatidyleserine (PS) and the acyl chain composition of PC in IPM-mimetic GUVs. Collectively, the favorable membrane binding properties of our sensors make them ideally suited for cellular cholesterol quantification.

Given its spatial resolution limitation, optical microscopy, even with super-resolution techniques, cannot resolve OPM and IPM lipid signals that are ≈ 5 nm apart. We overcame

this obstacle by spatially isolating two orthogonal cholesterol sensors, taking advantage of the fact that our D4-domain-based cholesterol sensors cannot cross the PM (Supplementary Fig. 2a). That is, those sensors added to the media exclusively detected cholesterol in the OPM, whereas those microinjected into the cells only monitored cholesterol in the IPM and the cytosolic leaflets of intracellular membranes. This allows for unambiguous simultaneous monitoring of OPM and IPM signals through multi-channel detection of spectrally orthogonal sensors. When extracellularly added to HeLa cells, DAN- or NR3-labeled WT and other mutant proteins gave strong signals at OPM, and ratiometric analysis of the fluorescence signals using *in vitro* calibration curves (Supplementary Fig. 1e–k) led to accurate, spatiotemporally resolved quantification of $[\text{Chol}]_o$ (Supplementary Fig. 2b). Most of them, however, yielded little to no detectable signal in the IPM and other intracellular membranes when microinjected into the cells (Supplementary Fig. 2c), suggesting that $[\text{Chol}]_i$ and cholesterol concentrations at intracellular membranes were much lower than $[\text{Chol}]_o$. When the two sensors with the highest cholesterol affinity (that is, NR3-YDA and NR3-YQDA) were microinjected, however, we clearly detected signals at the IPM, from which $[\text{Chol}]_i$ could be quantified in a spatiotemporally resolved manner (Supplementary Fig. 2d). For determination of both $[\text{Chol}]_i$ and $[\text{Chol}]_o$, we used two different sensors with partially overlapping dynamic ranges, which yielded similar results, confirming the accuracy of our method (Supplementary Fig. 2b,d). Although we focused on accurate quantification of $[\text{Chol}]_i$ and $[\text{Chol}]_o$, we also detected signals in intracellular membranes (Supplementary Fig. 2d). The average cholesterol concentration in the cytosolic faces of intracellular membranes (5.9 ± 2.1 mol%; Supplementary Fig. 2d) was comparable to the estimated cholesterol level (≈ 5 mol%) at the endoplasmic reticulum³⁴, giving further credence to the accuracy of our cholesterol quantification. In addition, cholesterol-binding-deficient mutants³⁵ of DAN-D434A and NR3-YDA failed to show OPM and IPM localization, respectively, when extracellularly added or microinjected, precluding the possibility that membrane signals derived from cholesterol-independent binding to membranes or membrane proteins (Supplementary Fig. 2e). Finally, actin disruption by latrunculin A did not affect $[\text{Chol}]_i$ quantification, indicating that the low $[\text{Chol}]_i$ value was not caused by a limited access of NR3-YDA to the PM as a result of the presence of the actin cytoskeleton (Supplementary Fig. 2f).

Transbilayer asymmetry of PM cholesterol revealed

When $[\text{Chol}]_o$ and $[\text{Chol}]_i$ in HeLa cells were simultaneously quantified employing DAN-D434A and NR3-YDA, respectively, we found that both $[\text{Chol}]_o$ and $[\text{Chol}]_i$ fluctuated spatiotemporally (see Supplementary Videos 1 and 2) and, most notably, the average $[\text{Chol}]_i$ was ~ 12 -fold lower than that of $[\text{Chol}]_o$ (Fig. 1d). This marked TAPMC was also observed in other mammalian cell lines, including HEK293 and NIH3T3 cells, and primary colon epithelial cells, although the degree of asymmetry varied to some degree (Supplementary Fig. 3 and Supplementary Table 2).

The average value of cholesterol concentration in the PM from our simultaneous quantification of $[\text{Chol}]_o$ and $[\text{Chol}]_i$ was ≈ 22 mol%, which fell in the range of the reported value of 19–40 mol%^{8–10}. To check the accuracy of our cholesterol quantification, we directly determined the total concentration of cholesterol in the PM of HeLa cells by

multiple reaction-monitoring-based quantitative mass spectrometry (MS). Specifically, we determined, under the same conditions, the absolute concentrations of all major lipid species in the PM isolated from HeLa cells by means of individual standard calibration curves constructed using external lipid standards (Supplementary Fig. 4a). We demonstrated the high quality of our purified PM fractions by western blotting analysis using various organelle markers; that is, when compared with total cell extracts, PM samples were largely devoid of cross-contamination of other organelles (Supplementary Fig. 4b). Notably, our MS method offered higher accuracy and precision than previously reported indirect cholesterol quantification using different quantification kits, which could introduce substantial variations and errors^{10,23}. Calculation of the total cholesterol concentration in the PM by our lipid quantification yielded 21.8 ± 1.1 mol% (Supplementary Fig. 4c and Supplementary Table 3), which is consistent with our sensor-based quantification of total PM cholesterol. This agreement supports the accuracy of our sensor-based quantification of $[\text{Chol}]_o$ and $[\text{Chol}]_i$.

