MASTER'S THESIS

The Lipid Raft Hypothesis:
Origins, Obscurities and Opinions

Galih

Programme : **Molecular and Cellular Life Sciences**
Graduate School of Life Sciences
Student Number : **3562247**
Supervisor : **Prof. dr. Bernd Helms**

Utrecht University
2011/2012

Table of Contents

Abstract

The plasma membrane has long been conceived as a homogenous lipid bilayer sheet that serves as a passive viscous matrix for membrane proteins. The hypothesis of lipid rafts has added more complexity in the lateral organization of the plasma membrane. Lipid rafts were originally defined as micrometer-sized lipid domains enriched in sphingolipids and cholesterol within cellular membranes that assemble specific sets of glycosylphosphatidylinositol-anchored and transmembrane proteins. These domains form platforms for membrane sorting in targeted-membrane trafficking and cell signaling processes. Experimentally, lipid rafts are partially resistant to non-ionic detergent solubilization in the cold and functionally dependent of the cholesterol level in the membrane. The extensive use of these simplistic biochemical definitions has accumulated a large body of inconsistent data that obscure the very concept of lateral membrane organization. More recent studies using less perturbing techniques in living cells have reshaped our understanding in the structure, function and dynamics of the plasma membrane. This current understanding became the essential principles of lateral membrane organization models that add new aspects previously uncovered by the original hypothesis of lipid rafts.

Introduction

The fluid mosaic model proposed by Singer and Nicolson about four decades ago set a major breakthrough in our understanding of the organization and dynamics of cellular membranes. This model was formulated after the observations of protein arrangement and diffusion on plasma membranes that were coupled with an understanding of how membrane proteins are associated with both leaflets of the membranes. According to this model, the plasma membrane is a two-dimensional arrangement of oriented lipids into a lipid bilayer that functions as a viscous solution of proteins (or lipoproteins). The lipids in the plasma membrane are always in a fluid state, hence allowing membrane proteins to diffuse randomly in the membrane. In addition, there is no long-range lateral organization in a fluid mosaic membrane (Singer and Nicolson, 1972) (**Fig. 1**). It is also well established that membrane lipids are not distributed equally to the outer and inner leaflets of the plasma membrane. Moreover, the membranes of various intracellular organelles possess unique lipid compositions (van Meer, 1989).

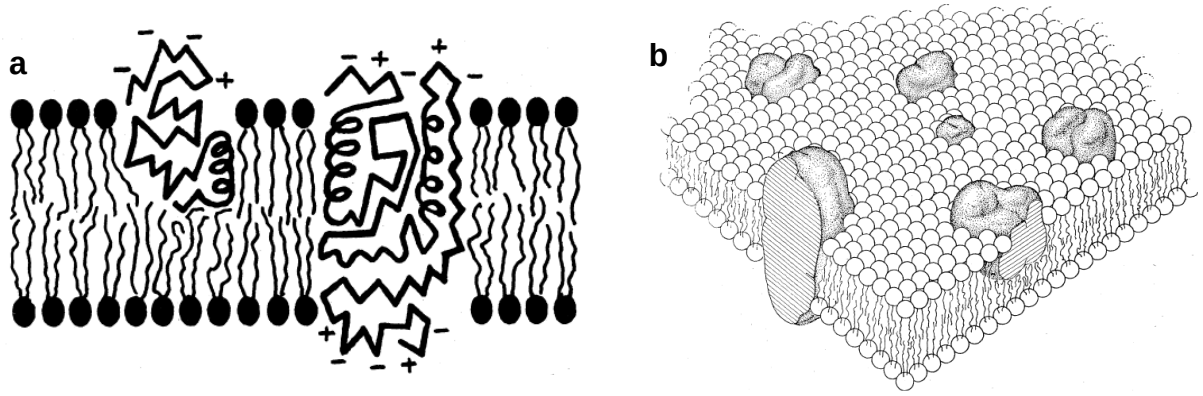

Figure 1. The structure of the plasma membrane according to the fluid mosaic model. **a.** The schematic cross-sectional view of the plasma membrane. The phospholipid head groups are depicted as filled circles, while the fatty acid chains are represented as wavy lines. The phospholipids are arranged as a discontinuous bilayer with their ionic and polar heads in contact with water. The integral proteins are shown as globular molecules partially embedded in, and partially protruding from, the membrane. The ionic residues (- and +) of the protein are on the protruding parts, while the non-polar residues are largely in the embedded parts. **b.** The schematic three-dimensional and cross-sectional views of the plasma membrane. The solid bodies with stippled surfaces represent the globular integral proteins, which at long range are randomly distributed in the plane of the membrane. At short range, some may form specific aggregates (Singer and Nicolson, 1972).

The view on cellular membranes has gradually changed from a laterally homogenous to laterally-organized lipid bilayer and from a structurally passive solvent to an active framework as new evidence for lipid self-association was revealed (Klausner and Wolf, 1980; Sankaram and Thompson, 1990; Thompson and Tillack, 1985). This emerging view was culminated with the proposal of the lipid raft hypothesis about 25 years after the

formulation of the fluid mosaic model. According to the hypothesis, lipids in the plasma membrane are heterogeneous in lateral distribution due to preferential association of sphingolipids and cholesterol into floating micrometer-sized domains called lipid rafts. These domains can actively and selectively assemble subsets of glycosylphosphatidylinositol (GPI)-anchored and transmembrane proteins to facilitate various cellular processes, including membrane trafficking and cell signaling (Simons and Ikonen, 1997). The alluring biological significance of lipid rafts has attracted many scientists from various disciplines to further study the involvement of lipid rafts in more diverse cellular processes. However, the accumulation of large and inconsistent data from the studies has caused serious doubt about the biological roles and even the existence of lipid rafts in cellular membranes (Munro, 2003).

In this review, I will initially present the origins of the lipid raft hypothesis derived from physical, chemistry, biochemistry, and cell biology studies in a chronological order to give insights into why and how the hypothesis was developed. I will summarize what features of the plasma membrane that were undisputed prior to the formulation of the lipid raft hypothesis, and how these features were incorporated into the raft model. In the following chapter, I will then discuss the cause of obscurities of lipid rafts coupled by their consequences in our understanding in the intracellular assembly and trafficking of lipid rafts. I will also point out the limitations of model systems for proving the existence of lipid rafts. In the last chapter, I will summarize how non-invasive techniques in investigating lipid rafts *in vivo* deepen our insights into the dynamics of membrane organization and how these insights have renewed interest in lipid domains and underpinned current opinions on lipid rafts in the form of membrane models that add new aspects of membrane lateral organization.

1. Origins of the lipid raft hypothesis

1.1. The main lipids in the plasma membrane

The plasma membrane is a semipermeable barrier that separates the interior environment from the exterior environment of a cell. This thin hydrophobic film composed primarily of lipids and proteins serves as a boundary defining the autonomy of individual cells. The plasma membrane of eukaryotic cells is formed from a highly diverse lipid species that can be categorized into three groups: phospholipids, neutral lipids and glycolipids (Bretscher, 1973) (**Fig. 2**). Phospholipids are the principle constituents of the lipid mass of eukaryotic plasma membranes. The quantitatively significant phospholipids are phosphatidylcholine, phosphatidylethanolamine, sphingolipids, phosphatidylserine, and phosphatidylinositol (Roelofsen et al., 1981; Zambrano et al., 1975). Neutral lipids are lipids that do not carry any charged group. A class of neutral lipids that is enriched at significant amounts in the plasma membrane is sterols, with cholesterol being the principle form found in vertebrates. Cholesterol is usually present at approximately one-half of the amount of total phospholipids in the plasma membrane (Colbeau et al., 1971). Glycolipids are lipids that contain carbohydrate residues. Glycolipids such as glycoglycerolipids and glycosphingolipids are minor lipid components of animal plasma membranes (Korey and Gonatas, 1963). Under normal conditions, intermediates in lipid metabolism constitute only small fractions of the lipid component of the plasma membrane (van Meer, 1989). As each of these lipid groups has unique molecular structures and physicochemical properties, each lipid group contributes to the nature of the plasma membrane in different ways.

The most abundant class of phospholipids in the plasma membrane is phosphatidylcholine. This phospholipid uses a glycerol molecule as a backbone to which two fatty acid residues and a phosphoryl-choline group are attached. The most common type of phosphatidylcholine has a saturated fatty acid residue in position *sn*-1 and an unsaturated fatty acid residue in position *sn*-2 of the glycerol backbone (Roelofsen et al., 1981). Phosphatidylcholine is the key building block of membrane bilayers as it spontaneously forms a bilayer structure in an aqueous environment. The fluidity of the bilayer structure depends on the acyl chains and temperature. Phosphatidylcholine with saturated and long acyl chains can be packed tightly. On the contrary, those with unsaturated and short acyl chains are loosely packed due to the presence of kinks in their acyl chains. Temperature affects the membrane fluidity by governing the diffusion rate of lipids in the membrane. Thus, saturated acyl chains and low temperature promote the formation of a rigid lipid bilayer, while unsaturated acyl chains and high temperature increase the propensity for the formation of a more fluid plasma membrane (Bretscher, 1973; Hubbell and McConnell, 1969).

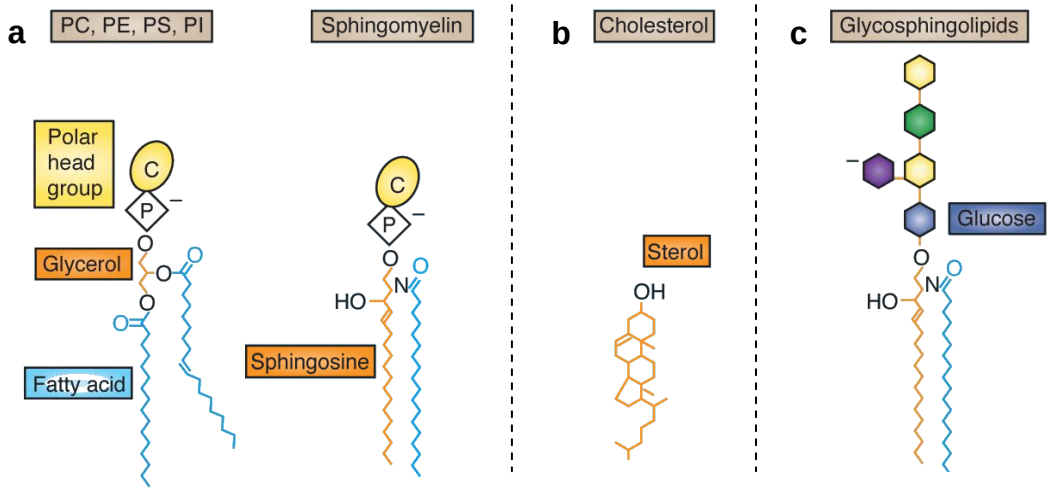

Figure 2. The main lipids in the plasma membrane of eukaryotic cells. The structures of the representative members of phospholipids (**a**), neutral lipids (**b**), and glycolipids (**c**). Glycerophospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) carry a glycerol backbone, while sphingolipids such as sphingomyelin carry a sphingosine backbone. Sterols such as cholesterol are based on a four-ring structure. Glycolipids, represented by glycosphingolipids, contain carbohydrate residues (Modified from: van Meer and de Kroon, 2011).

Another class of phospholipids, sphingolipids carries a sphingoid base instead of a glycerol molecule as a backbone (Stoffel, 1971). The most common sphingoid base found in animal tissues is sphingosine that consists of a carbon chain of fixed length, a double bond, a primary amine group, and two hydroxyl groups. A long saturated acyl chain can be esterified by an amide bond to the amine group of a sphingosine to form a ceramide. One of the hydroxyl groups of a ceramide, in turn, can be linked to a phosphoryl-choline group to form a sphingomyelin (Roelofsen et al., 1981). The orientations of the free hydroxyl and amide groups in the region between the polar head group and the non-polar hydrocarbon chains of sphingolipids have significance for the molecular interactions of sphingolipids in the membrane plane. The hydroxyl group is a hydrogen bond donor, while the amide group is a hydrogen bond acceptor. These functional groups are thought to permit sphingolipids to form hydrogen bonds with other sphingolipid molecules in the membrane plane (Boggs, 1987). This self association capacity increases the impermeability and rigidity of the plasma membrane where sphingolipids reside (Pascher, 1976).

Cholesterol is based on a four-ring structure that consists of three six-carbon rings and one five-carbon ring. One of the six-carbon rings has a double bond. All of the rings are joined together in a series to produce the rigid and planar part of the molecule. At one end of this ring configuration there is a free hydroxyl group and at the other end is a flexible hydrocarbon chain (Yeagle, 1985). These hydrophilic and hydrophobic poles determine the positioning of cholesterol molecules in the lipid bilayer. Cholesterol molecules span about half a bilayer with the hydroxyl group is situated next to the phospholipid ester carbonyl, while the four-ring structure is positioned alongside the acyl chains of neighboring phospholipids. The planar shape of cholesterol enables cholesterol molecules to fill the interstitial space between other membrane lipids. This property together with the rigid nature of the four-ring structure confer cholesterol a capacity to induce a tight packing of membrane lipids and an increase in the thickness of the lipid bilayer. As cholesterol inserts perpendicularly into the lipid bilayer, the lateral diffusion of membrane lipids and proteins is not dramatically perturbed. This membrane organization reduces passive permeability and increases the mechanical durability of the plasma membrane (Owicki and McConnell, 1980; Yeagle, 1985).

