Hydrophobic mismatch sorts SNARE proteins into distinct membrane domains

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The clustering of proteins and lipids in distinct microdomains is emerging as an important principle for the spatial patterning of biological membranes. Such domain formation can be the result of hydrophobic and ionic interactions with membrane lipids as well as of specific protein–protein interactions. Here using plasma membrane-resident SNARE proteins as model, we show that hydrophobic mismatch between the length of transmembrane domains (TMDs) and the thickness of the lipid membrane suffices to induce clustering of proteins. Even when the TMDs differ in length by only a single residue, hydrophobic mismatch can segregate structurally closely homologous membrane proteins in distinct membrane domains. Domain formation is further fine-tuned by interactions with polyanionic phosphoinositides and homo and heterotypic protein interactions. Our findings demonstrate that hydrophobic mismatch contributes to the structural organization of membranes.

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Since many years, the architecture of biological membranes has been subject of intense research. Presently, the concepts for the arrangement of membrane proteins, particularly in the plasma membrane of eukaryotic cells, are undergoing major changes. According to the classical Singer–Nicolson model, membrane proteins are viewed as separate entities floating in the lipid bilayer like icebergs in a sea. In contrast, it is now appreciated that many (if not all) membrane proteins are not evenly distributed across the membrane but rather organized in microdomains with a high local protein concentration, ranging in size between 2 and 200 nm (refs 1–4). Frequently, domain formation appears to be essential for the functions governed by these proteins, for example, by forming localized hotspots for signalling or for exo and endocytosis3–7.

SNARE proteins operating in exocytosis at the plasma membrane, in particular syntaxins 1 and 4, have served as convenient models for studying mechanisms responsible for protein clustering8–10. Syntaxins are tail-anchored membrane proteins mostly with a single hydrophobic transmembrane domain (TMD) at the C terminus11. In the plasma membrane, these proteins are organized in clusters with a diameter of ~70 nm that are dependent on the presence of cholesterol in the membrane, but are distinct from clusters formed by ‘classical raft’ residents such as caveolin or proteins linked to a glycosylphosphatidylinositol anchor5,12,13. In addition, clustering is dependent on the presence of plasma membrane-specific polyphosphoinositides (PI(4,5)P2 and PI(3,4,5)P3) that bind to a conserved juxtamembrane polybasic motif6,10,14–18. However, the physical principles underlying cluster formation within the bilayer are still unclear, with explanations including mechanisms as diverse as phase partitioning into cholesterol-enriched membrane rafts5,19–21, decreased solubility caused by the presence of cholesterol15,16, electrostatic interactions with the phosphoinositides10,18, and homophilic as well as heterophilic interactions between the proteins themselves involving either the hydrophobic TMDs or the hydrophilic domains8,9,22. In any case it is becoming apparent that membrane cholesterol plays a fundamental role in domain formation that is fine-tuned by the other factors but cannot be replaced by them. The causative mechanism of cholesterol is still unclear, with neither phase partitioning (yielding phase-segregated areas of much bigger size which exclude many of the cholesterol-dependent proteins4,23) nor specific binding of cholesterol to individual proteins (shown for only few of the cholesterol-dependent proteins) providing a satisfying explanation. A second important point is that closely homologous SNARE proteins such as syntaxin 1 and 4 are segregated into non-overlapping membrane domains8. Understanding how syntaxins segregate into distinct membrane clusters is essential for understanding their distinct roles in regulated (syntaxin 1) and constitutive (syntaxin 4) exocytosis.

