

## Revitalizing membrane rafts: new tools and insights

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**Abstract** | Ten years ago, we wrote a Review on lipid rafts and signalling in the launch issue of *Nature Reviews Molecular Cell Biology*. At the time, this field was suffering from ambiguous methodology and imprecise nomenclature. Now, new techniques are deepening our insight into the dynamics of membrane organization. Here, we discuss how the field has matured and present an evolving model in which membranes are occupied by fluctuating nanoscale assemblies of sphingolipids, cholesterol and proteins that can be stabilized into platforms that are important in signalling, viral infection and membrane trafficking.

### Caveola

A 50–80-nm, flask-shaped pit that forms in the plasma membrane and is enriched in caveolins, cavinins, sphingolipids and cholesterol.

Cell membranes contain hundreds of lipids in two asymmetric leaflets<sup>1</sup> and a plethora of proteins. For several decades, membrane research was dominated by the idea that proteins were the key factors for membrane functionality, whereas lipids were regarded as a passive, fluid solvent<sup>2</sup>. Introducing the lipid raft concept in 1997, we postulated that sphingolipid–cholesterol–protein assemblies could function in membrane trafficking and signalling<sup>3</sup>. These assemblies, or rafts, were thought to be characterized by their tight lipid packing, similar to the sterol-dependent, liquid-ordered phase in model membranes. The novelty of the raft concept was that it brought lipids back into the picture by giving them a function and by introducing chemical specificity into the lateral heterogeneity of membranes.

When we wrote our first Review in this journal<sup>4</sup>, the emerging raft field had become increasingly confused by ambiguous methodology and imprecise nomenclature. Caveolae, for example, became synonymous with rafts but clearly represented only a subset of membrane assemblies defined by the action of the protein caveolin<sup>5</sup>. Complicating matters further was the size of the sphingolipid–cholesterol–protein assemblies being studied, which were below the resolution of light microscopy. Only after cross-linking did raft proteins and lipid constituents cluster together to form micrometre-size, quilt-like patches.

Our focus in the first Review was to emphasize that rafts are small and dynamic and can be stabilized to form larger microdomains that function in membrane trafficking and signalling. We proposed that three types of assembly should be recognized in cell membranes — rafts, clustered rafts and caveolae (a subset of clustered rafts) — and that the residue remaining insoluble after detergent extraction should be called

detergent-resistant membrane (DRM) fractions. We also summarized the tools that were available for defining rafts and discussed their strengths and shortcomings. Obviously, what was known about lipid rafts and membrane organization at the time was dependent on the available methodology.

The rationale of the present Review is to summarize where we stand today and to highlight the important role that new technology has had in moving the field forwards (BOX 1). We describe how membrane rafts are now defined as dynamic, nanoscale, sterol–sphingolipid-enriched, ordered assemblies of proteins and lipids, in which the metastable raft resting state can be stimulated to coalesce into larger, more stable raft domains by specific lipid–lipid, protein–lipid and protein–protein oligomerizing interactions (FIG. 1). The lipids in these assemblies are thought to be enriched in saturated hydrocarbon chains. We describe advances in our understanding of how lipid rafts function as a membrane organizing principle in cellular processes such as T cell signalling, viral infection and membrane trafficking, and also try to identify issues that need to be resolved.

### Controversies then and now

A key issue ten years ago was the methodology used to define a raft component. An increasing number of papers used detergents as the main criterion for raft association; raft constituents were defined simply as the insoluble residue or DRM remaining after non-ionic detergent solubilization at 4°C. This criterion was usually combined with the use of methyl- $\beta$ -cyclodextrin to extract cholesterol from cell membranes. If the protein became detergent-soluble after cyclodextrin treatment, this strengthened the conclusion that it's

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### Box 1 | Changes in the raft concept

Our concept of rafts has shifted with the realization that the association of components is dynamic and sizes range from small, short-lived, nanoscale assemblies to more stable membrane domains, the size and lifetime of which also vary. The apical membranes of epithelial cells, for example, can behave as large percolating raft domains<sup>116</sup> or as a super-raft<sup>91,117</sup>. Similarly, the myelin sheets that oligodendrocytes produce to be wrapped around neuronal axons are another specific raft membrane type<sup>118</sup>. The situation is like that of logs in a river: one or several logs can function as a raft for one or more loggers and these can pile up into a raft jam. Discussing raft size as a criterion is irrelevant and the dynamics of raft lipids and proteins must be considered. Also, different methods and conditions used to study raft behaviour will give differing results. For example, fluorescence recovery after photobleaching (FRAP) may measure no difference in diffusion rates between raft and non-raft constituents, whereas stimulated emission depletion (STED) microscopy will<sup>30</sup> (FIG. 2).

The definition of a raft marker has evolved. Protein association with rafts was previously defined by sterol dependency and detergent resistance. Among putative raft lipids, the ganglioside GM1 was a commonly used marker because a fluorescently labelled probe was available. Patching of putative raft constituents was also done by antibody cross-linking in a way that would induce large raft clusters, potentially recruiting every membrane constituent with an affinity for the patched raft membrane environment.

Now when nanoscale assemblies are analysed, one does not expect every marker to be enriched, but instead only a limited subset of proteins and lipids<sup>16</sup>. When these assemblies are clustered into raft platforms, there is no obligatory reason why GPI-anchored proteins, GM1 or other raft constituents should be enriched. Only when larger patches are produced, such as in phase-segregated plasma membrane preparations, would such markers be considered useful.

DRM association was indicative of it being a raft component. Finally, if a biological process in living cells was disrupted by cyclodextrin treatment, the process was considered to be raft-based.

The uncritical use of these methods to study complex cellular processes caused an inflation of claims that were difficult to interpret and reconcile. Rightfully, this raised questions as to whether rafts were a real physiological phenomenon<sup>6,7</sup>. The main criticisms were of course directed towards the use of detergent resistance as a defining factor for raft components<sup>8</sup>. Whereas physiologically induced changes in DRM composition can reflect lateral biases in the membrane, detergent solubilization is an inherently artificial method giving different results depending on the concentration and type of detergent, duration of extraction and temperature<sup>9</sup>. Similarly, the other methods used to define raft-dependent processes were prone to artefacts and misinterpretations; for example, cyclodextrin treatment led to serious side effects such as lateral protein immobilization<sup>10</sup>. As plasma membranes can contain up to 40 mol% cholesterol<sup>11</sup>, it is perhaps not surprising that many cellular functions can be perturbed by cholesterol depletion<sup>12</sup>. For example, the plasma membrane can depolarize and Ca<sup>2+</sup> stores can be induced to empty<sup>13</sup>, leading to global cellular effects.

The difficulty of visualizing rafts in cell membranes was an important concern, discussed critically even ten years ago<sup>4</sup>. Although microscopically observable patches were present after cross-linking with exogenous ligands, the variability of the colocalization and sizes seen led to doubts about their relevance. Moreover, approaches used to study membrane protein diffusion, such as fluorescence recovery after photobleaching (FRAP)<sup>14</sup>,

and molecular complexes in membranes, such as Förster resonance energy transfer (FRET)<sup>15</sup>, led to mixed results and thus caused scepticism about the raft concept. These studies also brought to the fore the issue of what constitutes a raft marker<sup>16</sup> (BOX 1). There were claims that the lipid raft field was at a technical impasse because the physical tools to study biological membranes were lacking<sup>17</sup>.

### Advances with new technology

Despite the difficulties, the situation gradually clarified. The controversies inspired renewed efforts to find methodology that could detect and follow the behaviour of these small and elusive rafts. If they were present as postulated, one should be able to get glimpses of them before they clustered into more stable platforms. The problem required the development of high temporal and spatial resolution techniques to compare the location of different molecular constituents in the membrane.