We then treated HeLa cells with methyl- β -cyclodextrin (M β CD), which has commonly been used to deplete cholesterol from the PM³⁶. We treated the cells for 1-h treatment with 5 mM M β CD and found a 3.6-fold reduction in $[\text{Chol}]_o$, whereas $[\text{Chol}]_i$ was reduced by only 50% (Fig. 1e and Supplementary Table 2). By contrast, treatment of HeLa cells with 5 mM cholesterol-loaded M β CD (1:1 mol ratio) increased $[\text{Chol}]_i$ by 73%, with a negligible effect on $[\text{Chol}]_o$ (Fig. 1f and Supplementary Table 2). These data indicate that M β CD-mediated cholesterol depletion or repletion has distinctly different effects on $[\text{Chol}]_i$ and $[\text{Chol}]_o$ as a result of TAPMC, which may be important for understanding how these common treatments modulate various cellular processes.

Stimulus-dependent redistribution of PM cholesterol

The low average value of $[\text{Chol}]_i$ in quiescent cells suggests that an increase in $[\text{Chol}]_i$ ($[\text{Chol}]_i$) might be induced in a stimulus-dependent manner to trigger cell-signaling activities. We first monitored $[\text{Chol}]_i$ in HeLa cells in response to Wnt ligands on the basis of a report that a canonical Wnt ligand, such as Wnt3a, specifically facilitates cholesterol-dependent PM recruitment and activation of dishevelled (Dvl), a key scaffold protein in Wnt signaling¹⁵. Wnt3a treatment clearly induced $[\text{Chol}]_i$ in a dose- and time-dependent manner (Fig. 2a). Spatially averaged $[\text{Chol}]_i$ was quantitatively correlated with the spatially averaged decrease in $[\text{Chol}]_o$ ($[\text{Chol}]_o$) at a given time (Fig. 2b). These results suggest that cholesterol is rapidly redistributed between OPM and IPM in response to Wnt3a stimulation. This notion is further supported by the finding that the total cholesterol content of the PM, as determined by MS analysis of isolated PM of HeLa cells, remained essentially unchanged after Wnt3a treatment (Supplementary Fig. 4d). In addition, $[\text{Chol}]_i$ was spatiotemporally correlated with the increase in PM-bound enhanced GFP (EGFP)-tagged Dvl2 (Fig. 2c), verifying the direct link between the stimulus-dependent $[\text{Chol}]_i$ and Dvl PM recruitment. This also indicates that our cholesterol sensor does not substantially perturb the Wnt signaling system. The specific nature of the Wnt3a effect was demonstrated by the lack of $[\text{Chol}]_i$ by the non-canonical Wnt ligands Wnt5a and Wnt11 (Fig. 2a).

Mechanism of TAPMC

Cholesterol is known to spontaneously and rapidly translocate the lipid bilayer (flip-flop)^{37,38}. Thus, the observed TAPMC points to the existence of a directional cholesterol transport mechanism. Several types of lipid transporters and lipid transfer proteins have been implicated in cholesterol transport at the PM^{19,39} and in intracellular cholesterol transport^{5–7,40,41}. When we suppressed the expression of all major lipid transporters and cholesterol transfer proteins by siRNA (Supplementary Fig. 5a), inhibition of only two ATP-binding cassette (ABC) transporters, ABCA1 and ABCG1, had a significant effect on TAPMC (Fig. 3 and Supplementary Table 2). ABCA1 and ABCG1 have been reported to be involved in cellular cholesterol and phospholipid efflux onto high-density lipoprotein particles or apolipoprotein A1, but their mechanisms of action, including their potential role as lipid translocases, have not been determined^{19,42}. In addition, although ABCA1 is known to be localized in the PM, the exact cellular location of ABCG1 remains unknown⁴². We found that ABCA1 and ABCG1 single knockdown (KD) caused 2.3- and 1.6-fold reductions of the $[\text{Chol}]_o$ to $[\text{Chol}]_i$ ratio ($[\text{Chol}]_o/[\text{Chol}]_i$), respectively, whereas KD of both yielded a 2.7-fold decrease (Fig. 3a,b and Supplementary Table 2). This suggests that ABCA1 and ABCG1 have important and non-redundant roles in maintaining TAPMC. In all of the ABCA1 and ABCG1 KD cells, $[\text{Chol}]_i$ was comparable with $[\text{Chol}]_o$ (Supplementary Table 2). In addition, RNAi inhibition of three proteins that have been reported to be involved in intracellular trafficking of cholesterol^{41,43}, ORP5, Stard4 and NPC1, and the vesicle fusion inhibitor N-ethylmaleide had little effect on $[\text{Chol}]_o/[\text{Chol}]_i$ (Supplementary Table 2). These results support the notion that ABCA1 and ABCG1 are involved in direct transport of cholesterol from IPM to OPM.