In this study, we have investigated whether hydrophobic mismatch may be the underlying physical principle for many of the effects summarized above, with the effects of cholesterol being due to effects on membrane thickness. Hydrophobic mismatch means that the length of the hydrophobic part of a membrane-spanning macromolecule does not match the thickness of the hydrophobic core of the membrane. Such mismatch results in hydrophobic ‘defects’ at the boundary between protein and membrane lipid, which impose an energy penalty that can be minimized by clustering (Fig. 1a). The plasma membrane is a complex milieu with diverse thicknesses, amendable to quick changes, and we reasoned that the patterning of the plasma membrane might be caused by sequestering of lipids and proteins in membrane regions of matching hydrophobic thickness. Indeed, mismatch-driven clustering was previously shown to occur in a system employing synthetic peptides and artificial membranes24,25. Furthermore, hydrophobic mismatch was invoked as possible mechanism for the retention of membrane proteins in early compartments of the secretory pathway (such as the endoplasmic reticulum (ER)) since membrane thickness increases between the ER and the plasma membrane26. However, there appear to be many exceptions27,28, and thus hydrophobic mismatch is presently not being considered as a relevant factor contributing to membrane patterning and protein sorting. In this study, we show that hydrophobic mismatch between the length of TMDs and the thickness of the plasma membrane contributes to the clustering of proteins in the plasma membrane. We also show that hydrophobic mismatch can contribute to the segregation of structurally closely homologous SNARE proteins in distinct membrane domains even when the TMDs differ in length by only a single residue.

**Results**

**Clustering of syntaxin isoforms by hydrophobic mismatch.** To dissect the protein distribution in different membrane environments, we started by addressing syntaxin clustering in artificial liposomes with defined membrane thicknesses. First, we determined the effect of acyl-chain length and cholesterol on membrane thickness. To this end, we prepared ~100 nm sized large unilamellar vesicles (LUVs) composed of unsaturated phosphatidylcholine (PC) with stepwise increase in the length of the acyl chains (ranging from C14:1 to C20:1) either in the absence or in the presence of 30 mol% cholesterol. The thickness of these membranes was determined by imaging ellipsometry measurements (Fig. 1b), which is based on polarization changes of monochromatic light upon reflection on the bilayer29. As expected30, the membrane thickness increased by about 0.15 nm for each carbon unit added to the phospholipid acyl chains. Inclusion of 30 mol% cholesterol increased the membrane thickness by ~0.8 nm, independently of the acyl-chain length (Fig. 1b), indicating that cholesterol is a main modulator of membrane thickness.

We choose the SNAREs syntaxin 1 and syntaxin 4 to study the influence of hydrophobic mismatch on the distribution of membrane proteins for two reasons. First, the lengths of the hydrophobic segments of these SNAREs (21–23 amino acids) appear to be shorter than that needed to fully span the average hydrophobic core of a plasma membrane. This is particularly evident from the crystal structure of the neuronal SNARE complex where the TMD segments of the SNAREs synaptobrevin 2 and syntaxin 1 seem indeed not sufficiently long to traverse the average thickness of plasma membrane of eukaryotic cells (~4 nm). In a simulation, this resulted in defects in lipid packing30,31 and suggested that these SNAREs might prefer, or even organize, membrane regions of lipids with matching thicknesses. Second, these two syntaxins segregate in separate clusters although they are homologous and structurally very similar to each other. While it was shown previously that segregation depends at least in part on homophilic interactions between the cytoplasmic domains22, it is conceivable that the small differences in the length of the TMD segments (see below) may contribute to segregation. To isolate the effects on clustering within the membrane space from ‘secondary’ effects caused by protein–protein interactions in the hydrophilic space, we employed truncation mutants of syntaxins 1 and 4 with their cytoplasmically oriented domains deleted.

We tested whether syntaxin clustering is dependent on membrane thickness. For this purpose we measured clustering of the syntaxin 1 truncation mutant (sx-1TM) by Förster resonance energy transfer (FRET) using an approach very similar to that described by Murray and Tamm15,16. Two sx-1TM
populations labelled with spectrally separated fluorophores were mixed and incorporated into the liposomes described above, resulting in a high FRET signal in case of cluster formation (Fig. 1c). We found that in the absence of cholesterol, the FRET efficiency of sx-1TM was lowest (that is, least protein clustering) in membranes composed of C16:1 PC compared with C14:1, C18:1 and C20:1 PC (Fig. 1d). In the presence of cholesterol, clustering of syntaxin 1 was strongly enhanced (about 50%) in membranes composed of C16:1 PC, corresponding to a hydrophobic mismatch of about 3.4 nm, well below the average thickness of a