### Visualizing rafts with new microscopy techniques.

Single-molecule spectroscopy and microscopy techniques were, in principle, well-suited for this challenge because they should give access to the dynamic state of the membrane<sup>18–20</sup>. Indeed, an influx of novel methods, such as hetero- and homo-FRET and fluorescence polarization anisotropy, revealed that GPI-anchored proteins and other lipid-modified proteins form cholesterol-dependent, nanoscale clusters<sup>21,22</sup>. Single-particle tracking also revealed the existence of dynamic nanoscale domains, and, on this basis, resting rafts were proposed to consist of a few raft proteins<sup>23</sup>. Another tracking method employed dual-colour total internal reflection fluorescence (TIRF) microscopy and single quantum dot tracking to study the cholesterol-dependent diffusion behaviour of a GPI-anchored protein and showed that this protein dynamically partitioned into and out of cell surface clusters of the ganglioside GM1 (REF. 24). Furthermore, fluorescence correlation spectroscopy (FCS) analysis of how membrane proteins behave in live cells revealed cholesterol- and sphingolipid-based nanoscale domains, into which proteins and lipids dynamically partition or assemble with a timescale of tens to hundreds of milliseconds<sup>25</sup>.

Moreover, super-resolution optical microscopy methods have been introduced that provide resolution well beyond the diffraction limit<sup>26</sup>; these include stimulated emission depletion (STED) microscopy<sup>27</sup>, photo-activated localization microscopy<sup>28</sup> and stochastic optical reconstruction microscopy (PALM and STORM)<sup>29</sup>. STED microscopy showed that sphingolipids and GPI-anchored proteins are transiently trapped in cholesterol-dependent molecular complexes in live cells<sup>30</sup> (FIG. 2a). Super-resolution near-field scanning optical microscopy (NSOM)<sup>31</sup> was also used in combination with quantum dots to show that T cell stimulation triggers nanoscale organization of T cell receptors (TCRs) in live cells<sup>32</sup>. Together, these methods revealed different aspects of how the behaviour of lipids and proteins is correlated in the plasma membrane.

#### Förster resonance energy transfer

A fluorescence-based method for detecting interactions between fluorophores that are < 10 nm apart. It is dependent on the spectral overlap between donor and acceptor chromophores and uses non-radiative energy transfer from an excited donor molecule to excite an acceptor molecule.

#### Fluorescence polarization anisotropy

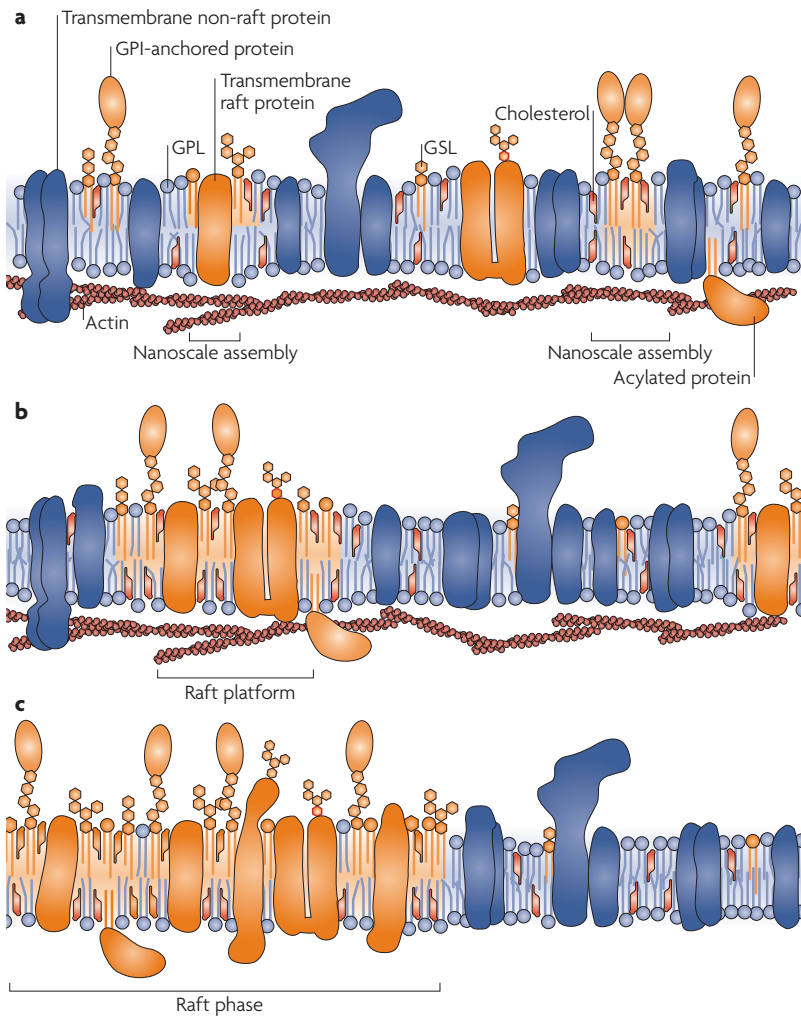
A technique to measure rotational diffusion using changes in fluorescence polarization that are due to fluorophore rotation.

#### GPI-anchored protein

(Glycosyl) phosphatidylinositol-anchored protein). One of a class of proteins that become post-translationally linked to GPI in the lumen of the ER.

#### Total internal reflection fluorescence (TIRF) microscopy

An optical technique based on evanescent wave illumination (~ 150 nm into the sample) that is created by a totally internally reflected beam at the glass–water interface.



**Figure 1 | Raft-based heterogeneity in cell membranes.** **a** | Nanoscale assemblies of sterols such as cholesterol, sphingolipids such as sphingomyelin and glycosphingolipids (GSLs), and proteins in the plasma membrane fluctuate in composition. GPI-anchored proteins, transmembrane raft proteins and acylated cytosolic proteins are postulated constituents of these assemblies, which can be modulated by actin filaments. Not much is known about the state of nanoscale assemblies in the cytosolic leaflet of the membrane. Transmembrane non-raft proteins are excluded from these assemblies. **b** | In response to external signals or the initiation of membrane trafficking events, raft platforms are formed from fluctuating assemblies through lipid–lipid, lipid–protein and protein–protein oligomerizing interactions. These platforms are important for membrane signalling and membrane trafficking. **c** | Micrometre-sized raft ‘phases’ can be induced at equilibrium. This state can be seen in model systems such as giant unilamellar vesicles (GUVs) and also in giant plasma membrane vesicles (GMPVs) or plasma membrane spheres released from the cell. Figure modified, with permission, from REF. 55 © (2010) the American Association for the Advancement of Science.

**Quantum dot**  
A nanoscale semiconductor crystal used as a label in fluorescence microscopy owing to its high emission and photostability.