Glycolipids contain a polar carbohydrate moiety linked by a glycosidic bond to the hydrophobic part of a lipid (Bretscher, 1973). Based on their core lipid moiety, glycolipids are classified into glycoglycerolipids (containing glycerol) and glycosphingolipids (containing sphingosine). Glycosphingolipids that carry sialic acid residue(s) are referred to as gangliosides (van Meer, 1989). Due to the presence of the carbohydrate moiety, glycolipids are localized primarily in the outer leaflet of the plasma membrane with carbohydrate residues protruding from the membrane. One of the notable properties of glycolipids is the structural diversity mainly in their carbohydrate moiety that provides the plasma membrane a unique surface characteristic. Consequently, glycolipids exhibit more species and tissue specificity than do any other lipid classes (Weinstein et al., 1970).

It is clear that the lipids in the plasma membrane of eukaryotic cells are extremely diverse. They are different not only in their hydrophobic parts, but also in their hydrophilic parts (Roelofsen et al., 1981). Like the plasma membrane, other cellular membranes also contain more lipid species than that is needed to form simple bilayers (Bretscher, 1973). The evolutionary advantages of possessing a plethora of lipid species in cellular membranes may be to provide a wide range of membrane functions. To maximize the functions, it is expected that membrane lipids are organized in a specific way for a particular function. Therefore, the next questions would be: How are lipids in cellular membranes distributed between the two leaflets? How are lipids distributed in various organellar membranes? How are lipids in each cellular membrane organized along the membrane plane?

1.2. The lipid distribution and organization in cellular membranes

The lipids in many cellular membranes are not distributed equally between the two leaflets, generating lipid asymmetry in the membranes. The lipid compositions of the two leaflets of the endoplasmic reticulum (ER) membrane are virtually the same, whereas those of the Golgi, plasma and endosomal membranes are very different (Rothman and Lenard, 1977). Phosphatidylcholine and sphingolipids are enriched in the luminal or exoplasmic leaflet, while phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are restricted to the cytoplasmic leaflet (Bretscher, 1973; Zwaal et al., 1975; Allan and Walklin, 1988). The cholesterol distribution between the two leaflets is not exactly known. Cholesterol is able to diffuse spontaneously from one leaflet to another since its hydrophilic moiety is much smaller than those of other lipids. Therefore, cholesterol is believed to be present in approximately equal proportions in both leaflets (Yeagle, 1985). All of the glycolipids are distributed in the exoplasmic leaflet of the membranes with their carbohydrate residues are oriented towards the outside of the membranes (Steck and Dawson, 1974; Roelofsen et al., 1981).

The lipid asymmetry in cellular membranes is passively governed by the intrinsic biophysical properties of lipids and actively maintained by lipid transporters in cells (Rothman and Lenard, 1977). This energy-dependent lipid translocation is essential because lipid asymmetry has biological significance. For instance, increased exposure of negatively-charged phosphatidylserine at the exoplasmic leaflet of the plasma membrane plays an important physiological role in local blood clotting reactions (Zwaal et al., 1989). The exposure of the complex carbohydrate moiety of glycolipids is thought to be an important part of the recognition of cell types and the interaction of cells with their vicinity (Weinstein et al., 1970). Moreover, vesicle budding requires lipid translocation to the cytoplasmic leaflet to create a lipid quantity imbalance that can induce the membrane bending (Pomorski and Menon, 2006). Nevertheless, the two leaflets should not be considered as two independent entities. According to the bilayer-couple theory, cellular membranes are bilayer couples possessing transbilayer crosstalk mechanisms (Sheetz and Singer; 1974).

The uneven lipid distribution can also be found among various organellar membranes. Organelles involved in the secretory pathway, such as the ER, Golgi and plasma membrane, have strikingly different lipid compositions. The ER membrane is enriched in phosphatidylcholine but depleted in glycolipids, while the plasma membrane contains significant amounts (up to 33 mol% of the total lipids) of glycolipids but less phosphatidylcholine (Zambrano et al, 1975). Sphingolipids and cholesterol are synthesized in the Golgi and ER, respectively. Cells continuously transfer sphingolipids and cholesterol to the plasma membrane to maintain membrane rigidity. Interestingly, this process generates increasing compositional gradients from the Golgi to plasma membrane for sphingolipids and from the ER through Golgi to plasma membrane for cholesterol (van Meer, 1989; Orci et al., 1981). Organelles engaged in the endocytic pathway, such as early endosomes and lysosomes, have similar membrane lipid compositions to that of the plasma membrane since a whole or at least part of their membranes are derived from the plasma membrane (Henning and Stoffel, 1973; Belcher et al., 1987).

Organelles that are not connected by the vesicular transport pathways maintain their simple lipid compositions. Mitochondrial membranes are devoid of glycolipids and poor in cholesterol, sphingolipids and phosphatidylserine. The mitochondrial inner membrane mainly consists of phosphatidylcholine, phosphatidylethanolamine and cardiolipin (Daum 1985). Similar to mitochondrial membranes, the peroxisomal membrane is depleted of sphingolipids. Its major constituents are phosphatidylcholine and phosphatidylethanolamine (Crane and Masters, 1986). This membrane lipid distribution indicates that all organelles contain at least some lipids that are synthesized elsewhere and obtain them by lipid transfer. The transfer, therefore, must be specific to maintain their unique lipid compositions. Lipids are transferred to organelles in the secretory and endocytic pathways mainly by membrane vesicles, whereas that to organelles that are not connected to the pathways most likely as monomers mediated by both soluble and membrane proteins (van Meer, 1989).

The lipids in cellular membranes also seem to show heterogeneous lateral organizations along the membrane plane. *In vitro* studies suggested that fluid-fluid immiscibility in a specified temperature and a lipid composition can give a lateral separation between gel and liquid phases in phospholipid membranes (Shimshick and McConnell, 1973; Hong-wei and McConnell, 1975; Hui and Parsons, 1975). In the late 1970s, it was recognized that cholesterol does not mix homogeneously with phosphatidylcholine. In fact, cholesterol proportions ranging from 5–25 mol% in phosphatidylcholine membranes are sufficient to induce the formation of two immiscible cholesterol-rich and cholesterol-poor regions (Estep et al., 1978; Mabrey et al., 1978). Furthermore, regions with different fluidity in the plasma membrane could be distinguished *in vivo* (de Laat et al., 1979). It was also shown that exogenously added *trans*-unsaturated and saturated fatty acids preferentially partition into solid-like domains, while *cis*-unsaturated fatty acids preferentially partition into fluid domains in the plasma membrane of lymphocytes (Klausner and Wolf, 1980). In 1982, Karnovsky and colleagues formalized the concept of lipid domains in the plasma membrane. According to this concept, the plasma membrane is not necessarily homogeneous, but may well be organized into co-existing gel and fluid domains.

At this point, the co-existence of lipids in more than one phase in the membrane plane was considered simply as the existence of lipid domains. Cholesterol levels were suggested to play an important role in the phase separation (Recktenwald and McConnell, 1981). Cholesterol has a striking property of ordering the lipid bilayer in one dimension. It is able to promote the tight packing of acyl chains, while allowing membrane lipids and proteins to diffuse laterally. This phase was termed cholesterol-rich liquid-ordered (l_o) phase that could co-exist with a cholesterol-poor liquid-disordered (l_d) phase (Ipsen et al., 1987; Ipsen et al., 1989) (**Fig. 3**). The l_o phase was believed to exist in most cellular membranes containing more than 25 mol% cholesterol as a fraction of total lipids (Thewalt and Bloom, 1992). Cholesterol has a strong preferential interaction with sphingolipids (Sankaram and Thompson, 1990), although sphingolipids at a small amount (<10 mol%) *per se* could cluster into dispersed microdomains in l_d phospholipid bilayers (Thompson and Tillack, 1985).

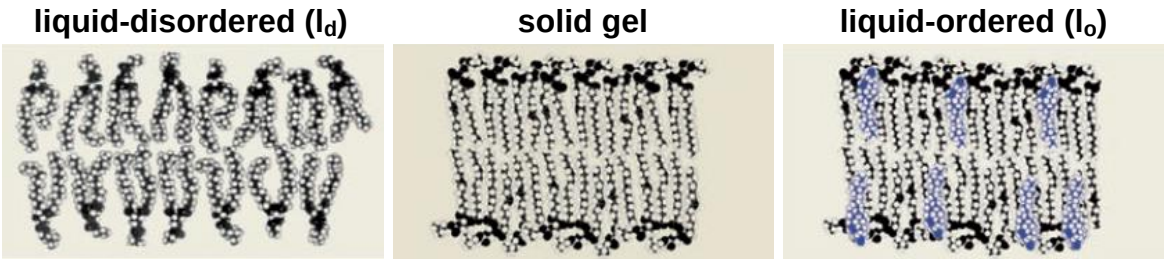

Figure 3. Lipid phases in membranes. Simple phospholipid bilayers above their melting temperature (Tm) form a fluid phase (liquid-disordered (l_d), sometimes referred to as liquid crystalline), while below their Tm form a solidified gel phase. The presence of cholesterol (shown in blue) promotes the formation of an intermediate phase termed liquid-ordered (l_o) (van Meer et al., 2008).

It is established that lipids are not distributed evenly in cellular membranes. The unequal lipid distribution between the two leaflets and among various organellar membranes have been shown to have biological significance. However, the direct links between lipid domains in the plasma membrane and cellular functions were not understood. An emerging speculation was that such domains are flexible and rapidly alterable recognition sites on cell surfaces (Brown and Thompson, 1987). Despite cholesterol and sphingolipids may seem to involve in the phase separation, the driving forces underlying the formation and maintenance of lipid domains were not exactly known. How lipid domains are distributed between the two leaflets also remains to be investigated. As biological membranes also contain significant amounts and diverse kinds of proteins, their possible interactions raised further questions. Do lipid domains associate with specific sets of proteins? Do lipid domains affect protein structure and function?

1.3. Lateral organization of plasma membrane lipids

Our understanding of lipid domains in the plasma membrane was revolutionized by the studies of the biogenesis of polarized plasma membranes in epithelial cells. The plasma membrane of polarized epithelial cells is differentiated into the apical and basolateral plasma membranes (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). The apical plasma membrane contains more glycosphingolipids but less phosphatidylcholine

compare to the basolateral plasma membrane (van Meer and Simons, 1982; Nichols et al., 1987; Hansson et al., 1986). The boundaries defining the apical and basolateral plasma membranes are tight junctions that encircle the apex of the cell (Simons and Fuller, 1985) and seal each cell to its lateral neighbors to form a permeability barrier between the cells (Diamond, 1977). Importantly, tight junctions maintain cell polarity by preventing lateral lipid and protein diffusion in the exoplasmic leaflet between the two plasma membranes. This suggests that the lipid differences between the apical and basolateral plasma membranes reside in their exoplasmic leaflets (van Meer and Simons, 1986).

The generation of the two plasma membranes with different lipid compositions, therefore, can be simplified by enriching sphingolipids (predominantly glycosphingolipids) and phosphatidylcholine in the exoplasmic leaflet of the apical and basolateral plasma membranes, respectively. The possibility that the local plasma membrane metabolism of glycosphingolipids and phosphatidylcholine play a significant role in the accumulation of the lipids in the corresponding plasma membranes can be ruled out since glycosphingolipids are synthesized in the Golgi and phosphatidylcholine is synthesized mainly in the ER. This implies that the newly synthesized lipids must be sorted and targeted to specific regions of the plasma membranes. If vesicular transport is the mechanism for distributing the correct lipids apically and basolaterally, it is expected that the lipid sorting takes place in the membrane compartment from which vesicles bud.

An investigation into the intracellular trafficking of sphingolipids by employing a fluorescent analog of sphingolipid precursor as a probe demonstrated that the newly synthesized sphingolipids are localized in the luminal leaflet of the Golgi membrane. These sphingolipids are then transferred directly to the apical plasma membrane (van Meer et al., 1987). This investigation underpinned a model proposed for a lipid sorting mechanism involving lipid domains. According to this model, the sorting of sphingolipids is carried out by the formation of (glyco)sphingolipid microdomains in the luminal leaflet of the trans-Golgi network (TGN) where vesicles exit for the cell surface. The domain formation is mediated by hydrogen bonds among sphingolipid molecules. Subsequently, the domains bud from the TGN into a membrane vesicle and fuse with the apical plasma membrane to introduce the sphingolipids to the exoplasmic leaflet. The luminal leaflet of the TGN membrane which buds into basolateral transport vesicles would, as a consequence, be depleted of the (glyco)sphingolipids and enriched in phosphatidylcholine. This model was the first clear example of a direct link between lipid domains and a cellular function (van Meer and Simons, 1988).