Figure 1 | Clustering of syntaxin isoforms by hydrophobic mismatch. (a) Positive and negative hydrophobic mismatch caused by differences between membrane thickness and TMD length. (b) Bilayer thickness determined by imaging ellipsometry of supported lipid bilayers composed of C14:1, C16:1, C18:1 and C20:1 PC with and without 30 mol% cholesterol (three independent experiments ± s.d.). Solid lines show linear regression analyses (slopes of 0.15 and 0.25 for without and with cholesterol, respectively). (c) Scheme of the clustering assay for sx-1TM in 100 nm sized liposomes using FRET. A mixture of sx-1TM was used that was N-terminally labelled with Rhodamine Red and Atto647N. (d) Clustering determined by FRET using liposomes composed of PC of increasing acyl-chain lengths, without (green) and with (pink) 30 mol% cholesterol. Independently, normalized lateral diffusion coefficients of sx-1TM labelled with Atto647N were determined by FCS (blue). Error bars: range from two independent reconstitutions, three technical repeats each. (e) Clustering of sx-1TM (labelled with Rhodamine Red) in supported lipid bilayers (C18:1 PC) in the absence (top) and presence (bottom) of cholesterol. The membrane was visualized with the lipophilic dye DiO. (Scale bars, 2 μm) (f) Domain organization and alignment of the TMD regions of syntaxin 1 and syntaxin 4. The TMDs and adjacent polybasic patches are marked in magenta and red, respectively. (g) Clustering of human sx-1TM (green) and sx-4TM (magenta) measured by FRET without cholesterol as in d. Solid lines show fits with quadratic curves. Error bars: range from two independent reconstitutions, three technical repeats each.
eukaryotic plasma membrane. Clustering increased with increasing membrane thickness independent of whether this increase was caused by longer acyl chains or by the inclusion of cholesterol in the membrane.

To directly visualize syntaxin clustering in dependence of cholesterol, we prepared supported artificial bilayers (C18:1 PC, with or without 30 mol% cholesterol) containing sx-1TM. In this experiment sx-1TM was labelled with the dye Atto647N to monitor its distribution, with the membrane being stained with the green fluorescent lipid analogue DiO (3,3′-dilinoleoylcarbocyanine; Fig. 1e). Clustering was clearly observable in the cholesterol-containing membranes, whereas it was much less conspicuous in the absence of cholesterol, in agreement with the results described above. Clearly, clustering is not due to cholesterol-induced phase separation of membrane lipids since these membranes did not contain any lipids with saturated fatty acids required for partitioning into Lo and Ld phases. Rather, our experiments reveal that the effect of cholesterol is caused by the increase of the membrane thickness, which results in clustering due to increased hydrophobic mismatch.

Interestingly, the TMD of syntaxin 4 is longer by 1–2 amino acids than that of syntaxin 1, and this difference in length is well conserved in mammalian species (Fig. 1f). Since the length of the TMD determines the hydrophobic matching with the surrounding lipid environment, it is expected that optimal matching (that is, lowest clustering) requires a thicker bilayer for syntaxin 4 than syntaxin 1. Therefore, we compared clustering of syntaxin 1 with that of syntaxin 4 as a function of membrane thickness, using an analogous truncation mutant for syntaxin 4 that lacked most of the cytoplasmic part (sx-4TM). Indeed, the local minimum of sx-4TM clustering was observed in C18:1 PC membranes instead of C16:1 PC membranes observed for sx-1TM (Fig. 1g). These FRET data fit well with a quadratic curve \( ax^2 + bx + c \). In this empirical model, \(-b/(2a)\) reflects the acyl-chain lengths with the lowest clustering which are 16.5 and 17.3 for sx-1TM and sx-4TM, respectively. Based on our imaging ellipsometry (Fig. 1b), these acyl-chain lengths correspond to membrane thicknesses of 3.6 nm for sx-1TM and 3.7 nm for sx-4TM. Together, our data indicate that the clustering of TMDs is indeed determined by the hydrophobic matching with the local lipid environment; with a length difference of even a single residue resulting in a shift towards an ~1-Å thicker membrane area. Despite this difference, both syntaxins are expected to cluster since the plasma membrane has an average thickness of around 4 nm (ref. 30), which would result in pronounced sequestering of these proteins to regions of decreased thickness.