We also asked whether a mechanism exists that slows or prevents the inward transport of OPM cholesterol, which would otherwise counterbalance the ABCA1/G1-driven outward cholesterol transport. Between two major lipids in the OPM, PC and SM, SM has a higher affinity for cholesterol¹². We therefore tested the possibility that SM-cholesterol association circumvents its rapid flipping by treating HeLa cells with sphingomyelinase (SMase), which converts SM into ceramide. Treatment of HeLa cells with SMase (0.2 units/ml for 1 h) caused a 2.8-fold reduction of $[\text{Chol}]_o/[\text{Chol}]_i$ (Fig. 3a,b and Supplementary Table 2), whereas hydrolysis of PC by PC-specific phospholipase C (5 units/ml for 15 min) had a negligible effect (Supplementary Table 2). The observed effect of SMase treatment was not a result of movement of cholesterol from PM to internal organelles, as our MS analysis revealed that the total cholesterol content of the PM remained essentially unchanged after SMase treatment (Supplementary Fig. 4d). In addition, this effect was not caused by the formation of ceramide that might affect the membrane binding of our cholesterol sensors, as DAN-D434A was insensitive to the presence of ceramide in the OPM-mimetic GUVs during calibration (Supplementary Fig. 1e). The combination of ABCA1/G1 double KD and SMase treatment reduced $[\text{Chol}]_o/[\text{Chol}]_i$ to ≈ 1.4 (Fig. 3a,b). Taking into account the fact that suppression of ABCA1/G1 expression was incomplete in the double KD HeLa cells (Supplementary Fig. 5b,c), these results suggest that the combination of the active outward cholesterol transport by ABCA1/G1 and inhibition of reverse transport through cholesterol-SM association is primarily responsible for TAPMC. Because ABCG1 was reported to

transport SM as well as cholesterol⁴⁴, it is also possible that ABCG1 contributes to TAPMC by facilitating asymmetric distribution of SM in the PM.

Mechanism of stimulus-induced [Chol]_i increase

We then determined the mechanisms by which Wnt3a specifically increases [Chol]_i. It has been reported that ABCA1 and ABCG1 activity is regulated by diverse mechanisms, including endocytosis, degradation and phosphorylation^{45,46}. MS analysis of ABCA1 and ABCG1 from Wnt3a-treated and untreated HEK293 cells detected phosphorylation at S884 and S1296 of ABCA1 as the only post-translational modification that occurred after agonist treatment (Supplementary Fig. 6). Phosphorylation of these residues, which are located in the long cytosolic nucleotide binding region⁴⁶, has not been reported, but a bioinformatics search⁴⁷ identified S884 and S1296 as potential phosphorylation sites by protein kinase A and protein kinase CKII, respectively.

To determine whether S884 and S1296 phosphorylation suppresses the cholesterol translocase activity of ABCA1, we ablated ABCA1 from HEK293 cells using CRISPR-CAS9 (Supplementary Fig. 5d) and measured the effect of reintroducing ABCA1 WT, an ATPase-inactive mutant (K939M/K1952M)⁴⁸, S884A(E), S1296A(E) or S884A(E)/S1296A(E) to these cells on agonist-induced [Chol]_i. ABCA1-null HEK293 cells had 2.5-fold higher basal [Chol]_i than WT HEK293 cells and did not show Wnt3a-induced [Chol]_i, unlike WT HEK293 cells (Fig. 3c and Supplementary Table 2). Transfection of ABCA1 WT into ABCA1-null HEK293 cells converted them into WT HEK293 cells in terms of basal [Chol]_i and Wnt3a-induced [Chol]_i; however, transfection of K939M/K1952M had a negligible effect on either parameter (Fig. 3c). This corroborates the importance of the ATP-dependent translocase activity of ABCA1 in TAPMC. Notably, cells transfected with S884A had the same low basal [Chol]_i as WT HEK293 cells, but showed no [Chol]_i in response to Wnt3a, whereas cells with S884E showed constitutionally high [Chol]_i, which did not increase further following Wnt3a stimulation (Fig. 3c). ABCA1-ablated cells transfected with S1296A showed some degree of Wnt3a-induced [Chol]_i, which was abrogated in cells with the S884A/S1296A double mutant (Fig. 3d). Similarly, cells with S1296E had lower basal [Chol]_i than cells with S884E and showed some degree of Wnt3a-induced [Chol]_i, which was again abolished in those with the S884E/S1296E double mutant (Fig. 3d). Collectively, these results suggest that S884 phosphorylation has a direct and crucial role in Wnt3a-induced ABCA1 inhibition and resultant [Chol]_i, whereas S1296 phosphorylation might have a complementary role.