Most proteins in the exoplasmic leaflet of the plasma membrane of polarized epithelial cells are glycoproteins (Bretscher and Raff, 1975). These proteins receive their first carbohydrate residues during their synthesis in the ER and their mature carbohydrate moiety in the Golgi (Walter et aI., 1984; Danielsen et aI., 1984). Interestingly, glycoproteins display similar behavior to sphingolipids. Both apical and basolateral glycoproteins remain together during transport through the Golgi before being sorted in the TGN (Rindler et al., 1985; Griffiths and Simons, 1986). Their delivery from the Golgi to the apical or basolateral plasma membranes has virtually the same kinetics as that of sphingolipids (Matlin and Simons, 1984). This finding raised the possibility that there is a close connection between the sorting of membrane lipids and proteins. The apically targeted sphingolipids and glycoproteins are thought to be sorted together into common transport vesicles that have similar properties to their destinations. The glycoproteins must be included in (glyco)sphingolipid microdomains in the luminal leaflet of the TGN. The inclusion might be assisted by sphingolipid-protein interactions because in some cases the proteins do not span the membrane (Simons and van Meer, 1988).

The hypothesis implies that there might be specific interactions between sphingolipids and apical proteins. Strikingly, an apical protein with a glycosylphosphatidylinositol (GPI) anchor that is restricted to the exoplasmic leaflet of the plasma membrane can be recovered in membrane fractions enriched in glycosphingolipids. These fractions are low-density membrane and resistant to cold non-ionic detergent (Triton X-100) solubilization (Brown and Rose, 1992). Detergent-insoluble membrane fractions that are rich in sphingolipids were shown to be similar to l_o phase bilayers both in terms of physical properties and in terms of enrichment in cholesterol and lipids with saturated acyl chains. As detergent-insolubility was found to correlate with lipid melting temperature, insolubility of cellular lipids might reflect their phase behavior. It was proposed that lipid domains are domains of ordered lipid. The association of GPI-anchored proteins with lipid-ordered domains was explained as a consequence of the ability of their saturated acyl chains to fit readily into tightly-packed domains (Schroeder et al., 1994).

In 1997, Kai Simons and Elina Ikonen extended the hypothesis of lipid microdomains to include cholesterol as a co-organizer and coined the term ‘lipid rafts’ for referring the domains. They proposed that the preferential association of sphingolipids with cholesterol is the main driving force for the assembly of lipid rafts. Glycosphingolipids are linked each other laterally by hydrogen bonds between their carbohydrate head groups. The void resulted from this association is filled by cholesterol molecules. Interactions between sphingolipids and cholesterol confer lipid rafts l_o phase wherein lipid acyl chains are longer and more saturated. These tight

assemblies would prefer lipids with saturated acyl chains to those with unsaturated ones (i.e. phosphatidylcholine), hence forming moving entities in l_d phospholipid bilayers. To maximize the tight packing, the cytoplasmic leaflet of lipid rafts was speculated to also carry lipids with saturated acyl chains. The lipid assembly into lipid rafts is dynamic, hence allowing individual lipids to associate with or dissociate from lipid rafts. Specific sets of proteins associate with lipid rafts by either their attachments to saturated acyl chains or transmembrane domains. Raft-associated proteins can be embedded in the domains or at the interface between lipid rafts and the fluid regions of glycerolipids (**Fig. 4**).

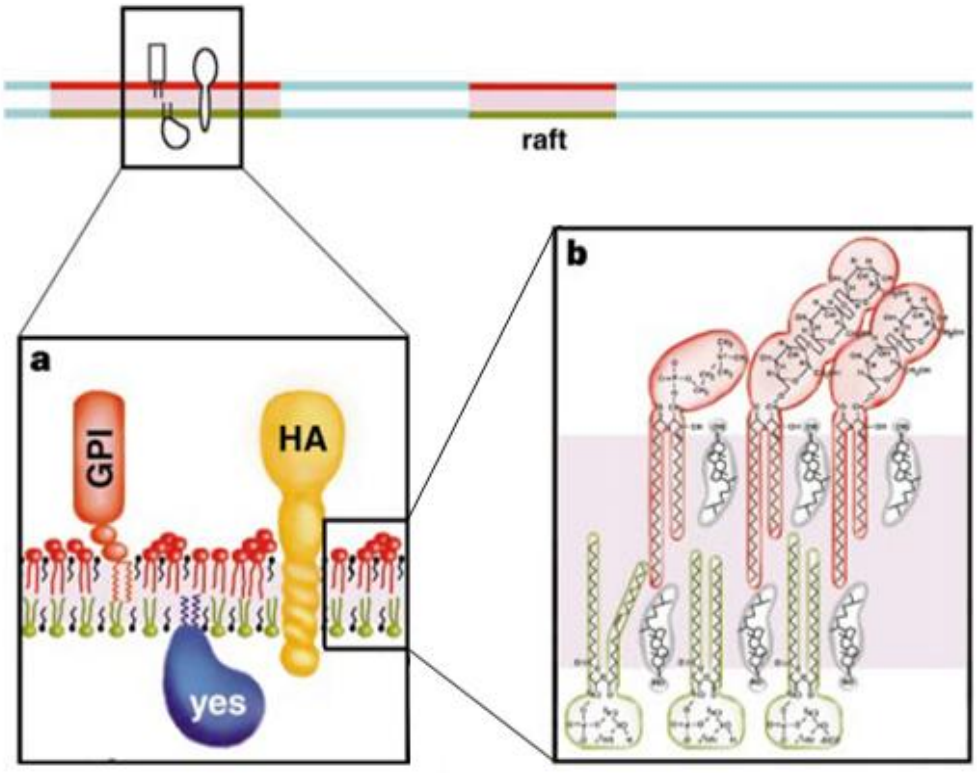

Figure 4. Original depiction of the organization of lipid rafts in the plasma membrane. Lipid rafts (red) have different lipid organization both in the exoplasmic and cytoplasmic leaflets of the plasma membrane from the other regions (blue) of the membrane. **a.** Raft-associated proteins can attach to lipid rafts by their GPI anchors, acyl tails (yes) or transmembrane regions (haemagglutinin, HA). **b.** The lipid bilayer in lipid rafts is asymmetric with sphingomyelin (red) and glycosphingolipids (red) enriched in the exoplasmic leaflet and glycerolipids in the cytoplasmic leaflet. Cholesterol (grey) is present in both leaflets, filling the void under the head groups of sphingolipids or extends the interdigitating acyl chain in the opposing leaflet (Modified from: Simons and Ikonen, 1997).

The hypothesis was also generalized with lipid rafts as a principle of the lateral organization of plasma membrane lipids that functions in membrane trafficking and cell signaling. These roles are due to the capacity of lipid rafts to selectively include or exclude specific sets of membrane lipids and proteins. In membrane trafficking, lipid rafts may function as sorting centers and attachment platforms for proteins directed to particular subcellular compartments. Although lipid rafts are mainly involved in the apical route of membrane traffic, their roles in the endocytic route were also postulated. Lipid rafts therefore may be present in organellar membranes involved in the secretory and endocytic pathways of membrane traffic beyond the ER. In cell signaling, lipid rafts may help to ensure specificity and fidelity during signal transduction. Lipid rafts increase the local concentrations of receptors to facilitate their interactions with exoplasmic ligands and cytoplasmic effectors. Thus, lipid rafts could accelerate the signaling process and prevent unwanted crosstalk between pathways (Simons and Ikonen, 1997). In short, the lipid raft hypothesis was originally proposed as a mechanism of lipid sorting to explain the generation of polarized plasma membranes of epithelial cells. The hypothesis was then developed into the notion of lipid rafts as a principle of the lateral organization of plasma membrane lipids that facilitates diverse cellular processes. The model extended by Kai Simons and Elina Ikonen became the cornerstone of the lipid raft model in the plasma membrane. In this review, the model is considered as the original hypothesis of lipid rafts.

2. Obscurities of lipid rafts

2.1. Operational definitions of lipid rafts

The lipid raft hypothesis suggests the existence of small and dispersed regions in the plasma membrane that are rich in glycosphingolipids and cholesterol, and capable to mediate membrane trafficking and cell signaling. The early biochemical criteria used to define lipid rafts were formulated from the discovery that the proposed raft-resident proteins, GPI-anchored proteins can be recovered in membrane fractions that contain significant amounts of glycosphingolipids and cholesterol (Brown and Rose, 1992). These fractions are remnants after membrane solubilization with non-ionic detergent Triton X-100 in the cold, so that they were termed detergent-resistant membranes (DRMs) (Brown and London, 1998). DRMs were believed to be derived from lipid rafts *in vivo* (Ahmed et al., 1997; Schroeder et al., 1998). In addition, DRMs exhibit similar properties to l_o phase of the lipid bilayer (Schroeder et al., 1994). The natures of lipid packing of l_o phase confer lipid rafts the capacity to selectively include or exclude membrane components to facilitate the cellular processes. As cholesterol is the key lipid of l_o phase, cholesterol was thought to be essential for the functionality of lipid rafts. This was the starting point of adopting partial resistance to solubilization with non-ionic detergents in the cold and cholesterol-sensitive functional membrane complexes as operational definitions of lipid rafts. These definitions have been extensively used as experimental guides for investigating the putative appearance, composition and functionality of lipid rafts.

DRMs isolated from mammalian cells have the appearance of vesicles of 0.1–1 μm. These DRMs were proposed to originate mostly from the plasma membrane with little fractions were derived from organellar membranes (Brown and Rose, 1992; Brown and London, 1998). An electron microscopy study showed that the unilamellar appearance of the vesicles is independent of the type of non-ionic detergents used in their isolation (Radeva and Sharom, 2004). These observations supported the original notion of lipid rafts as micrometer-sized and dispersed lipid domains in the plasma membrane. Prior to isolation, however, detergent-resistant regions of the plasma membrane appeared as a continuous sheet with holes once occupied by the detergent-soluble regions of the plasma membrane (Mayor and Maxfield, 1995; Hao et al., 2001; Sengupta et al., 2011) (**Fig. 5**). This striking finding indicates that DRMs do not exist as scattered structures in the plasma membrane. Instead, the detergent-resistant regions occupy the majority of the surface area of the plasma membrane. Importantly, it does not fit to the original hypothesis of small and dispersed lipid rafts, hence initiating the doubt about the existence of lipid rafts in the plasma membrane.

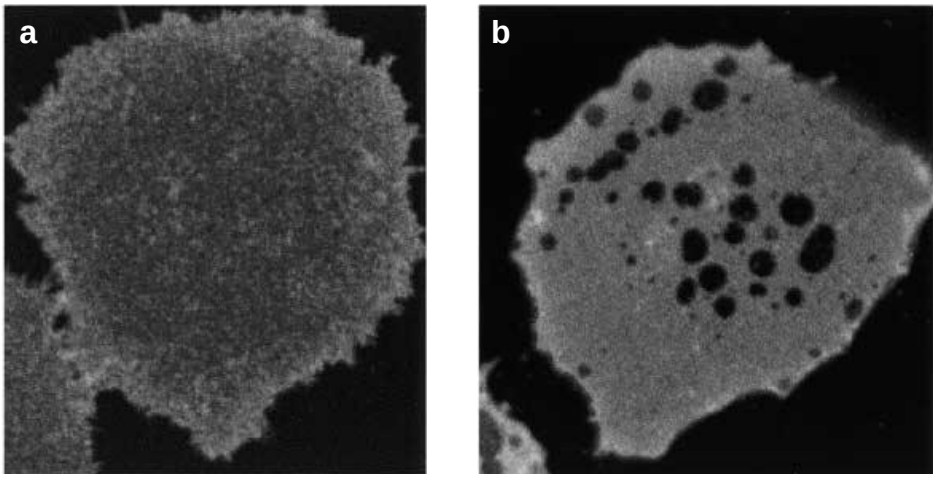

Figure 5. The majority of the plasma membrane is resistant to Triton X-100 solubilization. Micrographs of cells stained with a fluorescent monoclonal antibody to a GPI-anchored protein (folate receptor), without (**a**) or with (**b**) solubilization with Triton X-100 for 30 minutes at 4°C, before being rinsed and imaged (Hao et al., 2001).

DRMs isolated from various cell types by using particular detergents showed cell type-dependent variations in lipid compositions (Schuck et al., 2003). In addition, the ability of detergents that vary in their ionic strength, such as Triton X-100, Pluronic F-127, sodium cholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Tween 20, and Brij 96V, to enrich sphingolipids and cholesterol over glycerophospholipids in DRMs differ considerably. In fact, only Triton X-100 is able to enrich lipids expected to be found in lipid rafts (Jakop et al., 2009). Triton X-100 also tends to solubilize phospholipids located in the cytoplasmic leaflet of the plasma

membrane more than Brij 98 and Lubrol WX (Schuck et al., 2003; Delaunay et al., 2008). Besides showing variation in lipid compositions, DRMs prepared by using different detergents also contain different set of proteins (Castelletti et al., 2008; Williamson et al., 2010). Given that proteins are deemed to be associated with lipid rafts if they can be isolated together with DRMs, the use of different detergents suggests that more and more proteins should be considered as the residents of lipid rafts. To accommodate the newly claimed components of lipid rafts, the definition of lipid rafts has been subjected to continuous amendments (Shaw, 2006).