Synergy of ionic interactions and hydrophobic mismatch. Next we investigated how clustering caused by hydrophobic mismatch is influenced by phosphoinositides. Syntaxin 1 is known to interact with PI(4,5)P\(_2\) and/or PI(3,4,5)P\(_3\), via a conserved polybasic motif directly adjacent to the hydrophobic TMD (Fig. 1f).\(^6,10,15,16\) Furthermore, both phosphoinositides are highly accumulated in at least a fraction of syntaxin 1 clusters in the plasma membrane\(^6,10,17,18\). Using two-colour super-resolution stimulated emission depletion (STED) microscopy, we confirmed the enrichment of PI(4,5)P\(_2\) in syntaxin 1 clusters within plasma membrane sheets prepared from neuroendocrine PC12 cells (Fig. 2a). Syntaxin 4 also contains a polybasic motif at the membrane interface and, indeed, PI(4,5)P\(_2\) was also enriched in syntaxin 4 clusters (Fig. 2b). In the plasma membrane, the density of PI(4,5)P\(_2\) clusters (13.9 ± 1.6 clusters per \(\mu\)m\(^2\)) was approximately three times higher than the cluster density of syntaxin 1 (4.5 ± 0.4 clusters per \(\mu\)m\(^2\)) and syntaxin 4 (5.4 ± 0.7 clusters per \(\mu\)m\(^2\)), which is not surprising when considering that PI(4,5)P\(_2\) interacts with many other proteins in cells\(^{12,33}\). We then reconstituted sx-1TM labelled with Rhodamine Red (donor fluorophore) and sx-4TM labelled with Atto647N (acceptor fluorophore) in LUVs and measured interaction by FRET (Fig. 2c). The presence of 1 mol% PI(4,5)P\(_2\) in LUVs composed of brain PC caused an increase of the FRET efficiency, indicating that the TMDs of the two syntaxin isoforms co-clustered in the membrane when PI(4,5)P\(_2\) was present. This clustering corroborates with our previous findings showing that interactions with polyanionic phosphoinositides can cluster syntaxin 1 refs 10,18. Clustering was also observed in the presence of both PI(4,5)P\(_2\) and cholesterol (Fig. 2d) showing that interactions with phosphoinositides act synergistically with hydrophobic mismatch. To further dissect how electrostatic repulsion between the polybasic linker region affects clustering by hydrophobic mismatch, we repeated our FRET assay in the presence of high concentrations of NaCl. Membrane clustering of sx-1TM was strongly promoted when electrostatic interactions were screened under the presence of 1 M NaCl. This indicates that the repulsion of the polybasic linkers of syntaxins counteracts hydrophobic mismatch and thereby limits clustering even in the presence of PI(4,5)P\(_2\) (Fig. 2e).

For a better understanding of the membrane clustering, we carried out coarse-grain molecular simulations. In these simulations, clustering of sx-1TM in membranes composed of longer (C20:1) and shorter (C12:1) acyl chains was observed already at very short simulation time scales (~100 μs). The simulation qualitatively reproduced the experimentally observed minimum of sx-1TM clustering in C16:1 membranes (Supplementary Fig. 1). However, a detailed analysis revealed that hydrophobic mismatch is not the only mechanism driving clustering. In fact the mismatch between the sx-1TM peptide and the membrane seems to be always negative even in thin membranes (Supplementary Fig. 2a,b). In such thinner membranes, syntaxin clustering was primarily promoted by increased homotypic protein–protein interactions, which for membranes with shorter acyl chains were facilitated by the much larger rotational tilting angle relative to sx-1TM tilting angles in thicker membranes (Supplementary Figs 1b and 2c,d)\(^34\). In membranes with longer acyl chains, protein clustering was mainly caused by the more pronounced hydrophobic mismatch (Supplementary Fig. 2a), which causes a corresponding free energy penalty ΔAG (Supplementary Fig. 2b), proportional to the protein–lipid mismatch interface. While strictly a free energy difference, the effective TMD extension (\(d\)) of the mismatch interface is rather constant for a given membrane and, therefore, the length of the protein–lipid interface mainly determines this free energy. Accordingly, and intuitively, we will refer to ΔAG/d as a ‘line tension’.