Physiological significance of TAPMC

To determine the physiological importance of TAPMC, we examined the correlation between [Chol]_i and the Wnt signaling activity in HeLa cells, as measured by a luciferase-based TOP-FLASH assay. Before cell stimulation, the Wnt signaling activity of HeLa cells was linearly proportional to [Chol]_i (Fig. 3e and Supplementary Table 2). Stimulation of WT HeLa cells with Wnt3a (50 ng/ml) caused a fourfold increase in the Wnt signaling activity over unstimulated cells (Fig. 3f). Notably, the same treatments enhanced the signaling activity for ABCA1 and/or ABCG1 KD cells with higher basal [Chol]_i values to significantly greater degrees. In particular, ABCA1/G1 double KD cells exhibited 3.5-fold

larger responses than WT HeLa cells (Fig. 3f). This suggests that constitutively high $[\text{Chol}]_i$ caused by mutations or other factors under pathophysiological conditions leads to disproportionately higher Wnt signaling responses and, consequently, dysregulated cell activation. This idea was supported by our finding that some colorectal cancer cells with mutations in Wnt signaling proteins showed much higher basal $[\text{Chol}]_i$ than normal colorectal cells (Supplementary Fig. 3 and Supplementary Table 2). In addition, pre-treatment of ABCA1/G1 double KD HeLa cells with mevastatin, which inhibits *de novo* cholesterol biosynthesis, greatly attenuated the agonist-induced Wnt signaling activity (Fig. 3f) by lowering $[\text{Chol}]_i$ (Supplementary Table 2). Collectively, these results support the importance of maintaining the TAPMC and low $[\text{Chol}]_i$ in tight regulation of cell signaling leading to cell proliferation and growth. They also suggest that this signaling activity can be suppressed by pharmacologically lowering $[\text{Chol}]_i$.

DISCUSSION

We found that $[\text{Chol}]_o$ and $[\text{Chol}]_i$ can be simultaneously and directly determined with high accuracy and sensitivity, and in a spatiotemporally resolved manner, using a new set of orthogonal cholesterol sensors. Our work represents a technical and conceptual breakthrough in two respects. First, our systematic protein engineering generated tunable sensors that seamlessly cover a wide range of cholesterol concentration, which proved to be critical for accurate determination of dynamic TAPMC. Most naturally occurring lipid binding domains or proteins are not ideally suited for lipid sensors because they have relatively low specificity and affinity and a narrow dynamic range, and may interact with other proteins. Although a tandem repeat of lipid-binding domains has been commonly used to enhance their membrane affinity by avidity effect, this approach can only partially solve the problems associated with naturally occurring lipid-binding domains. Our systematic approach of generating tunable lipid sensors, which is universally applicable to any lipid quantification, should greatly facilitate lipid sensor design and determination of dynamic transbilayer asymmetry of lipids. Second, we were able to overcome the diffraction limit in optical microscopy by simultaneously quantifying $[\text{Chol}]_o$ and $[\text{Chol}]_i$ using orthogonal membrane-impermeable sensors. This approach can be applied to monitoring dynamic transbilayer distribution of any lipid in the PM and potentially in other cellular organelle membranes with the development of specific organelle delivery methods.

Our *in situ* quantitative imaging analysis in various mammalian cells led to the first unambiguous demonstration of TAPMC, which has long remained controversial^{2,3}. The high $[\text{Chol}]_o$ to $[\text{Chol}]_i$ ratio that we observed in diverse mammalian cells is generally consistent with the reported roles of cholesterol in the PM: that is, high $[\text{Chol}]_o$ would decrease PM permeability^{5,6} and might facilitate membrane domain formation in the OPM¹², whereas low $[\text{Chol}]_i$ would keep its cell signaling activity in check before agonist stimulation^{14–16,18,19}. The signaling role of IPM cholesterol has been often associated with the formation of cholesterol-rich membrane domains¹². Stimulus-induced $[\text{Chol}]_i$ and excellent correlations between $[\text{Chol}]_i$ and the cellular Wnt signaling activity, however, seem to indicate that individual cholesterol molecules have roles in signaling regardless of the presence of cholesterol-rich domains. Notably, our results demonstrate that TAPMC and

stimulus-induced PM cholesterol redistribution are crucial for cell homeostasis and tight regulation of cell growth and proliferation.

The link between cholesterol and cancer has been known for a long time⁴⁹. Although it was recently reported that 27-hydroxycholesterol links hypercholesterolemia to breast cancer⁵⁰, the mechanisms underlying the cholesterol-cancer correlation have not been fully elucidated. Our results suggest a potential direct link between [Chol]_i and cancer, a possibility of treating cancer through cholesterol modulation, and the potential use of [Chol]_i quantification as a diagnostic test for cancer.

Our results also suggest that the current methods to define, identify and characterize cholesterol-binding proteins have to be modified. Diverse membrane and soluble proteins have been reported to bind cholesterol through biochemical^{14,15}, cellular^{14–17,19}, structural¹¹ and MS-based chemoproteomic¹³ analyses under steady-state conditions. Our results indicate that the identification of functionally important cytosolic proteins that respond to [Chol]_i will require comparing PM affinity of proteins before and after stimulation. It is also possible that TAPMC and stimulus-induced PM cholesterol redistribution are involved in many other inter- and intra-cellular processes. Obviously, further studies are necessary to fully elucidate the mechanisms and physiological consequences of our findings. Nevertheless, our results provide a conceptual basis and experimental tools for studying the transbilayer dynamics, intracellular trafficking and regulatory function of cellular cholesterol and other lipids.