In parallel with its extensive use, the membrane solubilization with non-ionic detergents in the cold as a method in the preparation of DRMs has been subjected to careful and thorough examinations. Provided that the degree of membrane resistance to detergent solubilization depends on the stability of interactions among membrane components relative to their interactions with detergent molecules, it is affected by membrane composition and the type of detergent. In fact, a single detergent can be used to generate DRMs with various compositions as increasing concentration of the detergent gradually extracts one lipid or protein after another (Babiychuk and Draeger, 2006). Nevertheless, membrane lipid-to-detergent ratio is often not well defined and controlled during the experiment by investigators (Edidin, 2003). In addition, an accumulating body of evidence surprisingly suggested that Triton X-100 treatment to the plasma membrane induces the formation of ordered domains in a homogeneous fluid membrane (Heerklotz, 2002) as well as a profound lipid rearrangement including a rapid rearrangement of the ganglioside GM1 and cholesterol into the newly formed structures (Giocondi et al., 2000; Ingelmo-Torres et al., 2009). These discoveries have cast serious doubts on the hypothesis of lipid rafts.

Sensitivity to cholesterol deprivation has been widely used to demonstrate the involvement of lipid rafts in particular cellular processes. The reduction of cellular cholesterol level with lovastatin and β-methyl-cyclodextrin was accompanied by a decrease in the transport rate of the apical marker protein influenza virus hemagglutinin from the TGN to the plasma membrane. By contrast, the transport of the basolateral marker vesicular stomatitis virus glycoprotein and the ER-to-Golgi transport of both membrane proteins were not affected. The cholesterol reduction also led to an increase in Triton X-100 solubility of hemagglutinin and a missorting of the protein to the basolateral plasma membrane. Another glycoprotein that is apically secreted, gp-80 was also missorted following the cholesterol reduction, suggesting that gp-80 also uses a raft-dependent mechanism for apical sorting. This was the first direct evidence that supported the functional significance of cholesterol in the sorting of apical membrane proteins as well as of apically secreted glycoproteins (Keller and Simons 1998).

The major drawback of cholesterol depletion with cholesterol-chelating agents in assigning the functions of lipid rafts is that cholesterol depletion may cause non-specific effects. For example, despite clathrin-coated pit internalization is a process independent of lipid rafts, cholesterol depletion using β-methyl-cyclodextrin leads to a failure of clathrin-coated pits to induce curvature in the plasma membrane and detach from the membrane (Subtil et al., 1999). Moreover, cholesterol depletion with β-methyl-cyclodextrin inhibits Ca^{2+} influx known to be induced by T-cell receptor (TCR). However, this manifestation is not due to the perturbation of T-cell signaling after the disruption of lipid rafts, but the depletion of intracellular Ca^{2+} stores and the plasma membrane depolarization of T cells (Pizzo et al., 2002). Cyclodextrins also seem to extract cholesterol from both raft and non-raft domains of the plasma membrane and alter the distribution of cholesterol between plasma and organellar membranes (Zidovetzki and Levitan, 2007). Importantly, reduction in cholesterol levels induces the formation of micrometer-sized lipid domains in the plasma membrane and causes molecules in the more ordered domains to be extracted differently by non-ionic detergents in the cold (Hao et al., 2001; Babiychuk and Draeger, 2006). Therefore, any consequence following the reduction in cellular cholesterol level is not necessarily caused by the loss of lipid rafts. This complication in the interpretation of the effects may result in a misleading assignment of lipid raft functions.

2.2. Discrepancies in the assembly and trafficking of lipid rafts

The operational definitions have also been applied to study the assembly and trafficking of lipid rafts. According to the original hypothesis, the assembly of lipid rafts takes place in the luminal leaflet of the TGN (van Meer and Simons, 1988). This hypothesis is supported by the fact that apically-directed membrane proteins that are synthesized in the ER become detergent resistant only after entering the Golgi. The delay in acquired detergent insolubility implies that lipid rafts are assembled in the Golgi (Brown and Rose, 1992). In polarized epithelial cells, the newly assembled lipid rafts are transported from the TGN to the apical plasma membrane. Lipid rafts were also proposed to play more general roles in the post-Golgi membrane trafficking, including endocytosis that recycles some of raft-associated proteins back to the Golgi (Simons and Ikonen, 1997) (**Fig. 6**). The documentation of intermediate vesicles carrying a fluorescence-tagged GPI-anchored protein from the Golgi

to plasma membrane and those delivering the protein back to the Golgi has underpinned the proposed routes of the trafficking of lipid rafts (Nichols et al., 2001). Furthermore, the comprehensive lipidome of immunoisolated carriers between the TGN and plasma membrane has provided additional direct experimental support for a post-Golgi raft pathway that transports proteins towards the plasma membrane as raft-forming lipids are selectively enriched in the carriers (Klemm et al., 2009).

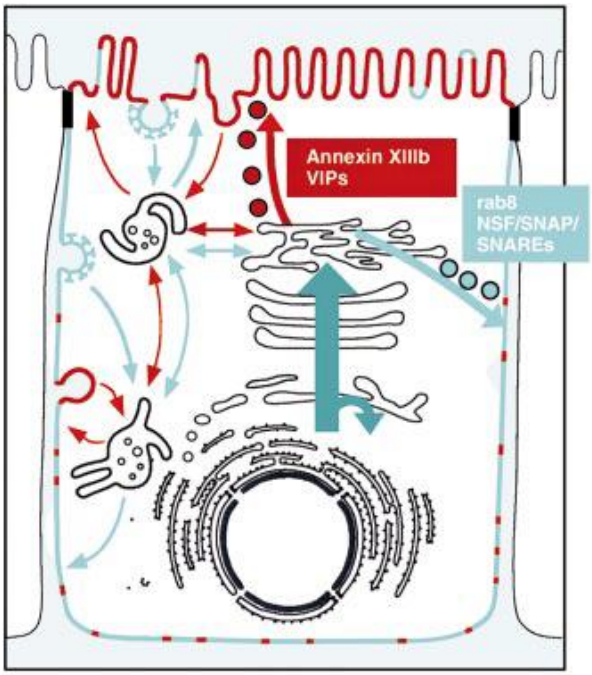

Figure 6. The postulated post-Golgi trafficking of newly assembled lipid rafts in polarized epithelial cells. The red regions of the plasma membrane are sphingolipid-cholesterol rafts and the blue areas are phosphatidylcholine-enriched regions. Sorting in the raft circuit (in red) is based on sphingolipid-cholesterol microdomains functioning as sorting platforms to which vesicular integral membrane proteins (VIPs) associate as stabilizers or as linkers to other proteins in the raft. The other circuit (in blue) employs sorting signals in the cytoplasmic tails as well as adaptor and coat proteins binding to them. The NSF-SNAP-SNARE-Rab system is used for vesicular docking and fusion in this blue circuit; in the red circuit specific annexins may be involved. VIPs are also in the basolateral vesicles in polarized epithelial cells but their physical state is different from that in apical vesicles, where they are part of a large protein-lipid complex (Simons and Ikonen, 1997).

A large body of studies has shown the existence of lipid rafts, or similar structures, even earlier in the secretory pathway of vesicular transport. Following the Triton X-100 solubilization of isolated Golgi membranes, Golgi-derived detergent-insoluble complexes (GICs) can be isolated together with proteins whose Golgi localization is sensitive to the ER-to-Golgi protein transport inhibitor brefeldin A. These data suggest that the major fraction of lipid rafts in the Golgi is at the *cis*-Golgi cisternae (Gkantiragas et al., 2001). Interestingly, putative raft lipids (i.e. sphingomyelin and cholesterol) and all GIC proteins analyzed were excluded from COPI-vesicles facilitating the retrograde transport from the *cis*-Golgi cisternae to ER (Brügger et al., 2000). Therefore, GICs were proposed to be involved in membrane sorting at early stages of the secretory pathway (Gkantiragas et al., 2001). A study on B-cell receptor (BCR) processing also demonstrated that lipid rafts occurs in the *cis*-Golgi cisternae. The receptor is always present in DRMs isolated from B-cell lysates, yet it is neither expressed at the plasma membrane nor fully *O*-glycosylated. These findings indicated that the folding, glycosylation, or retention of BCR into lipid rafts occurs in the *cis*-Golgi cisternae (Mielenz et al., 2005).

Despite the fact that the ER contains relatively low levels of sphingolipids and cholesterol (Patton and Lester, 1991), lipid rafts were also thought to be present in the ER. In baker yeast, inhibition of ceramide synthesis caused a defective ER-to-Golgi transport of GPI-anchored proteins without affecting the transport of non-GPI-anchored proteins. It is proposed that GPI-anchored proteins may be sorted from non-GPI-anchored proteins in the ER by a selective recruitment in a lipid raft-like structure enriched in ceramide (Horvath et al., 1994; Skrzypek et al., 1997). In addition, the lipid raft association and the ER-to-Golgi transport of a DRM resident protein Gas1p was abolished when the synthesis of sphingolipids and ergosterol was impaired. The protein, therefore, might be sorted in lipid platforms analogous to those in mammalian cells (Bagnat et al., 2000). In human, both the α-helix-rich prion protein (PrP^{C}) and β-structure-rich insoluble conformer (PrP^{Sc}) are associated with DRMs (Vey et al., 1996). PrP^{C} is associated with DRMs as an immature precursor in the ER (Prusiner et al., 1998; Sarnataro et al., 2004). The presence of lipid rafts in the ER is also supported by the finding that ER-resident proteins erlin-1 and erlin-2 can be enriched in DRMs of the ER (Browman et al., 2006). These results have caused a debate in the subcellular location where lipid rafts are first assembled.

Consistent with the proposal that lipid rafts are implicated in the endocytosis of membrane proteins, most of GPI-anchored proteins are internalized into endosomes. Most of them are recycled back to the plasma

membrane with rates at least 3-fold slower than those of recycling receptors. This endocytic retention is regulated by the level of cholesterol in cellular membranes, indicating that the sorting and retention are due to their association with lipid rafts in endosomes (Mayor et al., 1998). It has been suggested that lipid rafts are involved in endocytosis that occurs via caveolae pathways as the 22-kDa component protein of caveolae membrane coats caveolin or VIP21 is associated with vesicles thought to carry glycolipids, GPI-anchored proteins and apical transmembrane proteins from the TGN to plasma membrane of epithelial cells (Rothberg et al., 1992; Kurzchalia et al., 1992). Caveolae are flask-shaped invaginations of 50–70 nm of the plasma membrane (Palade, 1953; Parton, 1996), formed by self-associating caveolin molecules that form hairpin loops in the membrane (Fra et al., 1995).

Experimentally, caveolae display putative lipid raft characteristics, such as having a special membrane composition enriched in sphingomyelin, glycosphingolipids, cholesterol (Sargiacomo et al., 1993), and GPI-anchored proteins (Mayor et al., 1994). Caveolae are also resistant to non-ionic detergents solubilization in the cold and light in buoyant density on sucrose gradients (Sargiacomo et al., 1993; Chang et al., 1994; Parton, 2003). Moreover, the maintenance and functionality of caveolae depend on the presence of cholesterol in the membrane (Simionescu et al., 1983; Rothberg et al., 1990; Schnitzer et al., 1994; Murata et al., 1995). Based on these characteristics, caveolae were considered as a specific form of lipid rafts with a morphologically identifiable structure (Simons and Ikonen, 1997; Kurzchalia and Parton, 1999). Nevertheless, the relationship between caveolae and lipid rafts is not completely agreed upon. Immunofluorescence and electron microscopy studies demonstrated that GPI-anchored proteins may be enriched in caveolae only after cross-linking. Furthermore, GPI-anchored proteins are intrinsically detergent-insoluble in the plasma membrane, so that their co-isolation with caveolin is not reflective of their native distribution (Mayor and Maxfield, 1995). *In situ* coating of the surface of endothelial cells in rat lung with cationic colloidal silica particles was able to separate caveolae from DRMs enriched in GPI-anchored proteins (Schnitzer et al., 1995). Importantly, DRMs can be isolated from almost all mammalian cell types including those that do not have caveolae (Wu et al., 1997). This line of evidence suggests that caveolae and lipid rafts are distinct lipid microdomains.

Lipid rafts have also been proposed to play a role in flotillin-dependent endocytic pathways as scaffold proteins flotillins together with other members of the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain-containing protein family are constitutively associated with DRMs even in cell types that also posses caveolae (Schulte et al., 1997; Glebov et al., 2006; Bickel et al., 1997; Lang et al., 1998; Stuermer et al., 2001; Reuter et al., 2004; Neumann-Giesen et al., 2004). Like caveolins, the SPFH domain-containing proteins oligomerize in membranes and appear to form a protein scaffold for lipid-based membrane domains (Browman et al., 2007). Flotillins are localized mainly at the plasma membrane, but also found in endosomal compartments, phagosomes, exosomes, and the Golgi (de Gassart et al., 2003; Dermine et al., 2001; Stuermer et al., 2001; Gkantiragas et al., 2001). Given that SPFH domain-containing proteins are associated with DRMs, their vast subcellular distribution indicates the existence of lipid raft-like microdomains in all those subcellular compartments.