We then further characterized the influence of homotypic TMD interactions on SNARE clustering. It was demonstrated previously that syntaxin 1 TMDs homodimerize in membranes despite the electrostatic repulsion of the cationic linker. This homodimerization depends on specific protein–protein interactions in the hydrophobic phase that can be disrupted by alanine substitutions of three hydrophobic side chains within the TMD (M267A, C271A and I279A)\(^35\). To examine whether homodimerization contributes to homophilic clustering during hydrophobic mismatch, we reconstituted the corresponding sx-1TM mutant peptides and measured clustering by FRET. In membranes composed of C14:1 PC and C18:1 lipids (that is, both thinner and thicker than required for optimal hydrophobic matching of syntaxin 1), the dimerization mutant clustered comparable to wild type (Fig. 2f), indicating that at these conditions homotypic interactions were not required for
These findings agree with the results from our simulations, where although clustering of the dimerization mutant was reduced compared with wild-type sx-1TM, the pronounced rotational mobility in thin membranes facilitated clustering of even the sx-1TM mutant. Here the larger oligomers were absent and the shape of the cluster distribution resembled the wild-type size distribution in membranes with longer acyl chains (Supplementary Fig. 2c,d). Together, these results demonstrate that the observed minimum of clustering in membranes without cholesterol is caused by a competition between protein–protein interactions and hydrophobic mismatch. For membranes with more pronounced negative mismatch (such as the plasma membrane environment for syntaxins), clustering is mainly driven by the line tension that results from the increased hydrophobic mismatch free energy.

### Segregation of syntaxins 1 and 4 by hydrophobic mismatch

In the final set of experiments, we asked whether the small difference...
We then asked if hydrophobic mismatch can also cause segregation of syntaxin 1 and 4 in the complex environment of a plasma membrane. To this end, we transfected PC12 cells with truncation mutants of both syntaxins (sx-1TM and sx-4TM, similar to the fragments used in abovementioned experiments) N-terminally fused to EGFP and mCherry, respectively. When membrane sheets from these cells were analyzed by two-colour super-resolution STED microscopy, segregation of the two mutants in separate clusters was observed (Fig. 3c–e). To test if this segregation was due to the difference in length of the TMDs, we generated syntaxin 1 TMDs that were either two amino acids longer (sx-1TM+VG) or three amino acids shorter (sx-1TM−IFG) than wild type. In line with the hydrophobic mismatch hypothesis, clusters of the shorter sx-1TM−IFG strongly segregated from sx4-TM clusters (Fig. 3e). In contrast, the longer sx-1TM+VG, with a similar length of TMD as sx-4TM, showed significantly more co-localization with sx-4TM. Thus, our data show that increasing or reducing hydrophobic mismatch by altering the length of TMDs by only a few residues contributes not only to protein clustering but also to segregation into separate clusters.