ONLINE METHODS

Materials

Acrylodan and thrombin were purchased from Invitrogen. NR3 was synthesized and characterized as described previously³³. Most lipids, including porcine brain ceramide, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), cholesterol, liver phosphatidylinositol (PI), and porcine brain sphingomyelin (SM), and lipid standards for MS analysis were purchased from Avanti Polar Lipids. Cholesterol-2,2,3,4,6-d₆ (cholesterol-d₆) was purchased from CDN isotopes. 1,2-dipalmitoyl-phosphatidylinositol-4,5-bisphosphate (PI45P₂) and latrunculin A were from Cayman Chemical Co. All cell lines including human colon epithelial cells (CCD 841 CoN) were purchased from ATCC. Sphingomyelinase (SMase) from *B. cereus*, phosphatidylcholine-specific phospholipase C (PC-PLC) from *C. perfringens*, N-ethyl maleimide (NEM), and mevastatin were from Sigma. Rabbit primary polyclonal antibodies used for this study include: anti-human ABCA1 (Abcam; Cat. No. ab10180), anti-human ABCG1 (Abcam; Cat. No. ab36969), anti-human GAPDH (cytosol marker; EMM Millipore; Cat. No. ABS16), anti-human Na⁺K⁺-ATPase (PM marker; Cell Signaling; Cat. No. 3010), anti-human calnexin (ER marker; Cell Signaling; Cat. No. 2433), and anti-human Bcl2 (mitochondria; Cell Signaling; Cat. No. 2872). The rabbit secondary horseradish peroxidase (HRP)-conjugated antibody (Cat. No. 7074) was from Cell Signaling. Recombinant mouse Wnt3a was purchased from R&D Systems.

Cholesterol sensor preparation

The original PFO D4 domain (amino acids 391–500) construct was subcloned into the pGEX 4T-1 vector and single and multi-site mutations were then introduced to improve cholesterol affinity and protein stability as described in Supplementary Figure 1a. All D4 proteins were expressed as GST-tagged proteins in *E. coli* BL21 RIL codon plus (Stratagene) cells and purified using the GST-affinity resin (GenScript). These proteins were labeled at the single Cys site (C459) by acrylodan or NR3 as described^{27,33}.

Spectrofluorometric measurements

Horiba Fluorolog-3 spectrofluorometer was used for all cuvette-based fluorescence measurements. Lipid sensors (typically 500 nM) were added to large unilamellar vesicles with various lipid compositions and the emission spectra of DAN and NR3 were measured with excitation wavelength set at 380 nm and 580 nm, respectively, in a single-photon excitation mode. Vesicle binding of the proteins was monitored in terms of an increase in fluorescence emission intensity (F) (at 445 nm for DAN and at 635 nm for NR3). For each protein, F was normalized against the maximal fluorescence increase (F_{max}) and F/F_{max} was plotted against the cholesterol concentration ([Chol]) in vesicles. Nonlinear least-squares analysis of the plots using the equation; $F/F_{max} = 1/(1 + \text{Exp}((K_{1/2} - [\text{Chol}])/S))$ yielded $K_{1/2}$ (= [Chol] yielding half maximal binding) and S (slope or stiffness) values and the theoretical curves were constructed using these parameters.

Calibration of lipid sensors by fluorescence microscopy

All fluorescence microscopy measurements were carried out in a two-photon excitation mode using a custom-built multi-photon, four-channel microscope equipped with two femtosecond-pulsed laser sources (Newport)⁵¹. *In vitro* calibration of all cholesterol sensors was performed using POPC/SM/cholesterol (70- x /30/ x : x = 0–40 mol%) and POPC/POPE/POPS/PI/cholesterol/PI45P₂ (20/40- x /30/9/ x /1: x = 0–40 mol%). These OPM- and IPM-mimetic GUV were mixed with the sensors in the concentration range of 0–500 nM. DAN-labeled and NR3-labeled sensors were two-photon excited at 780 nm and 900 nm, respectively. 480 nm and 560 nm band pass filters were employed for the blue channel and the green channel, respectively, whereas 620 nm and 670 nm band pass filters were used for the orange channel and the red channel, respectively. For DAN-labeled sensors, blue channel fluorescence signals derive from membrane-bound sensors only whereas green channel signals are from both membrane-bound and sensors. Likewise, orange channel fluorescence signals derive from membrane-bound sensors only whereas red channel signals are from both membrane-bound and sensors for NR3-labeled sensors. For each cholesterol concentration, 10 GUVs were selected for image analysis by Image-Pro Plus (Media Cybernetics). For data analysis, the region of interest (the membrane in our case) was selected by setting a threshold intensity (or brightness) value on the basis of the intensity distribution profile of the image. Since the orange channel (or blue channel for DAN-labeled sensors) always gives stronger membrane signals than the red channel (or green channel for DAN-labeled sensors) for NR3-labeled sensors, we first selected the mask from the orange (or blue) channel and superimposed it onto the same image in the red (or green) channel. The estimated membrane region of the vesicle was validated by comparing it with the