The unique roles of lipid rafts in essential cellular processes and the simple operational definitions have triggered multidisciplinary research related to lipid rafts. The sheer number of literature published, in turn, has shifted the focus of the research from lipid rafts to DRMs (Munro, 2003). Combined with serious limitations of the operational definitions, it created a large body of inconsistent data that threaten the very concept of membrane lateral organization. Considering the complexity of cellular membranes and the perturbing nature of DRM formation, it is unlikely that DRMs reflect some pre-existing structure or organization in the membranes (Zurzolo et al., 2003). Therefore, DRMs should not be assumed to resemble biological rafts in size, structure, composition, or even existence (Heerklotz, 2002). Thus, to understand the natures of lipid rafts without the use of detergent solubilization as a means to infer the native membrane organization, alternative systems and analytical tools are needed (Schuck et al., 2003).

2.3. Limitations of the studies of lipid rafts in model systems

One of the fundamental tenets of the original lipid raft hypothesis is that the formation of lipid rafts is driven solely by preferential interactions between sphingolipids and cholesterol, forming l_o lipid domains in the midst of l_d lipid bilayer (Rietveld and Simons, 1998). This means that lipid rafts can be modeled in membranes composed of appropriate mixture of lipid species under suitable conditions. The potential existence of lipid rafts in cellular membranes has been shown by the findings that l_o lipid domains can phase separate from l_d lipids in vesicles composed of a mixture of phosphatidylcholine and cholesterol (Korlach et al., 1999) or all plasma membrane lipids at room temperature (Baumgart et al., 2007). Their existence was also reflected by the fact that the

compositions of mammalian plasma membranes were found to be tuned to reside near a miscibility critical point at physiological temperatures (Veatch, 2008).

Accumulating evidence strongly supports the lipid raft hypothesis to a remarkable degree as lipid microdomains with characteristics resembling those of lipid rafts can be generated in model membranes. Lipid domains of several micrometers in diameter were observed in supported lipid bilayers formed from a mixture of phosphatidylcholine, sphingomyelin and cholesterol. The domains were thicker and more resistant to non-ionic detergent solubilization than non-domain regions. Moreover, their appearance and size were dependent of the cholesterol concentration (Rinia et al., 2001). As expected, the diffusion rate in the domains was slower than that in non-domain regions (Dietrich et al., 2001). The domains were not artifacts caused by the supporting matrix since the domains could also be formed in vesicles of the same lipid mixture. In these model membranes, the domains were circular with smooth and rounded borders to minimize the line tension between the co-existing fluid phases. The circular shape of the domains quickly reassumed after they were deformed, suggesting a characteristic of l_o lipid domains (Samsonov et al., 2001) (**Fig. 7**). Consistent with the lipid raft hypothesis, the exogenously added ganglioside GM1 was highly enriched in the domains that were resistant to non-ionic detergent solubilization. These data demonstrated that sphingolipids and cholesterol can segregate to form more ordered, detergent-resistant lipid domains into which glycosphingolipids partition (Dietrich et al., 2001).

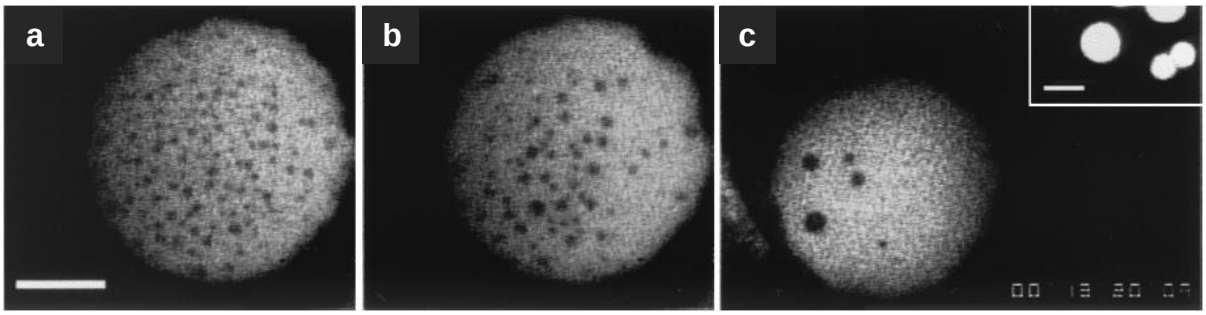

Figure 7. The formation of lipid domains in model membranes. The membranes contain phosphatidylcholine/ phosphatidylethanolamine (2:1), sphingomyelin/cholesterol (15 mol% each) and rho-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (5 mol%). **a.** The rho-DOPE was excluded from small domains after the temperature was lowered to 25°C, below the Tm of sphingomyelin. Scale bar is 50 mm. **b.** Dark domains merged, and at a later time the domains were larger but fewer in numbers. **c.** Circular domains at a higher magnification. The inset shows, at the same magnification, 2.5 and 4 mm fluorescent microspheres. Scale bar is 4 mm (Samsonov et al., 2001).

The roles of cholesterol in organizing lipid rafts have also been carefully investigated in model membranes. Cholesterol showed a markedly higher affinity to sphingolipids than with common membrane phospholipids with similar hydrocarbon chain lengths. Its interaction with sphingolipids to generate l_o phase appeared to be facilitated by the functional groups of the sphingoid base that are able to form intermolecular hydrogen bonds (Li et al., 2001; Leventis and Silvius, 2001). Interestingly, cholesterol and several sterols (dihydrocholesterol, epicholesterol, and 25-hydroxycholesterol) promote the formation of lipid domains enriched in saturated phosphatidylcholine, while other sterols and sterol derivatives either have little effect on domain formation or strongly inhibit it (Xu and London, 2000). Even the same cholesterol could decrease the fluidity of phosphatidylcholine bilayers or disrupt the highly ordered gel phase of sphingomyelin to promote a more fluid membrane (Crane and Tamm, 2004). Taken together, cholesterol enhances the formation of segregated lipid domains by favoring interaction with straighter and stiffer hydrocarbon chains of saturated lipids and disfavoring interaction with the more bulky unsaturated lipid species in lipid bilayers where domain segregation does not occur in the absence of sterols (Simons and Vaz, 2004). On the other hand, cholesterol can transform gel-phase domains to l_o domains in lipid bilayers that are able to exhibit coexisting gel and fluid domains in the absence of cholesterol (Silvius, 2003).

Lipid rafts are dynamic l_o phase domains into which specific types of proteins are selectively sequestered while others are excluded (Rietveld and Simons, 1998). A class of proteins proposed to reside in lipid rafts is GPI-anchored proteins whose association depends on the GPI anchor of the proteins (Wang et al., 2001). The dynamic association of a GPI-anchored protein has been studied using atomic force microscopy. Sphingomyelin-enriched l_o domains spontaneously formed in supported lipid bilayers composed of equimolar phosphatidylcholine and sphingomyelin (Saslowsky et al., 2002). When added to the preformed domains, the GPI-anchored protein placental alkaline phosphatase (PLAP) showed preferential insertion into the domains

particularly appearing at their periphery. Interestingly, in the presence of cholesterol, the protein was seen to be homogeneously distributed in the domains. Cholesterol, therefore, might encourage the protein to pass the boundaries between co-existing lipid domains (Milhiet et al., 2002) and stabilize the boundaries at the same time (Megha and London, 2004; Wang and Silvius, 2003) (**Fig. 8**).

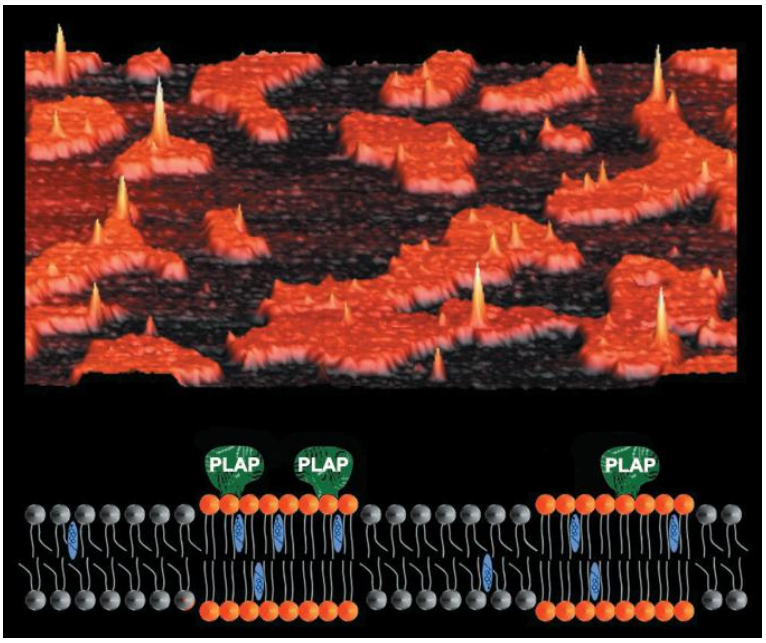

Figure 8. The sequestration of a GPI-anchored protein into l_o phase domains. Top: Atomic force microscopy shows sphingomyelin-enriched l_o domains (orange) protruding from a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) background (black) in a supported lipid bilayer. The height of the domains is ~7 Å. A GPI-anchored protein placental alkaline phosphatase (PLAP; yellow peaks) is shown to be almost exclusively domain associated. The bilayer is imaged under HEPES-buffered saline, and the width of the scan is ~2 μm. Bottom: The interpretation of the image at top. Sphingomyelin is shown by orange head groups with PLAP associated, while DOPC is shown by gray head groups. Cholesterol is shown by the blue insertions (Saslowsky et al., 2002; Henderson et al., 2004).

Besides GPI-anchored proteins, lipid rafts were also proposed to sequester certain transmembrane proteins. The transmembrane proteins soluble NSF attachment protein receptor (SNARE) and β-secretase (BACE) were thought to associate with lipid rafts as it is enriched in cholesterol-dependent microdomains in the plasma membrane and its activity is dependent of the level of sphingolipids and cholesterol, respectively. Both proteins, however, have an intrinsic preference for the l_d domain in model membranes (Bacia et al., 2004; Kalvodova et al., 2005), indicating that the proteins are excluded from lipid rafts instead. Surprisingly, two kinds of vesicles derived from the same plasma membrane but prepared by different techniques exhibited domain separation between l_o and l_d domains. Nevertheless, they had different capabilities to include raft transmembrane proteins in one phase together with the ganglioside GM1. It was found that the levels of lipid packing of the l_o domains were different significantly. These data suggested that raft transmembrane proteins partition into particular domains with certain degrees of lipid packing (Kaiser et al., 2009). Another possible explanation is that there are energetic constraints for transmembrane α-helices to partition into the tightly packed l_o domains as their amino acid side chains might have the potential disordering effect (Fastenberg et al., 2003).

Lipid rafts are generally assumed to exist in both exoplasmic and cytoplasmic leaflets of the plasma membrane. Lipid rafts in both leaflets are conceived to be overlap, so that they are coupled structurally and functionally. Given that both leaflets contain different lipid species, if they exist, the physical properties of lipid rafts in the cytoplasmic leaflet must be different from those in the exoplasmic leaflet (Devaux and Morris, 2004). The possibility of their existence has been exemplified by the findings that a lipid species similar to phosphatidylethanolamine, sialyllactosyl-phosphatidylethanolamine (SLPE) spontaneously forms complexes with cholesterol in Langmuir-Blodgett monolayer. At high cholesterol concentrations, SLPE may form l_o domains that are insoluble in Triton X-100 in the cold (Grzybek et al., 2009). By contrast, another study in multilamellar vesicles suggested that extrinsic factors, such as transbilayer penetration of long sphingolipid acyl chains, would be required to confer more highly ordered organization to the cytoplasmic leaflet to form lipid rafts (Wang and

Silvius, 2001). However, the existence of transbilayer connection in lipid rafts that would support this finding is still a matter of debate (Samsonov et al., 2001; Wu et al., 2004), leaving the presence of lipid rafts in the cytoplasmic leaflet of the plasma membrane remains obscure.

Provided that the basic lipid biochemistry operating in model membranes are the same with that in cellular membranes *in vivo* (Hancock, 2006), the preferential association of particular lipids in model membranes may reflect lipid lateral heterogeneity that occurs spontaneously as a function of membrane lipid composition in cells. Therefore, the natures of lipid rafts revealed in model membranes may provide a framework for understanding the organization of lipid rafts *in vivo* to some extent. Despite extensive works using model membranes, the size of lipid rafts and their existence in the cytoplasmic leaflet remains uncertain. In addition, as cellular membranes *in vivo* are constantly engaged in many cellular processes such as endocytosis and exocytosis, model membranes cannot be accurately used to study the dynamics of lipid rafts. Thus, to fill these gaps of information, the studies of lipid rafts in less perturbed plasma membranes are required.