Discussion

In summary, we have shown that hydrophobic mismatch due to cholesterol-mediated thickening of the membrane can drive clustering of proteins in distinct membrane domains and that this clustering can be modulated by homotypic interactions of the TMD and electrostatic protein–lipid interactions (Fig. 4). These data provide an alternative to previously established models of plasma membrane patterning4,37, allowing for drawing of three major conclusions. One, differences between membrane thickness and the length of the hydrophobic TMDs can drive clustering of membrane proteins. The resulting free energy penalty due to hydrophobic mismatch can be described in terms of a line tension, which is minimized by protein–protein clustering. Such hydrophobic mismatch not only explains protein clustering in the plasma membrane, but also may explain cargo sorting of proteins into specific intracellular compartments26,38,39. Indeed, theoretical calculations of hydrophobic mismatch carried out many years ago revealed that ‘embedded inclusions’ (that is, membrane proteins) perturb the membrane thickness, resulting in the length of the TMDs between syntaxin 1 and 4 might result in at least partial segregation into separate clusters because of hydrophobic mismatch between the TMDs. Thus, difference in length of the TMDs may contribute, in addition to the well-established homophilic interactions between SNARE motifs8,9,22, to the conspicuous segregation of the two syntaxins into different clusters (Fig. 3a). To address this question, we first reconstituted sx-1TM and sx-4TM in liposomes composed of a mixture of PC with different acyl-chain lengths (C14:1 to C20:1) and measured clustering by FRET. Clustering of sx-1TM with sx-4TM, but not of sx-1TM to sx-1TM and sx-4TM to sx-4TM, was lower compared with liposomes containing only C18:1 PC (Fig. 3b). This indicates that syntaxin TMDs preferentially cluster in regions containing lipids with matching hydrophobic thickness and a heterogeneous plasma membrane environment can drive segregation of membrane proteins with different lengths of TMDs in distinct membrane domains.

Figure 3 | Differences in the length of the TMDs contribute to segregation of syntaxin 1 and 4 to distinct clusters in the plasma membrane. (a) Two-colour STED microscopy of PC12 cell sheets immunostained for syntaxin 1 and syntaxin 4 shows segregation of endogenous proteins into separate clusters. (b) Reduced co-clustering of sx-1TM and sx-4TM in membranes composed of a mixture of PC with different acyl-chain lengths. FRET assay is similar to Fig. 1c, but now measuring clustering of TMDs in liposomes composed of either C18:1 PC or an equimolar mixture of C14:1, C16:1, C18:1 and C20:1 PC. All liposomes contained 1 mol% PI(4,5)P2 and 30 mol% cholesterol (± range from two independent reconstitutions, three technical repeats each). (c) Same as in a but now using PC12 cell sheets derived from cells expressing truncation mutants of syntaxin 1 and 4 (sx-1TM, sx-4TM) that are fused to mCherry and EGFP, respectively, and immunostained with antibodies against mCherry and EGFP. (d) Control experiment of PC12 cells co-expressing sx-1TM tagged with either mCherry or EGFP, showing co-localization (Scale bar, 2 μm). (e) Overlap of clusters in membrane sheets from PC12 cells transfected with various sx-1TM and sx-4TM mutants (all mCherry and EGFP tagged, respectively) measured by determining the Pearson-correlation coefficient. Sx-1FL and sx-4FL: full length constructs of syntaxin 1 and syntaxin 4, respectively (from a). Each analysis included at least 10 sheets from 3 independent transfections (**P<0.001, two-sided, unpaired t-test; error bars show s.e.m.). Scale bars, 2 μm.
overcoming electrostatic repulsion. We recently showed that head groups can balance the charges of the positive residues and ions. Here ions and the charged phosphoinositide further modulated by electrostatic interactions with phosphoinositides ('lipid rafts').

Three, clustering of proteins in the plasma membrane can be further modulated by electrostatic interactions with phosphoinositides and ions. Here ions and the charged phosphoinositide head groups can balance the charges of the positive residues found on the juxtamembrane regions of many proteins, thereby overcoming electrostatic repulsion.

Clustering by hydrophobic mismatch

Electrostatic sequestering by \( \text{PI(4,5)P}_2 \)

Figure 4 | Synergistic model of syntaxin clustering in the membrane.