membrane region in the differential interference contrast image of the vesicle. The total intensity of GUUV ($F_{B(\text{total})}$ and $F_{G(\text{total})}$ for DAN-labeled sensors and $F_{O(\text{total})}$ and $F_{R(\text{total})}$ for NR3-labeled sensors) were divided by the total area of the pixels that constitute each GUUV to yield the average intensities, F_B and F_G for DAN-labeled sensors and F_O and F_R for NR3-labeled sensors (counts/ m^2), which were then used to prepare the ratiometric calibration curves. For DAN-labeled sensors, nonlinear least-squares analysis of the (F_B/F_G) versus the cholesterol concentration ([Chol]) plot using the equation; $F_B/F_G = (F_B/F_G)_{\min} + ((F_B/F_G)_{\max} - (F_B/F_G)_{\min}) / (1 + \text{Exp}((K_{1/2} - [\text{Chol}])/S))$ yielded $K_{1/2}$, $(F_B/F_G)_{\max}$, $(F_B/F_G)_{\min}$ and S values. $K_{1/2}$, $(F_B/F_G)_{\max}$, $(F_B/F_G)_{\min}$ and S are [Chol] yielding half maximal binding (in mol%), the maximal F_B/F_G value, the minimal F_B/F_G value and the slope (or stiffness) constant, respectively. The theoretical calibration curve was then constructed using these values and [Chol] from an unknown sample was calculated using the calibration curve. The same calibration was performed for NR3-labeled sensors.

Lipid quantification

HeLa (or other) cells were seeded into 50-mm round glass-bottom plates and grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate (Life Technologies) and cultured in the plates for about 48 h before lipid quantification. Cells were cultured in RPMI supplemented with 10% FBS, 1% non-essential amino acids, and 1% penicillin/streptomycin in a humidified 5% CO₂ incubator at 37 °C. All cell lines were cultured bi-weekly and stocks of cell lines were passaged no more than ten times for use in experiments. For quantification of [Chol]_o and [Chol]_i in HeLa (or other) cells, and NR3-YDA (or NR3-YQDA) was delivered into the cells by microinjection and DAN-D434A (or DAN-D434A/A463W) was added to the growth media. Typically, 20–30 fl of 0.5–1 µM sensor solution was microinjected into the cell. All microscopy measurements and imaging data analysis were performed as described^{27,33}. The three-dimensional display of local lipid concentration was obtained using the surf function in MATLAB. The angular profile of photon counts in the cellular PM was calculated by Image-Pro Plus software. To circumvent cholesterol sensor endocytosis^{24,52,53}, particularly under Wnt signaling conditions, microinjection and cell imaging were performed at 23 °C.

siRNA knockdown of lipid transporters

HeLa or HEK293 cells were plated in 6-well culture plates at density of 1.5×10^5 cells/well and the next day, 10 pmole of dicer-substrate RNAs (DsiRNAs) (or the negative control) were transfected into these cells using Lipofectamine 2000 and oligofectamine for HEK293 cells and HeLa cells, respectively. After 48 h, transfected cells were harvested for cholesterol measurement, RT-PCR analysis, or western blot analysis.

Preparation of ABCA1-deficient cell lines by CRISPR-CAS9

gRNA oligonucleotides for human ABCA1 were phosphorylated, annealed, and cloned to the lentiCRISPR plasmid. HEK293 cells were co-transfected with lentiCRISPR, pVSVg, and psPAX to generate virus. After 60 h, viruses in the medium were collected, filtered and used to infect HEK293 cells. Infected HEK293 cells were selected in the culture medium

containing 0.5 µg/ml puromycin for 4 d. Successful ABCA1 knockout (KO) was confirmed by geno-typing of specific primers for genomic ABCA1 and ABCG1. HEK293 ABCA1 KO cells were plated in a 6-well plate at the cell density of 0.75×10^5 cells/ml. Cells were transfected next day with 2.5 µg ABCA1 WT or mutants. Cells were then used for cholesterol quantification after 48 h. Successful gene transfection was confirmed by western blot analysis of cell extracts.

Western blot analysis

Transfected cells or treated cells were lysed in cell lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton-X plus protease inhibitor and phosphatase inhibitors (1 mM Na₃VO₄, 1 mM NaF, 1 µg/ml leupeptin, 1 mM PMSF, 1.5 mM benzamidine, 2 µg/ml pepstatin)]. The same total amount of proteins (50 or 100 µg) from these cell extracts or isolated membranes (or organelles) were loaded onto each well of a 8% polyacrylamide gel and separated by sodium dodecylsulfate PAGE. The gel was transferred to a polyvinylidene difluoride membrane and the membrane was blocked by 5% nonfat milk (w/v) in 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05% (v/v) Tween 20 (TBST) for 1 h at 23 °C. Proteins were detected by incubating the membrane with various primary antibodies (1:250 to 1:1,000 dilution) in TBST buffer with 1% (w/v) milk. After 3× wash in the TBST buffer (5 min), the membrane was treated with the secondary HRP-conjugated antibody (1:5,000) in the TBST buffer with 1% (w/v) milk at 23 °C for 1 h. After 3× wash in the TBST buffer (5 min), the membrane was developed with an enhanced chemiluminescence (ECL) substrate (ThermoFisher).