3. Opinions on lipid rafts

3.1. *In vivo* studies revealed the natures of lipid rafts

The observations of lipid rafts in living cells without the use of invasive techniques have provided direct evidence for the existence of lipid rafts as well as deeper understanding of the organization and dynamics of lipid rafts *in vivo*. One of the non-invasive techniques is analyzing the envelopes of mammalian viruses to infer the lipid composition of specific regions in the plasma membrane from which they are derived. The cholesterol-to-phospholipid molar ratio and the degree of lipid order of HIV envelopes were found to be higher than those of the plasma membranes of their host cells, suggesting that the viruses select specific lipid domains within the plasma membranes of their host cells through which they emerge during viral maturation (Aloia et al., 1993). A growing body of data further underpins the conclusion as many of the viral and cellular components required for entry, assembly, and budding are concentrated in these domains (Waheed and Freed, 2009). Furthermore, the overall lipid composition of native HIV membranes purified from the medium of the infected T cell line by velocity gradient centrifugation resembles that of lipid rafts and significantly different from that of host cell membranes (Brügger et al., 2006). In addition, a lipidomic analysis of raft clusters in activated TCR domains isolated by immunoisolation using magnetic beads showed that the domains comprise a distinct molecular lipid composition similar to that of l_o phases in model membranes (Zech et al., 2009). Thus, it is clear that substantial lateral heterogeneity exists in the plasma membrane.

Visualization of GPI-anchored proteins employing direct immunofluorescence or fluorescent ligand analogues using a conventional fluorescence microscopy provided fairly uniform staining of living cells with little evidence of punctate patterns indicative of clusters packed with these proteins (Mayor et al., 1994). One of possible explanations for this finding is that if lipid rafts exist, they are too small (< 250 nm) to be observed using a light microscope (Anderson, 1998). Indeed, further measurements of the distribution area of GPI-anchored proteins using various techniques gave the sizes ranging from several nanometers to 250 nm (van Zanten et al., 2009; Lenne et al., 2006; Pralle et al., 2000; Varma and Mayor, 1998; Sharma et al., 2004). The sizes of clustered distribution of hemagglutinin and confinement area of sphingolipids also fall within the range (Hess et al., 2007; Eggeling et al., 2009) (**Fig. 9**). Nevertheless, the lateral diffusion measurements of a GPI-anchored protein, the ganglioside GM1, and a lipid probe with saturated acyl chains showed that the diffusion of those raft components is confined in wider areas ranging from 0.2 to 2 μm (Sheets et al., 1997; Schütz et al., 2000). This discrepancy in the size of lipid rafts can be reconciled if lipid rafts exist in hierarchically different length-scales (Jacobson et al., 2007).

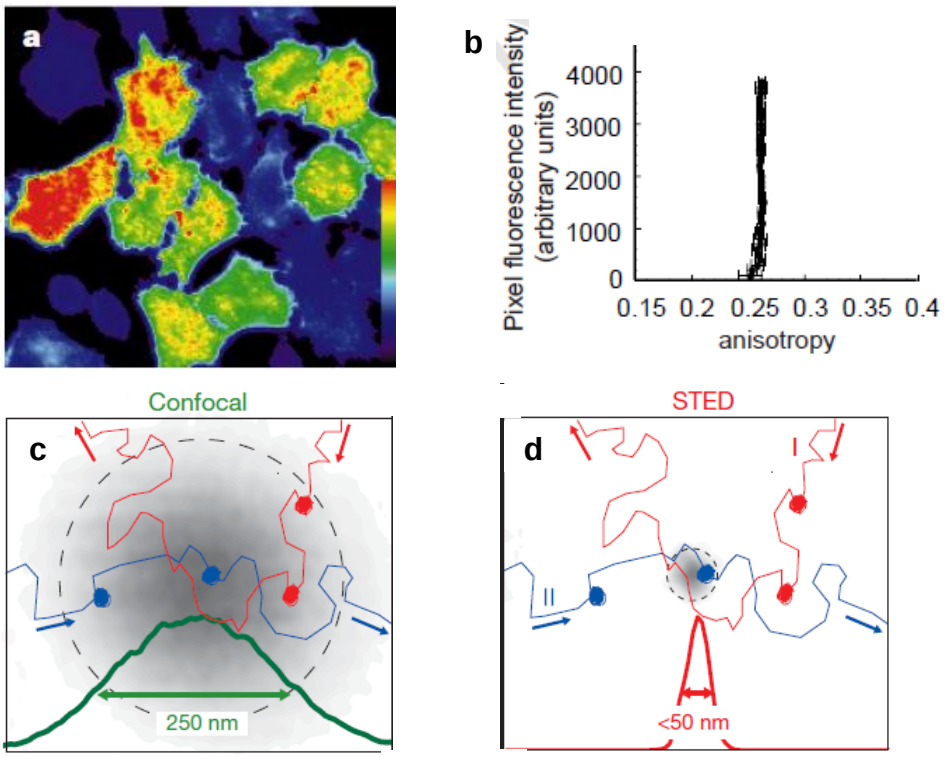

Figure 9. Lipid rafts are likely to be small. **a.** Distribution of fluorescence intensity at the surface of PLF-labeled GPI-anchored protein (folate receptor)-expressing cells. A fairly uniform staining of the cells indicates that if lipid rafts exist, they are too small (< 250 nm) to be observed using a light microscope. **b.** Distribution of fluorescence intensity and anisotropy values at the surface of the same cells. The anisotropy values of two pixels differing in fluorescence intensity are independent of fluorophore intensity, suggesting that the domains are submicron-sized (Varma and Mayor, 1998). Stimulated emission depletion (STED) microscopy time traces of single-molecule diffusion in live cell plasma membrane. Molecules may move freely and/or be transiently trapped on small spatial scales. The large detection area of a confocal microscope (**c**) cannot discern such details. However, the subdiffraction spot created by STED (**d**) is able to discriminate between lipids that diffuse freely (I) and those that are hindered (II) during their passage. This method revealed that sphingolipids and GPI-anchored proteins are transiently (10–20 ms) trapped in cholesterol-mediated molecular complexes dwelling within, 20-nm diameter areas (Eggeling et al., 2009).

Considering the large number of plasma membrane lipid species, the preferential interactions required to form lipid rafts would not be strong enough to create domains that are long-lived enough or large enough to be biologically significant (Edidin, 1997). In addition, a proximity-based study on three different GPI-anchored proteins and a glycosphingolipid endogenous to several different cell types revealed that lipid rafts might either exist only as transiently-stabilized structures or, if stable, comprise a minor fraction of the cell surface (Kenworthy et al., 2000). These notions raised a question whether small and transient domains could facilitate cellular functions. Interestingly, single-molecule tracking studies on the signaling mechanisms of GPI-anchored receptors CD59 in living cells demonstrated that three to nine CD59 molecules could cluster transiently which in turn provide a platform for signal transduction. The prolonged signal is likely generated by the sum of short-lived formation and activity of CD59 platforms. These studies also reported the involvement of actin in the platform formation and functionality (Suzuki et al., 2007a; Suzuki et al., 2007b) (**Fig. 10**). Taken together, it supports that lipid rafts are not in equilibrium with their membrane environment, but are actively-maintained structures.

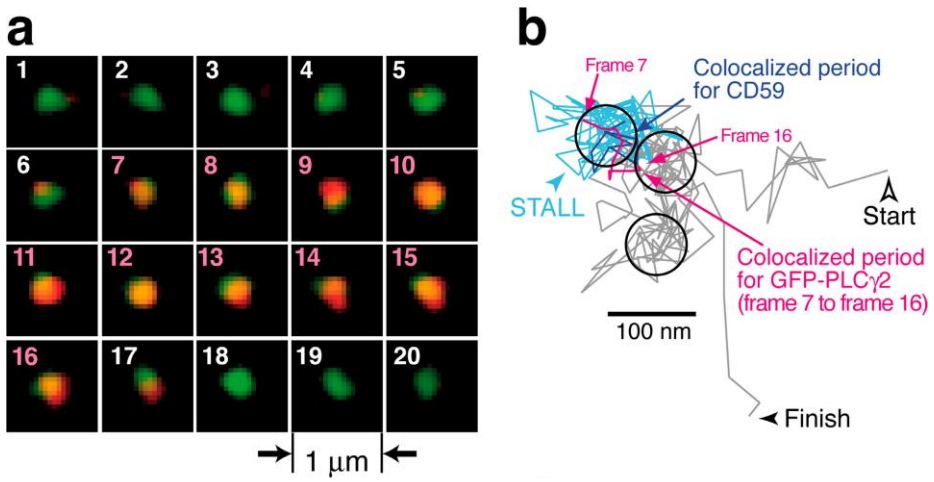

Figure 10. Lipid raft residents, GPI-anchored receptors CD59 cluster transiently to provide platforms for signal transduction. CD59 molecules form clusters and bind to F-actin, resulting in their immobilization, termed stimulation-induced temporary arrest of lateral diffusion (STALL). GFP-phospholipase C**γ**2 (PLC**γ**2) is recruited at CD59 clusters almost exclusively during the STALL period. (**a**) An image sequence showing superimposed video frames of simultaneous recordings of a CD59 cluster (green spot) and a single molecule of GFP-PLC**γ**2 (red spot). They were co-localized from frame 7 until frame 16 (pink frame numbers), which are within a STALL period. GFP-PLC**γ**2 suddenly appears from and returns to the cytoplasm. (**b**) A typical trajectory of a CD59 cluster (black), which includes three STALL periods (three circles). During one of the STALL periods (the blue part of the trajectory), a GFP-PLC**γ**2 molecule was recruited (magenta trajectory; the CD59 cluster trajectory during the co-localized period is shown in indigo). The co-localized period is completely included within the CD59 cluster's STALL period (Suzuki et al., 2007b).

Diffusion measurements showed that different membrane environments would have different effects on the lateral diffusion behavior of membrane components (Dietrich et al., 2002). The presence of lipid rafts would certainly affect the diffusion behavior of the molecules of which they are composed. Diffusion measurements, therefore, could be used to further elucidate the dynamics of lipid rafts. Currently, there are three models of lipid rafts based on diffusion measurements. (**1**) Lipid rafts diffuse as small and stable entities. The supporting observation of this model is that local measurements of the viscous drag on GPI-anchored and transmembrane proteins suggested that the proteins associate with lipid domains, which themselves are able to diffuse and remain stably associated over minutes in the plasma membrane (Pralle et al., 2000). (**2**) Lipid rafts as sites of transient protein trapping. This model was proposed after sphingolipids, GPI-anchored proteins, influenza virus hemagglutinin, ganglioside GM1, and lipid probes were shown to be transiently (less than 15 s, yet longer than the average diffusion coefficient of the molecule) confined in nanometer-sized areas in the plasma membrane, resulting in slowed diffusion rates (Eggeling et al., 2009; Sheets et al., 1997; Simson et al., 1995; Lenne et al., 2006; Shvartsman et al., 2003; Schütz et al., 2000). These areas were termed transient confinement zones (TCZs) (Dietrich et al., 2002). (**3**) Lipid rafts as receptor-cluster-induced complexes. According to this model, raft proteins may associate with small unstable rafts, allowing them to diffuse essentially as monomers under steady-state conditions. Upon stimulation, however, the raft-preferring receptors are clustered, leading to the formation of larger and stabilized lipid rafts (Subczynski and Kusumi, 2003; Kusumi et al., 2004). Each of these models implicitly assumes that the fraction of continuous non-raft membrane is larger than that of lipid rafts.

Lipid rafts in the exoplasmic leaflet may be readily fragmented if they are not backed by similar structures in the cytoplasmic leaflet (Devaux and Morris, 2004). A computational modeling of immunogold spatial point patterns suggested that lipid rafts in the cytoplasmic leaflet are also dynamic and actively regulated, similar to those in the exoplasmic leaflet (Plowman et al., 2005). However, multi-acylated proteins that are not lipid raft residents in the exoplasmic leaflet were found to co-cluster at the cytoplasmic leaflet of the plasma membrane. This finding suggested that the lipid organization of lipid rafts in both leaflets is potentially different (Zacharias et al., 2002). Nevertheless, these experiments provided convincing evidence for the existence of lipid rafts in the cytoplasmic leaflet of the plasma membrane.

In order to function as functional entities, a transbilayer connection is required for coupling lipid rafts in both leaflets. The cross-linking using antibodies and/or cholera toxin of raft elements on the exoplasmic leaflet of the plasma membrane was accompanied by the co-redistribution of protein tyrosine kinases in the cytoplasmic leaflet and the exclusion of non-raft proteins such as transferrin receptor (Harder et al., 1998; Pyenta et al., 2001). Moreover, antibody-mediated cross-linking of GPI-anchored proteins could also stimulate signaling in T cell

(Stefanová et al., 1991). Such signaling stimulation is dependent of sphingolipids and cholesterol levels (Lasserre et al., 2008). These observations have indicated that there are structural and functional connections between the two leaflets.

All of these studies point to the existence of small, dynamic, actively-regulated, and selective cholesterol-related heterogeneity in the plasma membrane of living cells. This emerging view suggests that functional lateral lipid organization in the plasma membrane is unlikely to be a result of equilibrium phase separation. In living cells, the pre-existing state of lipid assemblies caused by the intrinsic diversity of composition is likely to be small and dynamic. Functional assemblies that are larger and more stable are then induced upon requirement and in specific cellular contexts (Zurzolo et al., 2003). This view became the centre of current membrane models related to the lipid raft hypothesis.

3.2. Lipid raft-related membrane models

3.2.1. Lipid shell model

Antibody-mediated cross-linking of GPI-anchored proteins causes the migration of the proteins to pre-existing lipid domains, such as caveolae (Mayor et al., 1994; Rothberg et al., 1990). It is generally accepted that proteins are specifically targeted to lipid domains because they carry specific molecular addresses, commonly in the cytoplasmic domain, that direct them to these locations. In case of GPI-anchored proteins, however, they do not extend into the cytoplasm, hence do not have cytoplasmic targeting signals (Mineo et al., 1999). This implies that the molecular address directing the proteins to specific membrane domains is in the membrane anchor, the extracellular region, or both.