Syntaxin membrane clustering is induced by a combination of hydrophobic mismatch (increased by cholesterol-induced membrane thickening) and electrostatic interactions with ions and \( \text{PI(4,5)P}_2 \). The membrane clusters are further refined by protein–protein interactions in the aqueous space.

in a state with defined spacing between neighbouring 'inclusion' clusters. Membrane proteins can thus recruit lipids and other membrane proteins with matching hydrophobic thicknesses and are thereby able to pattern biological membranes.

Two, inclusion of physiological concentrations of cholesterol (~30 mol%) results in a substantial thickening (by ~0.8 nm) of the membranes and consequently profoundly increases membrane cluster formation by hydrophobic mismatch. This agrees well with previous studies in which leucine–alanine-rich repeat peptides were used as models for TMDs (refs 24,25). Our results provide a mechanistic explanation for cholesterol-induced syntaxin 1 domains reported by Murray and Tamm and also explain the cholesterol dependence of syntaxin 1 clustering observed in the plasma membrane.

However, in contrast to former interpretations, we now show that cholesterol-induced cluster formation is the result of hydrophobic mismatch and does not require partitioning into separate membrane phases or domains enriched in cholesterol and sphingolipids (‘lipid rafts’).

Three, clustering of proteins in the plasma membrane can be further modulated by electrostatic interactions with phosphoinositides and ions. Here ions and the charged phosphoinositide head groups can balance the charges of the positive residues found on the juxtamembrane regions of many proteins, thereby overcoming electrostatic repulsion.

Methods

Protein and lipids. Syntaxin 1 TMD (residues 262–297; sx-1TM) from Homo sapiens and syntaxin 1 TMD mutant (sx-1TM with the following mutations: M267A, C271A and I279A) were synthesized using Fmoc solid phase synthesis. The fluorescent dyes Atto647N NHS-ester (Atto-Tec) and Rodamine red succinimidyl ester (Life Technologies) were coupled to the N-termini of sx-TM. The detailed synthesis is described in ref. 10.

C18:1 (1,2-dioleoyl-sn-glycero-3-phosphocholine), C14:1 (1,2-dimyristoyl-sn-glycero-3-phosphocholine), C16:1 (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), C20:1 (1,2-diacetyl-sn-glycero-3-phosphocholine), brain PI(4,5)P2, doPI(4,5)P2 (1,2-dioleoyl-sn-glycerol-3-phospho-hydroxyl (1-mono-oleoyl-4,5'-bisphosphate)) and cholesterol were purchased from Avanti Polar Lipids. Bodipy-labelled PI(4,5)P2 (bodipy-FL-PI(4,5)P2, C16) was used as Echelon Biosciences, and Top-Fluor labelled PI(4,5)P2 was from Avanti Polar Lipids. The lipophilic fluorescent probe DiO was from Life Technologies.

Vesicle formation. LUVs were prepared from PC of different acyl-chain lengths (C14:1, C16:1, C18:1 and C20:1) with or without 30 mol% cholesterol and/or 1 mol% PI(4,5)P2. Lipid mixtures were prepared at a total concentration of ~30 mM lipids in chloroform as described in ref. 57. After removal of chloroform with a rotary evaporator, the lipid film was resuspended in methanol to the final concentration of 40 mM and fluorescently labelled peptides were added in 2,2,2-trifluoroethanol. The organic solvents were then evaporated and resuspended to 8 mM total lipid concentration in 50 mM HEPES buffer with 150 mM KCl (pH 7.4) unless otherwise indicated (that is, no salt or 1 M NaCl). The multilamellar vesicles (MLVs) were then extruded through polycarbonate filters with 100 nm pore diameter (Avanti Polar Lipids).

FRET measurements. For FRET analysis we used Rhodamine Red coupled to sx-TM (donor) and Atto647N coupled to sx-TM (acceptor). Protein-to-lipid ratio in our FRET measurements was 1:1000. Excitation was at 560 nm, and emission was collected from 570 to 700 nm with 1 nm slit widths on a FluorMax-2 (Horiba). We corrected for cross-talk residing from acceptor excitation with samples containing only the acceptor fluorophore. The obtained FRET spectra were normalized to the maximum donor emission at 580 nm. The FRET efficiency was calculated as the ratio of emission intensities at 660 nm (acceptor maximum) over 580 nm (donor maximum).