TOP-FLASH assay

1.5×10^5 HeLa cells were co-transfected with 1 µg TOP-FLASH firefly luciferase reporter plasmid and 0.1 µg constitutive Renilla luciferase plasmid pRL-TK. After 48 h, cells were treated with indicated chemicals for 24 h and lysed for measuring luciferase reporter gene expression by the Luciferase Assay kit (Promega). The data were normalized and averaged from triplicate measurements.

MS analysis

HEK293 cells transfected with GFP-tagged ABCA1 were treated with 50 ng/ml Wnt3a for 30 min and GFP-ABCA1 from these cells was isolated before and after stimulation by immunoprecipitation using a GFP antibody. After sodium dodecylsulfate polyacrylamide gel electrophoresis, each gel band containing GFP-tagged ABCA1 were digested with trypsin and digested peptides were extracted from the gel and analyzed using the Q-Exactive orbitrap hybrid mass spectrometer coupled with an EASY-nLC 1000 system (Thermo Fisher Scientific). The MS/MS scans were acquired at a resolution of 17,500 (at m/z 400) with an automated gain control target value of 1.0×10^6 and a maximum ion injection of 180 ms to get high quality of MS/MS spectrum. The acquired MS/MS spectra were searched against the Universal Protein Resource human protein database with the Sequest algorithm in Proteome Discoverer 1.4 (Thermo Fisher Scientific).

For lipid analysis, HeLa cells were harvested and PMs of cells were then isolated by sucrose-gradient ultracentrifugation and the purity of PM fractions was assessed by western

blot analysis using various membrane and organelle markers. PM lipids were extracted by chloroform/methanol (10:21, v/v) containing known amounts of non-natural or deuteriated forms of major lipid species of PM (PC-10:0/10:0, PE-10:0/10:0, PS-10:0/10:0, PA-10:0/10:0, PG-10:0/10:0, PI-8:0/8:0, SM-d18:1/12:0, and cholesterol-2,2,3,4,6-d₆) as internal standards. Quantitative lipid profiling of extracted samples was performed by ABI/Sciex 5500 QTRAP hybrid, triple quadrupole, linear ion trap MS equipped with a Turbo V ion source, together with the Analyst 1.5.1 software package (ABI/Sciex) and the Waters Acquity UPLC system with a Hypersil GOLD column (2.1 × 100 mm ID; 1.9 μm, Thermo Scientific). Multiple reaction monitoring conditions including transition and MS/MS collision energy were optimized to analyze target lipids in individual samples. To obtain accurate quantification results, external standard curves were constructed from the analysis of mixed lipid standards containing cholesterol-2,2,3,4,6-d₆ (Supplementary Fig. 4a). Standard curves were calculated by linear regression without weighting. Area of each lipid species was calculated by XIC (extracted ion current) using the Analyst 1.5.1 software package (ABI/Sciex). The analyte peak area values were normalized using the ratio of the calculated amount to the added amount of the internal standards, and lipid concentrations were then calculated using external standard curves. The s.d. of the relative peak areas was estimated from quintuple runs. The amount of each lipid was converted into molarity, from which mol% of cholesterol was calculated.

Statistical analysis

All *in vitro* studies were performed in triplicate or in quadruplicate to determine mean and s.d. values. For *in situ* cholesterol quantification, >100 cells were analyzed under each condition and among the majority of cells (that is, >60%) showing relatively similar lipid distribution patterns, a minimum of 50 cells were selected and cholesterol quantification in these cells were performed in triplicate. Spatially averaged values of [Chol]_o and [Chol]_i at a given time in each cell were used to calculate mean and s.d. values of [Chol]_o and [Chol]_i. *P* values were calculated from Student's *t* test.

Data availability

All imaging data are available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Simultaneous quantification of OPM ([Chol]_o) and IPM cholesterol ([Chol]_i) of HeLa cells by orthogonal cholesterol sensors