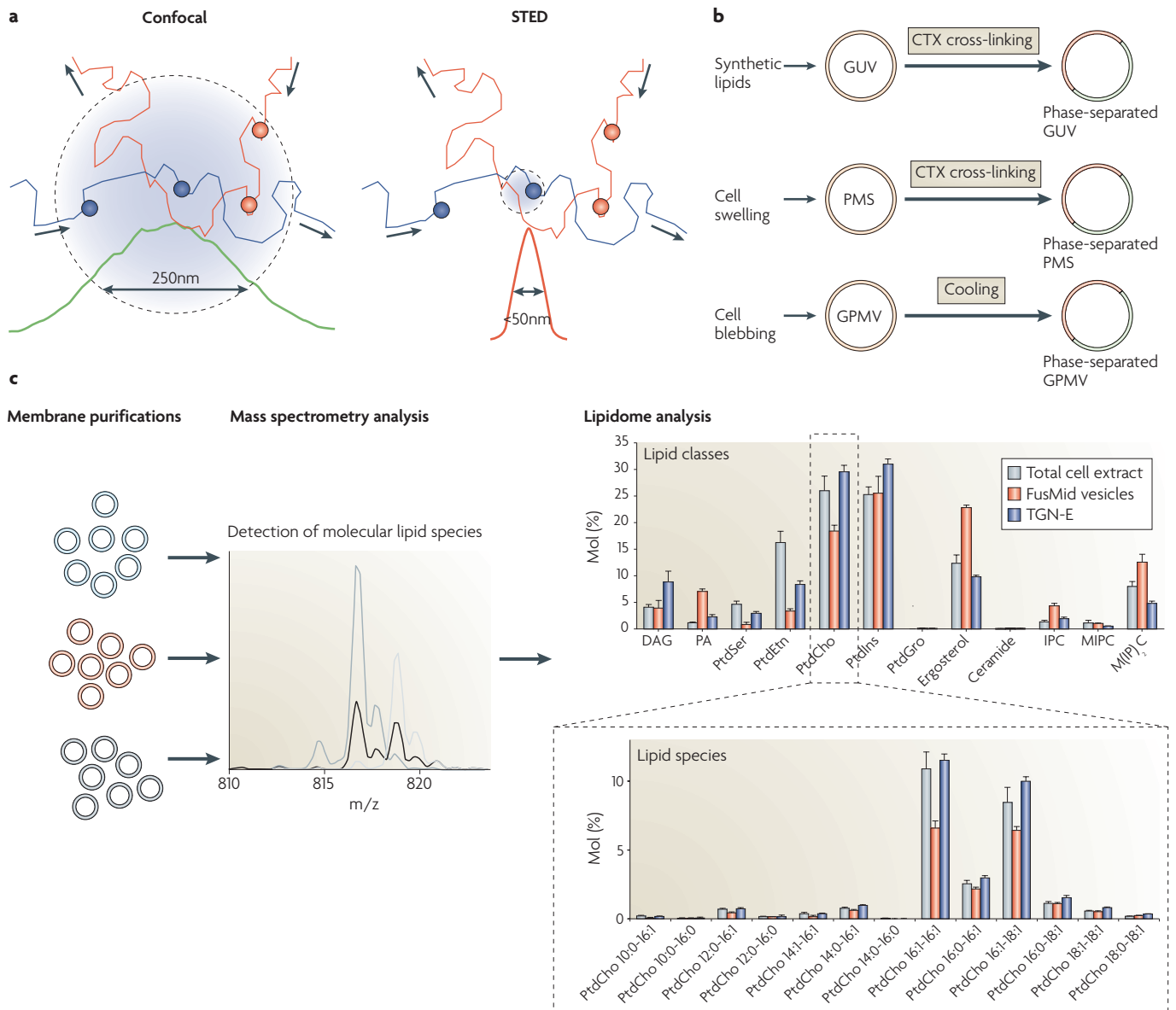
Despite the recent wealth of information gained from peering below the previously inviolable ‘diffraction limit’, all of the techniques mentioned above have their inherent disadvantages. FCS is diffraction-limited and therefore must be extrapolated<sup>25,33,34</sup> or combined with a super-resolution technique like STED<sup>30</sup> to reach the nanoscopic world. NSOM requires nanometre proximity to a tip or surface, which may influence the system under study<sup>35</sup>. A further issue is the use of labels that are bulky or chemically foreign to the cell, usually fluorescent tags,

beads or quantum dots. Although there are attempts to tackle the bulkiness of these tags<sup>36</sup>, it remains to be seen whether these tagged (often overexpressed) constructs faithfully mimic the behaviour of the unmodified native membrane components. Despite these limitations, data from these methods conclusively support the existence of nanoscale, cholesterol-assisted, dynamic and selective assemblies.

**Lipid diversity revealed by lipidomics.** Lipid analysis has dramatically improved in the past decade<sup>37</sup>. The field has been energized by the improved technical capabilities of tandem mass spectrometers that now allow fast quantitative profiling of lipidomes from minute amounts of sample. The analytical precision of modern instruments has made it possible to routinely identify lipids as molecular species and to analyse the compositional diversity of lipids in an unprecedented manner. Previously, most lipid analyses of membranes quantified composition in terms of lipid classes and not the molecular diversity in each lipid class (FIG. 2c). To understand how this diversity is used by the cell, sensitive and quantitative lipidomic techniques will have to be used to define the specificity that governs lipid assembly<sup>38</sup>. The first analyses of raft clusters in activated TCR domains<sup>39</sup>, raft transport carriers<sup>40</sup> and raft viruses<sup>41</sup> reveal selective enrichment of lipid species, as predicted by the raft hypothesis.

**Insights from biophysical analysis.** In parallel with biochemical and analytical studies of cell membrane organization, biophysicists have been characterizing model systems using monolayers and bilayers to explore liquid–liquid immiscibility (the inability to mix)<sup>42,43</sup>. There are two relevant phases of membranes: liquid disordered and liquid ordered. The liquid-ordered phase is typically enriched in raft lipids, such as sphingolipids, cholesterol and saturated phospholipids<sup>42</sup>. In three-component lipid mixtures reconstituted into giant unilamellar vesicles (GUVs) or supported bilayers<sup>44,45</sup>, varying sizes of the liquid-disordered and liquid-ordered phases can be seen with light and atomic force microscopes, depending on the composition<sup>46</sup>. The sizes of these domains can be altered from large to microscopically undetectable by adjusting the composition of the lipid mixture. Similarly, raft-like domains are visible in GUVs from isolated rat kidney membrane lipids<sup>47</sup>. Oligomerizing raft lipids (for example, by cross-linking a minor solute such as the ganglioside GM1, a typical raft lipid) was shown to have a dramatic effect in promoting phase separation<sup>48</sup> (FIG. 2b).

How do these findings in simple model systems relate to cell membranes? Given that cell membrane bilayers are asymmetric in their composition, the phase behaviour in each leaflet could be unique and coupled by interactions at the bilayer midplane<sup>49</sup>. Cell membranes are certainly not at equilibrium; they are continually perturbed by compositional fluctuations caused by lipid metabolism, the action of flippases, lipid transporters and membrane traffic, and the return to equilibrium can be slow. It has been argued that the chemical composition of the outer monolayer of cell plasma membranes endows them with



**Figure 2 | Novel methodology for the study of rafts.** **a** | High-resolution stimulated emission depletion (STED) microscopy (right) is one of several novel nanoscopic techniques that can go beyond the diffraction limit of standard confocal laser scanning microscopes (left). The large detection area of confocal microscopes (~250 nm) cannot discern details such as molecules moving freely or being transiently trapped on small spatial scales. However, STED is able to discriminate between molecules that diffuse freely (red) and those that are hindered (blue) during their passage through the subdiffraction spot (<50 nm). Red and blue lines indicate diffusion traces. **b** | Homogenous giant unilamellar vesicles (GUVs) can be prepared from defined lipids, and phase separation can be induced by cross-linking the ganglioside GM1 with cholera toxin (CTX)<sup>48</sup>. Plasma membrane spheres (PMS), produced from a cell swelling procedure, can also recapitulate the same effect in membranes containing the lipid and protein complexity of a plasma membrane<sup>53</sup>. Similarly, giant plasma membrane vesicles (GPMVs) produced by blebbing can be induced to phase separate by cooling<sup>51</sup> or CTX cross-linking<sup>122</sup>. **c** | A simplified workflow of a lipidome analysis. Biochemically purified membranes from *trans*-Golgi network (TGN)-derived (FusMid) vesicles (red), purified membranes from the donor organelle (TGN-E; blue), and total cell membranes (green) are analysed by mass spectrometry to reveal the amount of each molecular species in the sample. The sum of the species can be further analysed for specific features such as fatty acid length, saturation and abundance<sup>37,123</sup>. The mole percentage of each lipid class in yeast post-Golgi vesicles is shown together with the species distribution of the phosphatidylcholine (PtdCho) lipid class. Lipid species are denoted as, for example, PtdCho 10:0-16:1, in which a fatty acid with 10 carbon atoms and no double bonds and a fatty acid with 16 carbon atoms and 1 double bond are present. DAG, diacylglycerol; IPC, inositolphosphoceramide; MIPC, mannosyl-IPC; M(IP)<sub>2</sub>C, mannosyl-diinositolphosphoceramide; PA, phosphatidic acid; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine. Image in part **a** is modified, with permission, from *Nature* REF. 30 © (2009) Macmillan Publishers Ltd. All rights reserved. Data in part **c** is reproduced, with permission, from REF. 40 © (2009) The Rockefeller University Press.