GPI-anchored proteins have an intrinsic light buoyant density in sucrose gradients regardless of their association with lipid domains (Harder et al., 1998; Kenworthy and Edidin, 1998). Moreover, a cluster of only 80 molecules of sphingolipids and cholesterol (~7 nm in diameter) is able to confer a GPI-anchored protein such a property. Therefore, they have a light buoyant density not because they are in a lipid domain, but because they are encased in a shell of sphingolipids and cholesterol, termed lipid shell. The lipid shells and the proteins they surround exist as mobile entities in the membrane plane without the need to form a separate lipid phase. Lipid shells are proposed to have an affinity for pre-existing lipid domains, hence targeting the protein they encase specifically to these membrane domains (Anderson and Jacobson, 2002).

Lipid shells are conceived as the smallest aggregates in a hierarchy of laterally-organized lipids in the plasma membrane. The formation of lipid shells is initiated by the self-assembly of sphingolipids–cholesterol to form complexes, called condensed complexes. These complexes do not need to form a separated phase while residing in a relatively cholesterol-poor phase in the membrane. The complexes then associate with certain proteins that have the propensity to associate with them, forming lipid shells that surrounding a protein (**see Fig. 11b**). A lipid shell is stabilized by long-term interactions between specific lipids and selected proteins in the membrane. For proteins in the exoplasmic leaflet, such as GPI-anchored proteins, multiple lipid–lipid, glycan–lipid, and protein–lipid interactions may stabilize the association between the proteins and condensed complexes. Transmembrane proteins directly interact with condensed complexes through specific amino acids in the transmembrane region. For proteins resided in the cytoplasmic leaflet of the plasma membrane, electrostatic interactions between charged amino acids in a protein and the oppositely-charged head groups of phospholipids in condensed complexes may play a role in the lipid shell formation (Anderson and Jacobson, 2002; McConnell and Radhakrishnan, 2003) (**Fig. 11**).

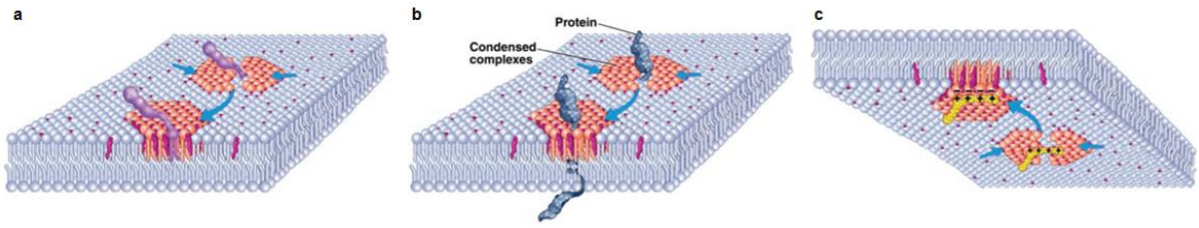

Figure 11. Proposed mechanism for lipid shell formation. Condensed complexes are formed by self-assembly of sphingolipids (orange) and cholesterol (magenta). The sphingolipid head groups protrude higher than the surrounding phospholipid head groups owing to the longer fatty acyl chains on the sphingolipids. The formation of lipid shells for GPI-anchored proteins (purple) (**a**), transmembrane proteins (blue) (**b**) and proteins in the cytoplasmic leaflet of the plasma membrane (yellow) (**c**) (Anderson and Jacobson, 2002).

According to the model, the pre-existing l_o lipid domains are formed by specialized cellular machinery (Anderson and Jacobson, 2002). Lipid shells together with the surrounded proteins have an affinity for l_o lipid domains because lipids composing lipid shells are molecularly compatible with those of lipid domains. This is the key in the sorting of membrane molecules into specialized lipid domains. Despite the possibility that transbilayer interactions exist between lipid shells situated on opposite sides of the membrane, the molecular sorting mediated by lipid shells is independent of transbilayer interactions. The sorting regulations may occur when the protein associates with condensed complexes and when the shelled protein associates with lipid domains. Once resided in the domains, protein and carbohydrate interactions between the shelled protein and resident molecules in these domains may increase the associating time of the shell in these sites. Caveolae or lipid rafts in the TGN and plasma membrane, in turn, transport proteins packed in lipid shells into vesicles to specific locations in the cell, such as for the biogenesis of the apical and basolateral plasma membranes in polarized epithelial cells (**Fig. 12**).

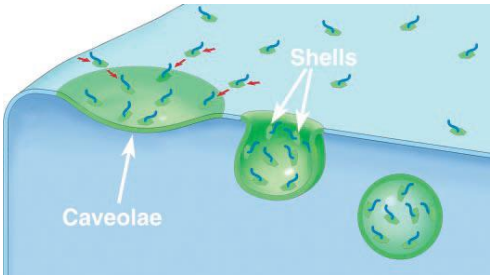

Figure 12. A sorting model of proteins encased by lipid shells to caveolae or lipid rafts. Once a protein associates with condensed complexes (green polygons), it is targeted (red arrows) to lipid domains like caveolae or lipid rafts (green invaginations). Caveolae or lipid rafts enriched in shelled proteins then bud from the membrane and carry their cargo to specific locations in the cell (Anderson and Jacobson, 2002).

Lipid shell model does not contradict with the original hypothesis of lipid rafts, instead lipid shells are considered to be the missing links in a membrane-sorting process essential for the generation of cell polarity. Furthermore, this model also explains how the sorting of peripheral proteins on the cytoplasmic leaflet may be carried out. This model is supported by the finding that there is no detectable clustering and overall enrichment of GPI-anchored proteins in the regions where TCR signaling is active. This indicates that biochemically-defined lipid rafts are in fact shells of raft lipids (i.e. glycosphingolipid and cholesterol) surrounding individual raft proteins (Glebov and Nichols, 2004). Another raft-resident protein, the influenza virus hemagglutinin does not partition into lipid rafts either, but it assembles into lipid-protein complexes exhibiting order from the nanometer to micrometer scales. Lipid shells might mediate this self-assembly process by enhancing the attraction between hemagglutinin trimers (Hess et al., 2005). In addition, the plasma membrane has higher protein occupancy than has generally been considered (Engelman, 2005). At such high protein concentrations in the membrane, there is only enough room for a few rings of lipids between individual proteins (Jacobson et al., 2007).

Nevertheless, this model also received criticisms (Simons and Vaz, 2004). The association of the first layer of lipids with the anchor of a membrane protein is anticipated to require a rather strong interaction of the shell lipids with the anchor. However, such a strong interaction has yet to be demonstrated. Moreover, the formation of the succeeding layers in the shell would require that the lipids of the shell have a rather strong tendency to associate. If this were the case, raft protein would not be required for the formation of lipid shells. Lastly, the concept of lipid shells as a solvation shell around the raft protein contradicts with their ability to associate with pre-existing larger lipid domains.

3.2.2. Actively-generated raft model

GPI-anchored proteins are normally endocytosed via a specialized pathway known as the GPI-anchored-protein-enriched early endosomal compartments (GEEC) pathway. Despite the fact that GPI-anchored proteins lack of cytoplasmic targeting signals, they must be segregated in the membrane prior to endocytosis (Mayor and Riezman, 2004). However, the detection of different GPI-anchored proteins with fluorescently-labeled monoclonal antibodies showed that these proteins are diffusely distributed over the plasma membrane. A possible explanation was that the GPI-anchored proteins are indeed diffusely distributed or the protein clusters are too small, i.e. below the resolution limit of the light microscopy (Mayor et al., 1994).

Further studies measuring the extent of energy transfer between GPI-anchored proteins showed that these proteins are not randomly organized but are organized in sub-pixel-sized cholesterol-dependent clusters at the surface of living cells (Varma and Mayor, 1998). These proteins are present as monomers and a smaller fraction (20-40%) as nanoscale (<5 nm) cholesterol-sensitive clusters. They are closely packed, so that they are almost touching each other. The distribution between monomers and clusters is concentration independent, implying that the clusters are actively-generated. The ability of cholesterol levels to modulate the fraction of clusters and monomers suggested that cholesterol homeostasis may in turn regulate this active process (Sharma et al., 2004).

The clusters are composed of at most four molecules of diverse GPI-anchored proteins. The interactions holding the GPI-anchored proteins together are likely to be weak since crosslinking of the proteins segregate them from pre-existing clusters. Interestingly, the crosslinking also prevents endocytosis of the crosslinked GPI-anchored proteins via the GEEC pathway. Considering that GPI-anchoring appears to be necessary for targeting proteins to a specific dynamin-independent, cdc42-regulated endocytic pathway (Sabharanjak et al., 2002), GPI-anchoring provides a mechanism for bringing diverse proteins within nanometer proximity of each other in small clusters. These clusters, in turn, serves as a sorting signal for specific endocytic routing, suggesting a mechanism for functional lipid-dependent clustering of GPI-anchoring (Sharma et al., 2004).

Provided that cluster formation is directly dependent of cholesterol and indirectly dependent of sphingolipids, nanoscale clusters were proposed to associate with actively-maintained domains enriched in sphingolipids and cholesterol or lipid rafts. Besides defining an endocytically active zone in the membrane, the pre-existing domains may be induced to form larger domains that are crucial for signal transduction. The ability of the small domains to be organized into larger structures has important implications for signaling, such as thresholding and a mechanism to reset the system. These observations suggest a hierarchical picture of an active lipid-dependent organization at different length scales that are exploited for distinct functions (Sharma et al., 2004; Mayor and Rao, 2004) (**Fig. 13**).

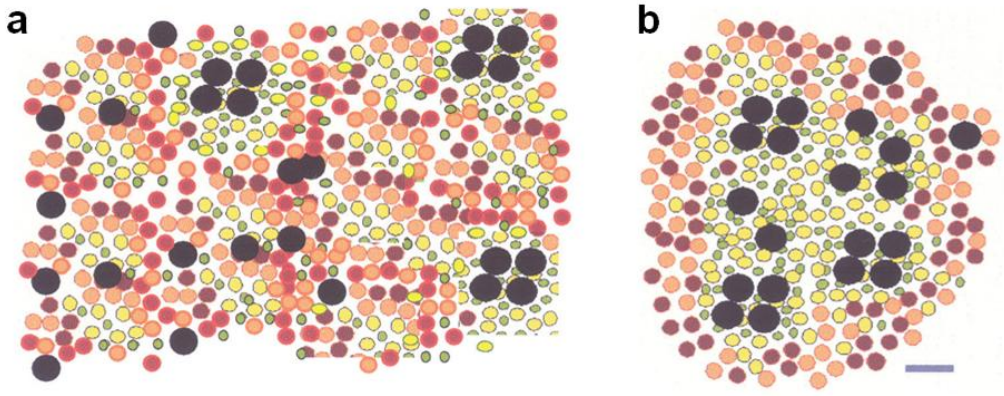

Figure 13. The spatial and temporal organization of lipid rafts as actively-generated entities. **a.** The pre-existing organization of raft components, showing small and dynamic clusters of GPI-anchored proteins that co-exist with their monomers. **b.** Actively-generated clustering of pre-existing lipid assemblies to form large-scale lipid rafts. Black circles: GPI-anchored proteins; red and pink circles, non-raft associated lipids; yellow circles, raft-associated lipids; green, cholesterol. Scale bar is ~5 nm (Mayor and Rao, 2004).

Besides being organized in nanoscale (<10 nm) clusters, GPI-anchored proteins are also organized at optically resolvable scales (≤ 450 nm) wherein nanoclusters are further concentrated. This indicates the existence of large-scale lipid domains that may share some properties expected of lipid rafts. However, they are

completely different in the mechanism of molecular complexation. Nanoclusters are found to be selectively enriched in flat regions of plasma membranes corresponding to the underlying cytoskeletal matrix. Furthermore, direct perturbations of cortical actin activity affect the construction, dynamics, and spatial organization of nanoclusters. Thus, lipid rafts of GPI-anchored proteins represent actively-constructed domains with a unique mechanism of complexation of cell-surface molecules regulated by cortical actin activity (Goswami et al., 2008).

The physical principle underlying the molecular complexation may be understood in terms of the framework of the plasma membrane as a membrane-cytoskeleton composite. Membrane proteins such as GPI-anchored proteins or transmembrane proteins can interact either directly or indirectly with the cortical actin network. Due to these interactions, they can transiently bind or unbind onto the cortical actin network. Subsequently, transiently bound molecules can be actively driven along the polar actin filaments resulting in local molecular clustering. The remodeling dynamics of the polar actin filaments may be regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) that control GTPases involved in actin polymerization. The remodeling dynamics of the polar actin filaments dynamically reorganizes this molecular clustering. Thus, cells using membrane organizing principle of the active polymer to locally control the nature and composition of lipid membranes (Goswami et al., 2008).