(a) Basic strategy. Binding of our cholesterol sensor (an engineered D4 domain labeled with a solvatochromic fluorophore, such as NR3) to the cholesterol-containing membrane led to major changes in spectral properties of the fluorophore. (b,c) Cholesterol dependence of DAN- (b) and NR3-labeled (c) sensors in binding to POPC/POPS/cholesterol (80–x/20/x: x = 0–40 mol%) large unilamellar vesicles. For sensors, we used QYDA, YDA, D434A/A463W, D434A and WT (from left to right). Vesicle binding of the proteins was monitored in terms of an increase in fluorescence intensity (ΔF) that was normalized against the maximal ΔF ($\Delta F_{\max}$). (d) Simultaneous quantification of [Chol]_o and [Chol]_i in resting HeLa

cells. **(e,f)** Effects of 1-h cholesterol depletion by 5 mM M β CD and cholesterol enrichment by 5 mM M β CD-cholesterol (1:1) adduct on [Chol]_o and [Chol]_i. Extracellularly added DAN-D434A and microinjected NR3-YDA were used for OPM and IPM sensors, respectively. Each image shows spatially resolved [Chol]_o or [Chol]_i on the cross-section of a representative cell at a given time. A pseudo-coloring scheme with red representing the highest and blue the lowest concentration is used to illustrate the spatial concentration heterogeneity. Scale bars represent 5 μ m. Spatiotemporally averaged [Chol]_o and [Chol]_i are displayed in the right panels. Cholesterol quantification was performed in triplicate in multiple cells. All data represent mean $\pm$ s.d. $n = 6$ **(b,c)**, 225 **(d)**, 125 **(e)** and 116 **(f)**.

Figure 2. Stimulus-induced increases in $[\text{Chol}]_i$ ($\text{D}[\text{Chol}]_i$) in HeLa cells

(a) Wnt3a dose dependence of $[\text{Chol}]_i$. Spatially averaged $[\text{Chol}]_i$ in response to 50, 25 and 12.5 ng/ml of Wnt3a (blue circles from top to bottom) was plotted as a function of time. 50 ng/ml of Wnt5a (green triangles) and Wnt11 (red squares) were used as controls. (b) Time course of spatially averaged $[\text{Chol}]_i$ (orange) and $[\text{Chol}]_o$ (blue) in response to 50 ng/ml Wnt3a. Notice the excellent synchronization and quantitative correlation between $[\text{Chol}]_i$ and $[\text{Chol}]_o$. (c) Angular profiles (that is, plots of the relative fluorescence intensity using a single PM location as a reference point) of $[\text{Chol}]_i$ (orange) and PM-bound EGFP-Dvl2 (blue) at 0, 15 and 30 min after Wnt3a stimulation. The EGFP fluorescence intensity of PM-bound Dvl2 was normalized using the maximal intensity value at 30 min. Notice the good spatial correlation between $[\text{Chol}]_i$ and Dvl2 PM recruitment. All data represent mean $\pm$ s.d. from quintuplicate measurements using multiple cells. $n = 135$ (a), 108 (b) and 49 (c) cells.

Figure 3. Mechanisms for PM transbilayer asymmetry of cholesterol and its physiological relevance

(a) Effects of lipid transporter RNAi and SMase treatment on $[Chol]_i$. Spatial distribution of $[Chol]_i$ in HeLa cells before and after ABCA1/G1 double knockdown (KD) and ABCA1/G1 double KD + SMase treatment (0.2 U/ml) is shown. Scale bars represent 5 μ m. **(b)** Effects of various treatments on the $[Chol]_o/[Chol]_i$ ratio. Simultaneous quantification of $[Chol]_o$ and $[Chol]_i$ was performed as described in Figure 1. The red line indicates $[Chol]_o/[Chol]_i = 1.0$. $P < 0.001$ for all data pairs. **(c)** Time courses of spatially averaged $[Chol]_i$ in HEK293 WT, ABCA1 knockout (KO), ABCA1 KO transfected with ABCA1 WT, ABCA1 K939M/K1952M, ABCA1 S884A and ABCA1 S884E, respectively, in response to 50 ng/ml of Wnt3a. **(d)** Time courses of spatially averaged $[Chol]_i$ in ABCA1 KO transfected with ABCA1 S1296A, ABCA1 S1296E, ABCA1 S884A/S1296A and ABCA1 S884E/S1296E in response to 50 ng/ml of Wnt3a. **(e)** Linear correlation between $[Chol]_i$ and the basal Wnt signaling activity in HeLa cells. The Wnt signaling activity in WT (1), ABCG1 KD (2), ABCA1 KD (3) and ABCA1/G1 double KD (4) HeLa cells was measured using the TOP-FLA SH assay. Relative activity was calculated as the ratio of the observed activity to that of HeLa WT. Linear regression was used to fit the data. **(f)** Wnt3a-induced (50 ng/ml) Wnt signaling activity for various HeLa cells. Fold increase in signaling activity was calculated as the ratio of the observed activity to that of unstimulated WT HeLa cells. $P < 0.001$ for all data pairs. All imaging data (**a–d**) represent mean $\pm$ s.d. from triplicate measurements in multiple cells. $n = 225$ (HeLa), 121 (ABCA1 KD), 116 (ABCG1 KD), 115 (ABCA1/G1 KD), 134 (SMase), 102 (ABCA1 KD + SMase), 109 (ABCG1 KD + SMase) and 107 (ABCA1/G1 KD + SMase). $n = 60$ for **c** and **d** cells. The activity data are mean $\pm$ s.d. from quadruplicate measurements.