**Box 2 | Critical behaviour in membranes**

Domain-forming lipid mixtures exhibit critical fluctuations near miscibility transition points before passing the boundary to stable microscopic liquid ordered–liquid disordered phase separation<sup>119</sup>. These fluctuations arise because the energy required to maintain regions of different composition becomes vanishingly small, and thermal motions lead to composition fluctuations over a wide range of time and length scales<sup>120</sup>. A remarkable similarity between plasma and model membranes is that the giant plasma membrane vesicles (GPMVs) produced by blebbing from plasma membranes display critical behaviour. In the two-phase region, the energetic cost (line tension) of the interface between two coexisting domains approach zero as the temperature approaches the miscibility transition boundary. Micrometre-scale fluctuations occur and suggest that the plasma membrane composition is tuned to a critical point. This behaviour can be extrapolated to physiological temperature, suggesting that, with the composition of the plasma membrane, the heterogeneity would correspond to less than the 50 nm-sized compositional fluctuations seen in some cell types<sup>121</sup>.

**Fluorescence correlation spectroscopy**

A technique that measures diffusion by correlating the fluorescence signal of a diffusing fluorophore with time.

**Stimulated emission depletion**

A nanoscopic technique that uses a red-shifted beam to deplete the emission of the periphery of the excitation spot and create a smaller excitation region, thus overcoming the diffraction limit.

**PALM and STORM**

(Photoactivated localization microscopy and stochastic optical reconstruction microscopy). Super-resolution microscopy techniques that use stochastically photoactivated fluorescent probes to reconstitute the full image from individual point spread functions.

**Near-field scanning optical microscopy**

A super-resolution technique that exploits the evanescent wave near the surface of the sample by placing the detector close to the sample.

**Glycosphingolipid**

A lipid that contains at least one sugar residue and a ceramide (*N*-acylated sphingoid).

**Palmitoylation**

The reversible covalent attachment of fatty acids to Cys residues of membrane proteins, which promotes their membrane association.

**Major histocompatibility complex**

A complex of genetic loci in higher vertebrates that encodes a family of cellular antigens that allow the immune system to recognize self from non-self.

the propensity for phase separation (if at equilibrium)<sup>50</sup>. Even if no microscopically observable phase separation can be detected, the propensity for sub-microscopic domain formation may still exist. Clearly, the phase segregation seen in liquid-phase model systems is a manifestation of liquid–liquid immiscibility. So if one were to leave a plasma membrane in a resting state (no lipid synthesis or metabolism, no exocytosis or endocytosis, no interactions with peripheral proteins and no actin cytoskeleton), would membrane components phase-separate into large domains with time?

This question has yet to be answered, but lipid-based phase separation into liquid-ordered-like and liquid-disordered-like phases has been seen in isolated giant plasma membrane vesicles (GPMVs) formed by membrane blebbing<sup>51</sup>, and the temperature- and cholesterol-dependence of this phase separation resembles that of simple model systems<sup>52</sup> (FIG. 2b). Furthermore, plasma membrane spheres (PMSs), produced using a swelling procedure and separated from the influence of cytoskeletal and trafficking events, showed sterol-dependent coalescence into a micrometre-scale phase on clustering by cholera toxin at 37°C (FIG. 2b). The selective lateral reorganization of proteins and lipids correlated with their predicted affinity for raft domains<sup>53</sup>.

This phase-separating behaviour is similar to the cholera toxin-induced phase separation seen in model membranes, suggesting that the protein and lipid composition of the plasma membranes is positioned close to a phase boundary and can be induced to phase separate by the clustering of raft components such as the ganglioside GM1.

Together, these studies have provided an exciting and unexpected new dimension in membrane research, showing that cell membranes of complex lipid and protein compositions can phase separate similarly to

simple model membranes and that the composition might be tuned close to a critical point (BOX 2). It should be pointed out that the two phases induced by cholera toxin in PMS behave differently from liquid-ordered and liquid-disordered phases in model membranes in terms of the amount of order and packing<sup>54</sup>. These differences in organization are perhaps not surprising, considering that the plasma membrane is crowded with proteins and specific lipid–protein interactions.

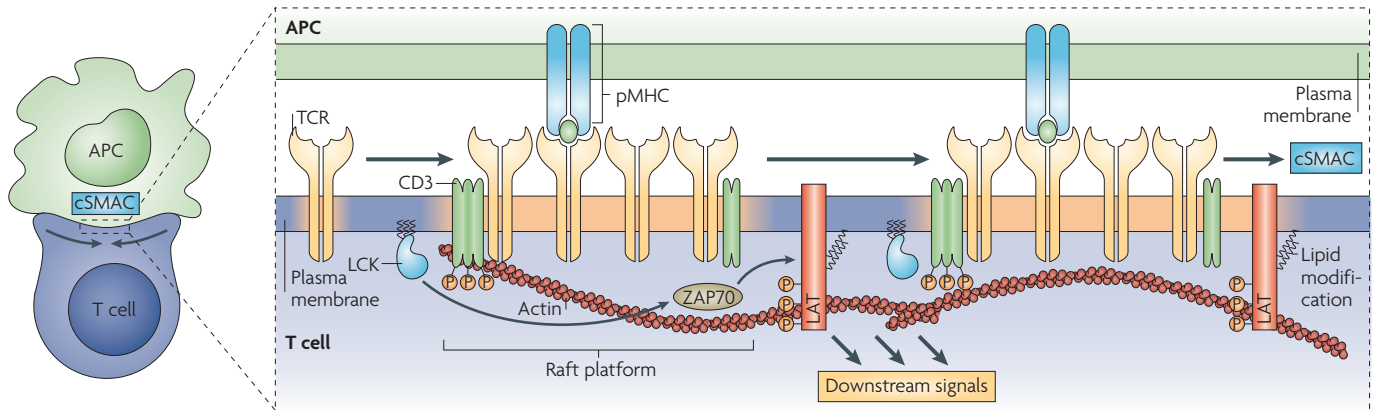
Hence, we are left with a description of plasma membranes that could correspond to three states, of which two are seen in living cells<sup>55</sup> (FIG. 1). The first state is represented by dynamic nanoscale assemblies that associate and dissociate on a subsecond timescale<sup>56,57</sup>. These can be clustered into the second state to generate more stable, selective and functional platforms. The third state is the complete micrometre-scale phase separation that is seen only in isolated membranes at equilibrium. How the critical fluctuations observed in GPMVs relate to associating and dissociating nanoscale assemblies in living cells is still unclear. Obviously, the dynamic behaviour will depend on the composition of the membranes, and therefore on the cell type that the plasma membrane is derived from (BOX 1).

**Biological roles of raft heterogeneity**

The recent advances of high-resolution microscopic methodology together with observations of large-scale phase separation in plasma membranes have confirmed the potential for lipid phase separation. To illuminate the possible roles of rafts in cellular functions, we focus on four areas of cell biology — TCR signalling, HIV assembly, endoplasmic reticulum (ER)-to-Golgi and post-Golgi trafficking to the cell surface and glycosphingolipid-mediated endocytosis — and review progress made in the past decade.