Protein reconstitutions in polymer-supported membranes. Glass cover slides used in microscopy were prepared as described in ref. 58. At the supported lipid bilayer was generated by spincoating. For spincoating, (at 100 x g) we prepared a 40 mM lipid mixture consisting of a 2:1 molar ratio of phospholipids to cholesterol. Molar ratios of DiO and sx-TM to phospholipids were 1:5,000 and 1:10,000, respectively. After spincoating 10 μl of the lipid solution, the lipid film was rehydrated in 1 ml of 50 mM HEPES buffer with 150 mM KCl (pH 7.4).

Cell culture and immunofluorescence. We used the pheochromocytoma cell line PC12 from R. norvegicus. Lipofectamin LTX reagents from Life Technologies were used for transfection and cells were analyzed 24 h post transfection. Native membrane sheets were generated by gentle sonication as described in refs 8,10 and
Fluorescence correlation spectroscopy. For FCS, we used home-built confocal beam-scanning microscopy set up with two-colour excitation by pulsed-diode lasers at 485 nm (pulse length 80 ps; LDH-P-485B, PicoQuant) and 635 nm (pulse length 80 ps LDH-P-485B, PicoQuant)61. Emission filters were 540 ± 20 nm for the green channel and 670 ± 30 nm for the red channel. We used a ×100 oil objective with 1.42 numerical aperture (Leica Microsystems). Avalanche single photon counting detectors were used (SPCM-AQR-13-FC, Perkin Elmer Optoelectronics). FCS curves were fitted to a model for two-dimensional diffusion and with the axial radius of the focal volumes $o_{ax} \text{d}$ defined as the point where the measured fluorescence drops $e^{-3}$ times relative to the maximum, of 200 and 250 nm for Bodipy-FL and Atto647N, respectively.

**Ellipsometry measurements.** Lipid stock solutions (c$_{lipid} = 1–10 \text{mg ml}^{-1}$) were prepared in chloroform and transformed into lipid films by removal of the solvent. Lipid films (1–10 mg ml$^{-1}$) were deposited on the surface of quartz substrates in a fluid chamber. Ellipsometry experiments were performed using an imaging ellipsometer Ep$\text{\textsuperscript{3}}$-SW from Accurion as described previously$^{62}$. This method offers the possibility to measure thin layer thicknesses in real time and to monitor the convective flow at defined temperature. The principal angle del determined by this method is proportional to layer thicknesses for sufficiently thin dielectric layers ($h < 30 \text{nm}$). Absolute height changes resulting from spread solid-supported lipid bilayers were computed from the angle del, which is linearly related to the height for thin layers (1 nm $< 0.01 \text{del}$) and assuming a refractive index of 1.5 for all the used lipids.

Molecular dynamics. The molecular dynamics simulations were performed with the GROMACS simulation package, version 4.5.5 ref. 66. We used the MARTINI coarse-grained model to simulate the lipids, amino acids and polar solvents$^{67,68}$. Within this model an unsaturated bond is modelled by a decreased equilibrium bond-angle ($120^\circ$ versus $109.5^\circ$), increased polarity and backbone flexibility. In all simulations, the system was coupled to a constant temperature bath with a relaxation time of 1.0 ps ref. 69. We performed our simulations at a temperature of 30 K. Periodic boundary conditions were applied to simulate bulk behaviour.

The time step used in the simulation was 20 fs. The dielectric constant in the simulation was $\varepsilon_r = 15$. The solvent model was updated every 10 simulation steps. The pressure was weakly coupled to 1 bar with a relaxation time of 0.5 ps. In analogy to the other studies done with the MARTINI model, the time scales quoted in this work were scaled by a factor of 4 to approximately correct for the faster diffusion rates of water and lipids in the coarse-grained model$^{60}$. The pressure was weakly coupled to 1 bar with a relaxation time of 0.5 ps.

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