**T cell signalling.** An early clue that rafts might affect T cell signalling was the observation that antibody-mediated cross-linking of GPI-anchored proteins (which do not span the membrane) could stimulate signalling<sup>58</sup>. Later, DRM analysis showed that factors important for T cell signalling were detergent-insoluble, whereas engineered palmitoylation-deficient proteins became detergent-soluble and impaired T cell activation. Cholesterol depletion inhibited T cell activation, whereas co-patching experiments using cholera toxin induced part of the T cell-activation programme and lead to microscopically observable domains containing essential T cell-activation proteins. Taken together, these data suggested that lipid rafts are involved in T cell signalling.

T cells are activated by conjugation with cognate antigen-presenting cells (APCs). TCRs interact with the antigenic peptide bound to the major histocompatibility complex (pMHC) on APCs<sup>59</sup>, and this leads to the phosphorylation of immunoreceptor Tyr-based activation motifs in the TCR multisubunit complex by lymphocyte cell-specific protein Tyr kinase (LCK; a Src family kinase) and subsequent recruitment and activation of the Tyr kinase 70 kDa ζ-associated protein (ZAP70) (FIG. 3). In turn, activated ZAP70 phosphorylates linker for T cell



**Figure 3 | T cell receptor activation.** T cell receptors (TCRs) in the resting state might associate with raft lipids to form nanoscale assemblies that stabilize into a raft platform (TCR microclusters) on activation of the T cell by the antigenic peptide-bound major histocompatibility complex (pMHC) group on an antigen-presenting cell (APC). Here, the TCR gets phosphorylated by the Src family kinase lymphocyte cell-specific protein Tyrosine kinase (LCK), and recruits and activates the Tyrosine kinase 70 kDa  $\zeta$ -associated protein (ZAP70). The main target of ZAP70 is linker for T cell activation (LAT), which initiates further downstream signalling. The TCR clusters are anchored to actin filaments and are transported towards the central supramolecular activation cluster (cSMAC), the central part of the immunological synapse. The TCR microcluster signalling is envisaged to take place in a raft platform<sup>39</sup>. For clarity, only a subset of the involved proteins is shown.

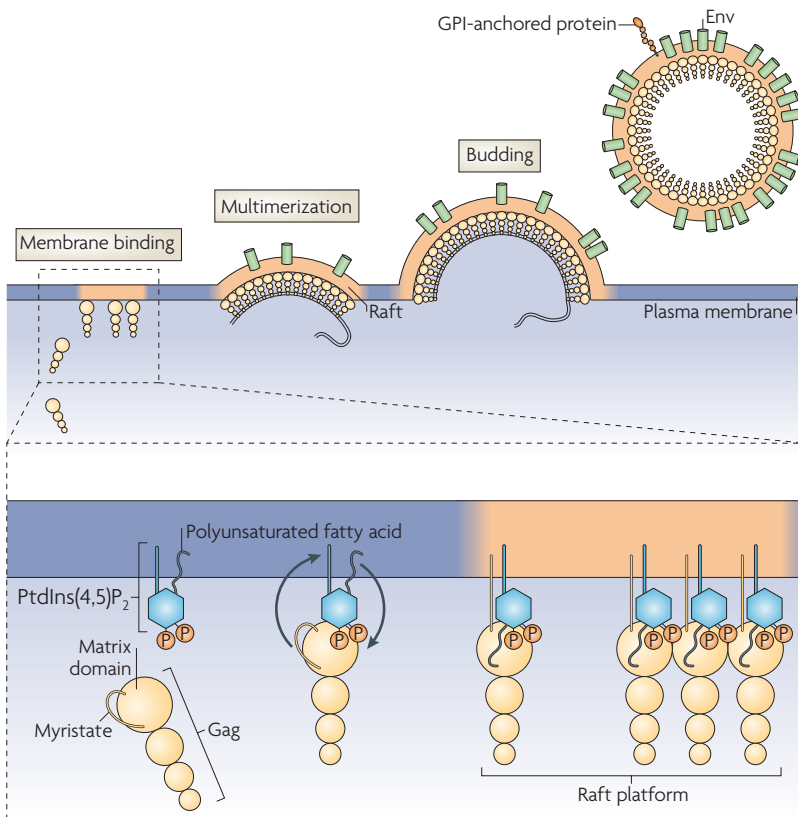
activation (LAT), a transmembrane adaptor protein that recruits several signalling molecules to initiate downstream events such as actin polymerization,  $\text{Ca}^{2+}$  influx, Ras activation and transcriptional changes. This cascade of events results in the formation of an 'immunological synapse' between the contacting cells, with a bull's eye structure that consists of a central supramolecular activation cluster (cSMAC) and a surrounding ring of adhesion molecules.

The model of T cell synapse formation by stimulated raft condensation generated considerable criticism. The effects of cholesterol depletion, for example, could be explained by the previously mentioned pleiotropic effects of this treatment on, for instance,  $\text{Ca}^{2+}$  influx<sup>13</sup>. Moreover, the immunological synapse itself, first considered to be a stabilized raft platform, was found to be a dynamic structure<sup>59</sup>. TCRs are first engaged and activated in TCR microclusters, which are subsequently transported radially to the centres of the contact by actin filaments, generating a cSMAC, in which many TCRs are already dephosphorylated and inactive<sup>60,61</sup>. Using dual-colour, single-molecule microscopy, microclusters have been observed with protein-protein interactions involving TCR, LAT, LCK and CD2 (but not the phosphatase CD45 (REF. 62)). The data suggested that diffusional trapping through protein-protein interactions created microdomains that include specific cell surface proteins to facilitate T cell signalling. A non-raft mutant of LAT lacking palmitoylation sites was also trapped by the microclusters, whereas a raft-associated construct, the lipid-modified amino terminus of LCK, was not concentrated in the microclusters<sup>63</sup> (BOX 1). Thus, there was no direct evidence for lipid raft involvement, but it should be noted that lipids were not included in the analysis.

An important issue in T cell signalling is how TCRs connect to 10–100 cognate pMHC molecules among a total cell surface pool of  $10^4$ – $10^5$  MHC molecules on an APC<sup>64</sup>. The kinetics of binding between TCRs and the

cognate pMHC have been evaluated using engineered proteins in solution, and only weak affinities and dissociation rates were seen. However, when binding was measured between a TCR in a T cell plasma membrane and the cognate pMHC integrated into another membrane, accelerated kinetics and a more than 100-fold higher affinity were observed<sup>65,66</sup>. These data explain the rapid and efficient recognition of an APC and also show that TCRs can serially engage a few cognate pMHCs in a large, self-MHC background. Cholesterol depletion was found to reduce the effective two-dimensional affinities between TCRs and pMHCs<sup>66</sup>.

One issue in the field is the oligomeric state of TCRs before interaction with pMHCs. There are mixed reports as to whether it is monovalent<sup>67,68</sup> or multivalent<sup>69</sup>, but high-resolution microscopy methods show complexes with up to 7–20 TCRs, which are manifested as protein islands that are 70–140 nm in diameter<sup>70</sup>. How can these findings be reconciled with monovalent TCRs? What is the molecular size of a resting state TCR? One suggested possibility is that the contact of a cell with a glass surface perturbs membrane organization such that weak signalling of TCRs occurs without ligation<sup>71</sup>. This is reminiscent of live-cell analysis by photonic force microscopy, which concluded that GPI-anchored protein domains have a cholesterol-dependent size of 50 nm<sup>72</sup>, much larger than the <10 nm measured by homo- and hetero-FRET<sup>21</sup>. In these experiments, beads coated with antibodies against the GPI-anchored protein were immobilized by an optical trap to measure the unhindered diffusion on the cell surface, and effects of the trap on the lifetime and size of the nanoscale, GPI-anchored protein assemblies cannot be excluded. These studies therefore highlight how experimental conditions can easily lead to divergent results. If the resting states of raft proteins were indeed associated with nanoscale assemblies of raft lipids, such as sphingolipids and cholesterol, then such variances in size could arise from the coalescent capacity of these



**Figure 4 | HIV assembly and release.** Gag binding induces the formation of raft platforms at the plasma membrane of the host cell and Gag multimerization drives the assembly of the virus particle. The viral Env protein is incorporated during the budding process and the virus detaches from the plasma membrane. To emphasize that not all host proteins are excluded from the virus particle, a GPI-anchored protein is shown included<sup>124,125</sup>. One possibility is that during raft platform formation, Gag binding to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) through its matrix domain releases the bound myristate group, which inserts into the plasma membrane<sup>83</sup>. The polyunsaturated fatty acid of PtdIns(4,5)P<sub>2</sub> has been proposed to flip into the hydrophobic cleft of the matrix domain, and multimerization of the Gag protein stabilizes the raft platform. Figure modified, with permission, from REF. 76 © (2009) Elsevier.

fluctuating structures. Stabilization of fluctuating nanoscale structures could mirror a physiological process in which increased access to LCK mediates the stimulation of TCR signalling<sup>73</sup>.

Lipidomics has been used to measure immunolysed TCR activation domains purified by coating magnetic beads with TCR-activating antibodies that induce TCR cross-linking. When the lipidome of the immuno-isolated TCR domains was compared to similarly cross-linked transferrin receptor plasma membrane domains from T cells, TCR domains were enriched in sphingolipids, saturated phosphatidylcholines (PtdChos) and plasmalogen phosphatidylserine (PtdSer)<sup>39</sup>.

Together, these data are difficult to explain without assuming that lipid heterogeneity is functionalized to activate T cells. What is missing are data on whether and how the lipid context around the receptors is changing from the inactive to the activated state and back again. This is a general issue in the raft field: how are the ‘lipid shells’, the lipids contacting the raft proteins, composed and organized<sup>74</sup>? Do lipid–protein interactions

allosterically change receptor conformations? The potential relevance of dynamic, nanoscale raft assemblies for other signalling processes in which rafts have been implicated is obvious but requires further analysis. An example is the STALL concept, in which clusters of GPI-anchored CD59 recruit the G protein subunit Gα<sub>12</sub> and the Tyr kinase LYN through protein–protein and protein–raft interactions, resulting in temporary immobilization of the cluster by binding to filamentous actin (F-actin) and signal activation by phospholipase Cγ2 (PLCγ2)<sup>75</sup>.

**Virus budding.** Many viruses acquire a membrane envelope when budding off from the host cell plasma membrane. Some viruses, including HIV<sup>76</sup> and influenza<sup>77</sup>, seem to do this by organizing a lipid raft domain around their nucleocapsid that includes viral glycoproteins and excludes most host cell surface proteins from the budding viral envelope. The Gag protein of HIV, the matrix domain of which assembles with the Env glycoprotein in the plasma membrane, becomes detergent-resistant while driving the budding process (FIG. 4); furthermore, budding is cholesterol- and sphingolipid-dependent. If labelled cholera toxin is applied to HIV-expressing cells, Gag, GM1 and the virus proteins co-patch in distinct clusters that segregate away from clusters of non-raft transferrin receptors<sup>76</sup>. These data suggested that the assembly of the virus envelope at the host cell plasma membrane involves the clustering of rafts. In support of this hypothesis, the lipidome of purified HIV particles showed that sphingolipids, cholesterol, plasmalogen phosphatidylethanolamine (PtdEtn), PtdSer and saturated PtdChos were enriched in the HIV membrane relative to total host cell membranes<sup>41</sup>. Comparison of the HIV lipidome with that of the host cell plasma membrane found that only cholesterol, the ganglioside GM3 and ceramide<sup>78</sup> were highly enriched in the virus envelope<sup>79</sup>. Consistent with the enrichment of raft lipids, the viral membrane was shown to have an ordered lipid packing by spectroscopy using the fluorescent probe laurdan<sup>80</sup>, which reports relative membrane order, with lipid composition being its main determinant, in an experimental setup that is not affected by geometry<sup>54</sup>.

The phosphoinositides phosphatidylinositol phosphate (PtdInsP) and PtdIns-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) are also enriched in the HIV envelope<sup>79</sup> and PtdIns(4,5)P<sub>2</sub> is bound by Gag. Structural models are now emerging for how the association of PtdIns(4,5)P<sub>2</sub> with Gag may drive membrane reorganization and viral budding. PtdIns(4,5)P<sub>2</sub> is negatively charged owing to two phosphates in the inositol headgroup and it contains both a saturated fatty acid and a polyunsaturated arachidonic acid. Why would a phospholipid with a polyunsaturated fatty acid be included in a raft domain? Proteins such as myristoylated Ala-rich C-kinase substrate (MARCKS) and growth-associated protein 43 (GAP43) that bind to PtdIns(4,5)P<sub>2</sub> have been proposed to polymerize and tightly cluster PtdIns(4,5)P<sub>2</sub> in the cytosolic leaflet of the membrane and partition PtdIns(4,5)P<sub>2</sub> into raft domains<sup>81,82</sup>. But the matrix domain of the Gag protein may do this differently<sup>83</sup>.

**Lipid shell**

A model proposing that specific membrane proteins bind to complexes of cholesterol and sphingolipids and laterally organize specific contacting lipids.

**STALL**

(Stimulation-induced temporary arrest of lateral diffusion). The cholesterol-assisted, sub-second trapping of GPI-anchored signalling proteins with downstream signalling proteins.

The matrix domain of Gag, like in MARCKS, has a cluster of basic amino acids that binds to PtdIns(4,5)P<sub>2</sub> and it also has a saturated myristate fatty acid at its N terminus buried in a hydrophobic cleft. The hypothesis has been put forward that Gag multimerizes during budding and, on binding to PtdIns(4,5)P<sub>2</sub>, the N-terminal myristate becomes exposed on the matrix domain surface to insert into the cytosolic leaflet of the plasma membrane, to be replaced by the polyunsaturated arachidonic acid flipping from the membrane into the myristate pocket on the matrix domain. This exchange would result in a multimeric cluster of saturated fatty acid tails that penetrate into the bilayer to promote raft clustering around the HIV Gag nucleocapsid<sup>83</sup>. Polyunsaturated fatty acids such as arachidonic acid have an extremely flexible and highly disordered structure, and poor packing with cholesterol could promote the flipping out of the polyunsaturated chain into the pocket on the matrix domain<sup>84</sup>. Viruses ingeniously make use of the cellular machinery to replicate in host cells. If this postulated mechanism of PtdIns(4,5)P<sub>2</sub> interaction with the matrix domain is correct, it is likely that native cellular proteins could make use of similar tricks for association with, or condensation of, raft domains.

#### Rafts in membrane trafficking

**ER-to-Golgi traffic in yeast.** Secretory proteins are inserted into the ER and transported by coat protein II (COPII) vesicles to the Golgi and subsequently to their final destinations. In the ER of yeast, secretory proteins are sorted into at least three types of ER exit site<sup>85</sup>. Two of these sites concentrate mainly soluble or transmembrane cargo, and early COPII machinery was found to be necessary for these functions. The third exit site also produces COPII vesicles, but COPII machinery is not required to concentrate its predominantly GPI-anchored cargo<sup>85</sup>. This concentration depends on the remodelling of GPI anchors with a saturated, long-chain fatty acid or a ceramide that confers detergent resistance<sup>86,87</sup>. Defects in GPI-anchor synthesis dramatically reduce the total sphingolipid levels<sup>88</sup>, and ER exit of GPI-anchored proteins depends on ceramide synthases<sup>89,90</sup>. Therefore, the sorting mechanism for this type of exit site might be a concentration of ceramides that attracts GPI-anchored proteins but tends to exclude most transmembrane proteins<sup>85</sup>.

**Post-Golgi traffic to the cell surface.** One of the main tenets of the lipid raft hypothesis was the prediction that the transport machinery in the *trans*-Golgi network (TGN) of epithelial cells sorts lipids and proteins into common carrier vesicles for targeted delivery to the apical or basolateral surface<sup>91</sup>. The postulated apical-surface raft carriers have remained elusive and have so far not been isolated. In yeast, there are also two main pathways to the cell surface<sup>92</sup>: one that transports plasma membrane proteins (including GPI-anchored proteins) directly, and one that uses endosomal compartments as an intermediate station to transport soluble secreted proteins such as invertase to the cell surface (akin to what has been suggested for the basolateral route

in epithelial Madin–Darby canine kidney (MDCK) cells). A genome-wide visual screen identified several enzymes involved in sterol and sphingolipid synthesis that are essential for the delivery of a raft marker protein to the cell surface<sup>93</sup>. Thus, the yeast raft lipids, which are the cholesterol homologue ergosterol and three classes of sphingolipids (inositolphosphoceramide, mannosyl-inositolphosphoceramide and mannosyl-diinositolphosphoceramide)<sup>94</sup>, were shown to regulate the delivery of detergent-resistant cargo to the plasma membrane. Consistent with the notion of raft-enriched carriers, immuno-isolation and subsequent quantitative analysis by mass spectrometry of TGN-derived vesicles isolated using a raft marker protein as bait showed that ergosterol and the most complex yeast sphingolipid, mannosyl-diinositolphosphoceramide, were selectively enriched and were the most abundant lipids in the transport vesicles<sup>40</sup> (FIG. 2). These were the first data to give direct experimental support to the hypothesis that raft cargo proteins are delivered from the Golgi complex to the cell surface in a raft carrier. Interestingly, laurdan analysis of the immuno-isolated yeast carrier vesicles showed that their membranes were more condensed than the membrane of the donor compartment, supporting a change in membrane packing during sorting. The protein and lipid-sorting process therefore probably involves raft clustering to drive segregation in the membrane of the TGN<sup>95</sup>. Biophysical studies of yeast raft lipids have shown that they can phase separate into liquid-ordered and liquid-disordered domains in GUVs<sup>96</sup>. Therefore, the formation of the raft-selective 100-nm carrier vesicles at the yeast Golgi membrane is envisioned as an interplay between raft clustering (induced by unidentified proteins), line tension and curvature, and additional machinery, including tensional forces generated by actin-based motors (FIG. 5b). Curvature is probably facilitated by bending proteins, such as the yeast BAR (Bin–Amphiphysin–Rvs) protein Rvs161 (reduced viability on starvation protein 161), which was identified in the plasma membrane delivery screen.

**Glycosphingolipid-mediated endocytosis.** Toxins and viruses have proven to be important tools for the study of endocytosis as they can be taken up by various internalization mechanisms<sup>97</sup>. Here, we consider Shiga toxin and the polyoma virus Simian virus 40 (SV40), both of which bind glycosphingolipids as surface receptors to become internalized by a glycosphingolipid-mediated clustering process.

Shiga toxin uses both clathrin-mediated and non-clathrin-dependent endocytosis<sup>98</sup>. Non-clathrin-dependent endocytosis has been studied in detail. The homopentameric Shiga toxin can bind up to 15 copies of its receptor globotriaosylceramide (Gb3)<sup>99</sup>. Binding on the plasma membrane causes the formation of narrow tubular invaginations that use the dynamin scission machinery to be released from the plasma membrane into the cytoplasm<sup>100</sup>. Tubule formation requires no energy but is regulated by membrane tension. Studies using phase-separated GUVs showed that the binding

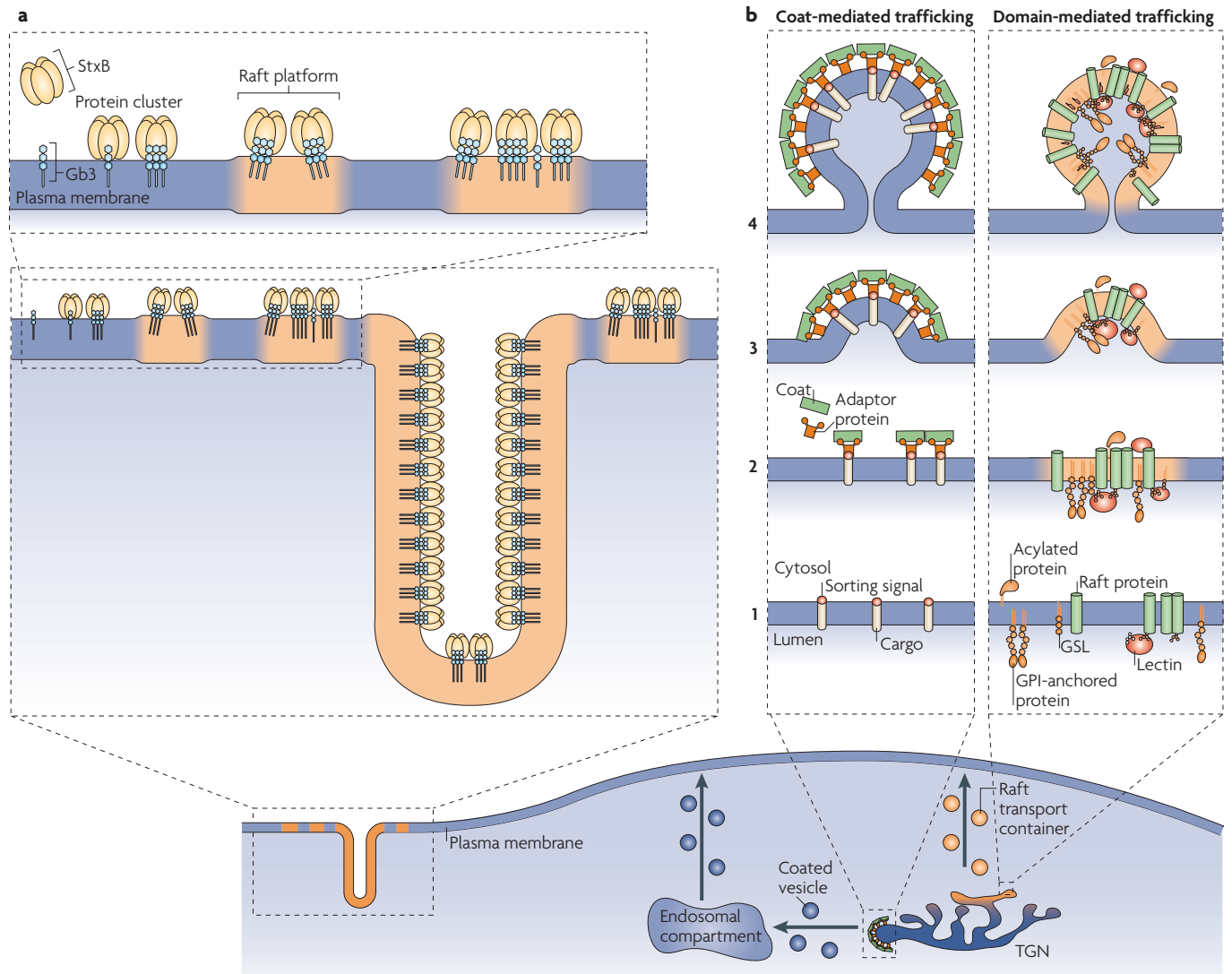
#### Myristate

A group that is attached to a protein through an amide bond by the irreversible, co-translational process of myristoylation. This is an important modification for membrane targeting.

#### Shiga toxin

One of a family of protein toxins produced by bacteria that can cause dysentery.





**Figure 5 | Rafts in membrane trafficking. a** Glycosphingolipid-mediated endocytosis. The pentavalent Shiga toxin B (StxB) binds to its receptor, the glycosphingolipid Gb3, inducing a clustering process that leads to invagination of the plasma membrane into the cytosol. **b** Post-Golgi traffic to the endosomal compartments in yeast or the basolateral plasma membrane in epithelial cells is sorted by adaptor proteins and coat proteins such as clathrin (shown as coat-mediated trafficking). Proteins containing the appropriate cytoplasmic sorting signals (1) are recognized by adaptor proteins on which the coat proteins assemble (2). For clathrin-mediated sorting, membrane bending (3) and subsequent budding (4) is driven by the formation of the coat, aided by specific bending proteins (not shown). Proteins sorted into a raft transport container (shown as domain-mediated trafficking) are clustered into a raft platform as described in FIG. 1. The platform includes raft constituents and excludes non-raft proteins and lipids. Membrane bending is facilitated by the line tension between the domains. Sorting is brought about by clustering agents, which might be luminal lectins or cytosolic peripheral proteins. GSL, glycosphingolipid. Figure in part **a** is modified, with permission, from REF. 102.

of Shiga toxin to Gb3 takes place in the liquid-ordered phase, which induces a more condensed phase and segregation of Gb3 into high-density clusters. Moreover, Shiga toxins may locally create an asymmetric stress in the external leaflet of the bilayer that leads to bending towards the protein; this effect seems to depend on wedge-shaped Gb3 species<sup>100</sup> (FIG. 5a). Additionally, the toxin molecules in the Gb3 cluster can experience attractive interactions, arising solely as a result of membrane bending<sup>101</sup>. These effects could together drive the formation of tubules<sup>102</sup>.

Furthermore, tubule fission has also been proposed to occur by a line energy-driven mechanism<sup>103</sup>. When dynamin is inhibited in cells, long tubules are created in response to Shiga toxin, which can be released in a cholesterol-dependent manner by conditions that favour domain formation such as cooling or actin polymerization<sup>103</sup>. The scission is therefore proposed to derive from physical pinching as the newly created domains minimize the unfavourable interaction with the bulk membrane, and not from the ‘pinching’ activity of GTPases.

**Line energy**

The energy arising from the unfavourable interaction or ‘tension’ of two phase-segregating membrane domains. It is the product of line tension and interaction length.

Similarly to Shiga toxin, the SV40 virus binds a glycosphingolipid, the ganglioside GM1 (REF. 97). In this case, the binding is mediated by the pentameric capsid protein VP1. After binding, the GM1 patch invaginates towards the cytoplasm, usually enclosing one virus into a vesicle destined for the ER. Binding of SV40 can also occur in caveolae, but these mostly remain at the cell surface<sup>97</sup>. In phase-separated GUVs containing GM1, the viral structures bind to the liquid-ordered phase<sup>104</sup>. Unlike native GM1 molecules containing long acyl chains that support line tension-driven tubulation, synthetic GM1 molecular species with short hydrocarbon chains partition to the liquid-disordered phase and impair internalization and infection when introduced into host cells lacking the native version of this glycosphingolipid. The saturation level of the hydrocarbon chains affects GM1 partitioning in a similar manner. Finally, the spacing of multivalent binding by toxins and the virus seems to be important as antibody clustering fails to induce internalization<sup>104</sup>.

Studies of GPI-anchored proteins using clathrin-independent internalization pathways have shown that proteins with artificial, unsaturated lipid anchors<sup>105</sup> can be endocytosed together with GPI-anchored proteins that have normal, saturated anchors, although it is unclear how. The linkers could form hydrogen bonds with more 'raftophilic' molecules, such as native GPI-anchored proteins, and the protein ectodomains could help drive internalization by curvature-mediated interactions<sup>101</sup>. An alternative explanation is that GPI-anchored proteins use a simple, nonspecific, default (or bulk) process for the uptake of lipid-bound proteins present in the exoplasmic (luminal) leaflet of the plasma membrane<sup>105</sup>.

The emerging principles from these studies are that raft clustering can lead to formation of membrane domains that can bud off to form raft carriers that distribute specific sets of lipids and proteins to different post-Golgi destinations in the secretory and endocytic pathways. These mechanisms differ from those used by the well-known coat-mediated transport carriers, but the machinery governing their generation is poorly understood.

### Moving forwards with rafts

Although many cell functions take place in membranes where proteins and lipids are intimately mixing, research in this field has long avoided the study of how lipids and proteins function together. This neglect is now being remedied by the realization that membranes are made functional by lipid–lipid, lipid–protein and protein–protein interactions. For example, it has been recently shown that proteins adjust their transmembrane length and composition to the specific physical properties of the different subcellular membranes in which they reside<sup>106</sup>, which themselves have evolved highly specific lipid compositions<sup>1</sup>. Cell membranes are lipid bilayers, crowded with proteins occupying around 20% of the bilayer area<sup>107</sup>. This means that lipids and proteins in membranes should really be studied as collectives<sup>55</sup>. Membrane proteins alter their lipid environment not only by binding specific lipids but also by influencing their surrounding lipid environment<sup>108</sup>. Therefore, one area of increasing

importance will be the study of lipid–protein interactions that regulate the nanoscale raft protein assemblies and how they can coalesce to form functional platforms. This work will require sophisticated and difficult image technology, as well as biochemistry that must overcome the technical hurdles that plague work with hydrophobic proteins and lipids. The well-known asymmetry of the bilayer and the proteins spanning the membrane add additional barriers to reconstituting membrane organization and function *in vitro*. In addition, most saturated lipids that are thought to underlie raft formation reside in the exoplasmic leaflet of the membrane, and the principles of raft organization in the cytosolic leaflet remain unknown<sup>109</sup>.

One issue we have not discussed is the role of cortical actin in regulating membrane organization. Clearly, actin can shape the lateral distribution of membrane components. For example, transient binding of a clustered GPI-anchored protein requires actin and depends on CSK-binding protein (CBP), ERM-binding protein 50 (EBP50), Src-family kinase phosphorylation and cholesterol<sup>110</sup>. Actin also seems to nucleate raft-based heterogeneity, both at the nanoscale level<sup>111,112</sup> and as a scaffold. Here, actin is functional, for example during high-affinity immunoglobulin- $\epsilon$  receptor (FcERI) immobilization<sup>113</sup> or TCR signalling<sup>114</sup>. We suggest that the cell, not aware of our present distinction between biophysics, biochemistry and structural mechanics, couples the specificity of peripheral decorating agents, such as actin, to those of cholesterol and sphingolipids, with the goal of making heterogeneity functional in the lateral dimension<sup>115</sup>. Realizing the power of this synergy will likewise require the coming together of these fields in membrane research.

The emergence of lipidomics methodology is an important advance. Until now, few studies have existed in which the diversity of membrane lipid composition has been analysed. We need to go beyond lipid classes and also analyse molecular diversity. With the new mass spectrometers and with an increasing availability of lipid standards, quantitative lipidomics is becoming possible that could characterize for the first time the molecular lipid composition of different organelle membranes. This possibility demands improved methodology for fractionating and purifying subcellular membrane compartments. However, the superior sensitivity of the mass spectrometric methods will allow analysis of minute samples. The analysis of membrane protein–lipid complexes will also become routine. Elucidation of the fine-tuned composition of membranes by lipidomics and proteomics is bound to open up new perspectives for basic biology and biomedical research.

During the past ten years, this field has been energized by the introduction of new and sophisticated methodology that is providing unprecedented resolution in space and time. The combination of methods and ideas will continue to challenge our views on how membranes are organized. A decade from now, we will have more accurate insights into how cells use their vast lipid and protein variability to construct functional membrane collectives.

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#### Competing interests statement

The authors declare [competing financial interests](#): see web version for details.

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