The actively-maintained complexes may be a generic mechanism for local nanoclustering of variety of cell-surface molecules such as GPI-anchored proteins (Sharma et al., 2004), Ras-isoforms (Plowman et al., 2005) and gangliosides (Fujita et al., 2007). This indicates the dynamic organization of membrane lipids and lipid-anchored proteins into complexes of different size scales. At the nanoscale, these complexes could greatly enhance the rates of chemical reactions in living cells (Hancock, 2006). Whereas organization at the larger scale coupled to the cortical actin network, might facilitate the induction of functional membrane domains responsible for sorting and signaling functions (Mayor and Rao, 2004).

3.2.3. Fence-picket model

The observation of diffusion behavior of a transmembrane protein Anion Exchanger 1 (AE1 or band 3) in erythrocyte ghosts showed that the protein can diffuse freely inside the cytoskeletal protein meshwork. Its diffusion beyond the mesh is determined by the spectrin dimer population in the network since it can only take place through cytoskeletal gates that open as spectrin tetramers dissociate to dimers (Tsuji and Ohnishi, 1986). A further critical examination of the rotational diffusion of band 3 showed that the meshwork restricts its translational diffusion over the mesh without influencing its rotational diffusion inside the mesh. Moreover, association or aggregation of band 3 reduces rotational diffusion much more than translational diffusion. This difference indicates the existence of control mechanisms of the cytoskeletal protein meshwork that imposes a non-specific barrier effect on translational diffusion, which termed a cytoskeletal fence model (Tsuji et al., 1988) (**Fig. 14a**).

The cytoskeletal fence model was supported by the studies of various transmembrane proteins employing laser tweezers (Edidin et al., 1991), single-particle tracking (Kusumi et al., 1993; Tomishige et al., 1998), and atomic force microscopy (Takeuchi et al., 1998). These studies showed that the barrier to lateral movement is the dynamic membrane-associated cytoskeleton network covering the cytoplasmic surface of the plasma membrane, except in special functional domains such as coated pits and cell adhesion structures. This network forms compartments of approximately 500-700 nm in diagonal length confining the short-range diffusion of membrane proteins. Their long-range diffusion is achieved by successive intercompartmental hops (Sako and Kusumi, 1994).

The binding and confining effects of the membrane skeleton on the movement of membrane proteins can play a pivotal role in the molecular organization of the plasma membrane, especially in controlling mechanisms for the formation of supramolecular assemblies. Cells actively regulate thermal movements that drive the movements of membrane proteins by varying the structure of the membrane skeleton using free energy released by the hydrolysis of ATP to construct supramolecular complexes. Furthermore, cell also able to move membrane proteins trapped in the compartments or those bound to the skeletal network simultaneously in energetically and temporally efficient manner by gross movements of the membrane-skeleton network (Kusumi and Sako, 1996).

In addition to transmembrane proteins, membrane lipids also have been subjected to diffusion behavior studies. A typical non-raft lipid, unsaturated phospholipids showed a confined diffusion within compartments of 230 nm in diameter for 11 ms on average before hopping to adjacent compartments. This suggests that the plasma membrane is compartmentalized with regard to lateral diffusion of phospholipids. The compartmentalization is dependent of the actin-based membrane skeleton, but not of the extracellular matrix,

extracellular domains of membrane proteins, or the cholesterol level. Meanwhile, about 30% of various kinds of transmembrane proteins are immobilized on the membrane skeleton meshwork (Ryan et al., 1988). These proteins serve as pickets along the membrane skeleton fence confining the movement of phospholipids through both steric hindrance and circumferential slowing. Phospholipid long-range diffusion is largely limited by the hop rate across the compartment boundaries. Such compartmentalization may be necessary for the localization of intracellular signals at the point where the extracellular signal has been received. This model was termed the anchored-protein picket model (Fujiwara et al, 2002) (**Fig. 14b**).

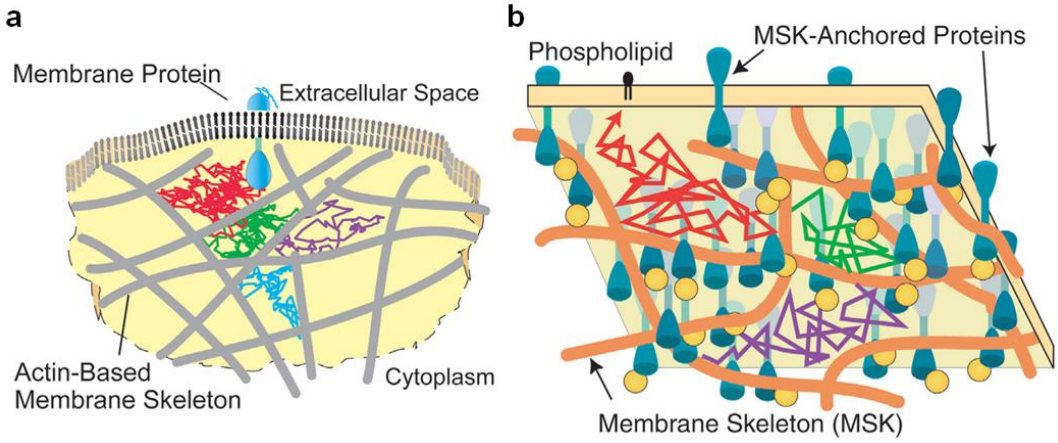

Figure 14. The membrane skeleton fence model and the anchored-protein picket model. **a.** The membrane skeleton fence model. Transmembrane proteins are protruding out into the cytoplasm. Their cytoplasmic domains collide with the membrane skeleton, inducing temporary confinement of the proteins in the membrane skeleton mesh (compartments). Transmembrane proteins could hop from a compartment to an adjacent one when the actin filament temporarily and locally breaks, when the membrane-to-skeleton distance fluctuates to allow the passage of the transmembrane protein, and/or the protein has by chance kinetic energy enough to push through the barrier when it is in the boundary area. **b.** Anchored-protein picket model. Various transmembrane proteins anchored to and lined up along the actin-based membrane skeleton mesh act as rows of pickets. These rows of pickets temporarily confine phospholipids and proteins in the membrane skeleton mesh (compartments) due to the steric hindrance (Kusumi et al., 2004).

The active organization of the plasma membrane as modeled by the fence-picket model has fundamental implications for lateral organization of the membrane. In accord with the notion of actively-generated rafts, the singe-particle tracking utilizing a high-speed video camera to measure the hop frequency also revealed that GPI-anchored proteins form a small raft containing only a few raft lipids with a lifetime of less than 1 ms. These small rafts can coalesce and cluster to stabilized or signaling rafts (Suzuki et al., 2007a; Suzuki et al., 2007b). It is proposed that monomeric GPI-anchored proteins associate with small and unstable rafts in unstimulated cells. GPI-anchored or transmembrane receptors with some affinity to cholesterol and saturated alkyl chains form oligomers upon activation by ligand binding and oligomerization. Oligomerized receptors may then induce small but stable rafts around them. This process generates a long-lived confinement of the crosslinked species, which depends on actin polymerization and cholesterol levels. Next, signaling machinery including those in the cytoplasmic leaflet of the membrane are recruited, resulting in a stable signaling platform (Subczynski and Kusumi, 2003).

This model can accommodate the possible existence of lipid rafts in the cytoplasmic leaflet of the plasma membrane. The recruitment of signaling molecules in the cytoplasmic leaflet implies the coupling of the exoplasmic- and cytoplasmic -leaflet rafts. Ligand binding induces GPI-anchored receptors to cluster, forming an exoplasmic-leaflet stabilized raft. The coupling of exoplasmic- and cytoplasmic-leaflet rafts may involve diffusional collision of the cytoplasmic-leaflet raft containing lipid-anchored signaling molecules with the exoplasmic leaflet raft. This collision is followed by transient entrapment probably due to an interaction based on lipid interdigitation. The coupling may also be mediated by transmembrane proteins with some affinity to the exoplasmic-leaflet raft. The association of transmembrane proteins with exoplasmic-leaflet raft could lead to recruitment and concentration of the cytoplasmic-leaflet rafts which contain signaling molecules that also have some affinity to the transmembrane protein. These two possibilities are not mutually exclusive, but are likely to work synergistically (Kusumi et al., 2004).

3.2.4. Dynamic nanoscale assembly model

The studies of lipid rafts *in vivo* suggest the existence of lateral membrane heterogeneity that is small, dynamic, actively-regulated, selective, and cholesterol-dependent. This led to a notion of lipid rafts as fluctuating nanoscale assemblies of sphingolipids, cholesterol, and proteins that can be stabilized to coalesce, forming platforms to focus and coordinate membrane activities such as membrane trafficking and cell signaling. In this model, lipid rafts are heterogeneous in size and lifetime in the plasma membrane. Lipid raft stability or lifetime is a function of size. Additional stability is provided by a raft-stabilizing protein, protein-protein interactions of constituent proteins, and saturated lipid anchors (Hancock, 2006).

The smallest entities are nanoscale assemblies of sterol- and sphingolipid-related lateral organization. These nanoscale assemblies are dynamic and metastable. They can be accessed and/or modulated by GPI-anchored proteins, certain transmembrane proteins, acylated cytosolic effectors, and cortical actin. Non-raft proteins do not possess the chemical or physical specificity to associate with these resting-state rafts. Following activation events (i.e. multivalent ligand binding), nanoscale assemblies can be stabilized to coalesce into raft platforms. Raft platforms constitute a more ordered assemblage that may be nucleated by scaffolding elements such as cortical actin. They would still have a size below the resolution of light microscopy, but could already mediate membrane trafficking and cell signaling. In the absence of membrane trafficking, protein modifications such as phosphorylation may increase the number of protein–-protein interactions, and multivalent clustering of raft lipids leading to the coalescence of raft platforms into microscopically observable raft phases. Raft phases are independent of the influence of cortical actin. Membrane constituents are laterally sorted according to preferences for membrane order and chemical interactions (Lingwood and Simons, 2010; Simons and Sampaio, 2011) (**Fig. 15**).

There are two fundamental concepts underlying the model. First, the dynamic raft-based membrane heterogeneity exists at the nanoscale and can be functionally coalesced to more stable membrane-ordered assemblies. Studies on the dynamics of molecular lipid composition in T-cell activation have demonstrated that lipid raft components are not associated in the plasma membrane of resting cells. Upon T-cell activation, they are selectively immobilized in nanoscale clusters together with specific receptors, indicating heterogeneity of lipid rafts (Drbal et al., 2007; Zech et al., 2009). This provides direct evidence for the existence of dynamic raft-based membrane heterogeneity in the plasma membrane.

Second, sphingolipid-cholesterol assemblage potential provides membranes with a lateral segregation propensity that can be organized at little energetic costs by proteins. Indeed, clustering of the plasma membrane glycosphingolipid Gb3 by its ligand Shiga toxin promotes the formation of energy-independent tubular invaginations of sphingolipid-biased membrane composition. These membrane invaginations even can be formed upon inhibition of dynamin or actin functions (Römer et al., 2007). In addition, when removed from underlying cytoskeletal influence and membrane trafficking, plasma membranes can be induced to produce large-scale raft-based phase separation by Cholera Toxin B Subunit (CTB)-mediated clustering of GM1 at physiological temperature. This coalescence was found to be cholesterol-dependent and selectively re-organized proteins and lipids according to their affinity for lipid rafts. This underscores the inherent capability of the plasma membrane to be selectively stimulated to generate larger-scale lateral reorganization of membrane structure and function. Furthermore, this coalescence cannot be explained by only those interactions that define l_o formation in model membranes. Therefore, additional interactions between proteins and lipids are needed for the formation of larger-scale lipid rafts (Lingwood et al., 2008).

In the plasma membrane, certain lipids of varying chain length are distorted vertically to counter hydrophobic mismatch condition induced by the surface of the transmembrane proteins that in direct contact with the lipids. The lipid species best adapted to the matching condition will be resided at the protein-lipid interface (Mouritsen and Bloom, 1993). On the contrary, hydrophobic mismatch condition can also be induced by cholesterol that forces neighboring hydrocarbon chains into more extended conformations, increasing membrane thickness and promoting membrane components segregation (García-Sáez et al., 2007). The significance role of protein in lipid domain formation has been asserted from the studies of signaling machinery recruitment in T-cell activation (Douglass and Vale, 2005; Burack et al., 2002). It is proposed that specific protein interaction with membrane-ordering raft lipids serves as a functionalizing connection to the sphingolipid-cholesterol basis for raft assembly. Membrane proteins bind and/or enrich certain lipids through chemical and physical specificities.

A transmembrane raft protein specifically interacts with sterol and glycosphingolipid in raft-based heterogeneity. This interaction lubricates the inclusion of the protein to raft-based heterogeneity and promotes the assembly of functionalized raft membrane. The assembly of proteins into rafts may be accompanied by conformational changes that modify protein activity. However, protein–lipid interactions alone cannot describe

lipid rafts, because these do not account for the preferentially connecting lipid–lipid interactions that have so convincingly been demonstrated in model membranes. It is postulated that during raft activation, protein–lipid interactions are coupled to lipid-order-based sorting, generating heterogeneity serving to functionalize, focus, and coordinate the activity of membrane constituents. Thus, sphingolipid-cholesterol assemblage potential forms a core raft connectivity that can be precisely modulated by proteins (Lingwood and Simons, 2010).

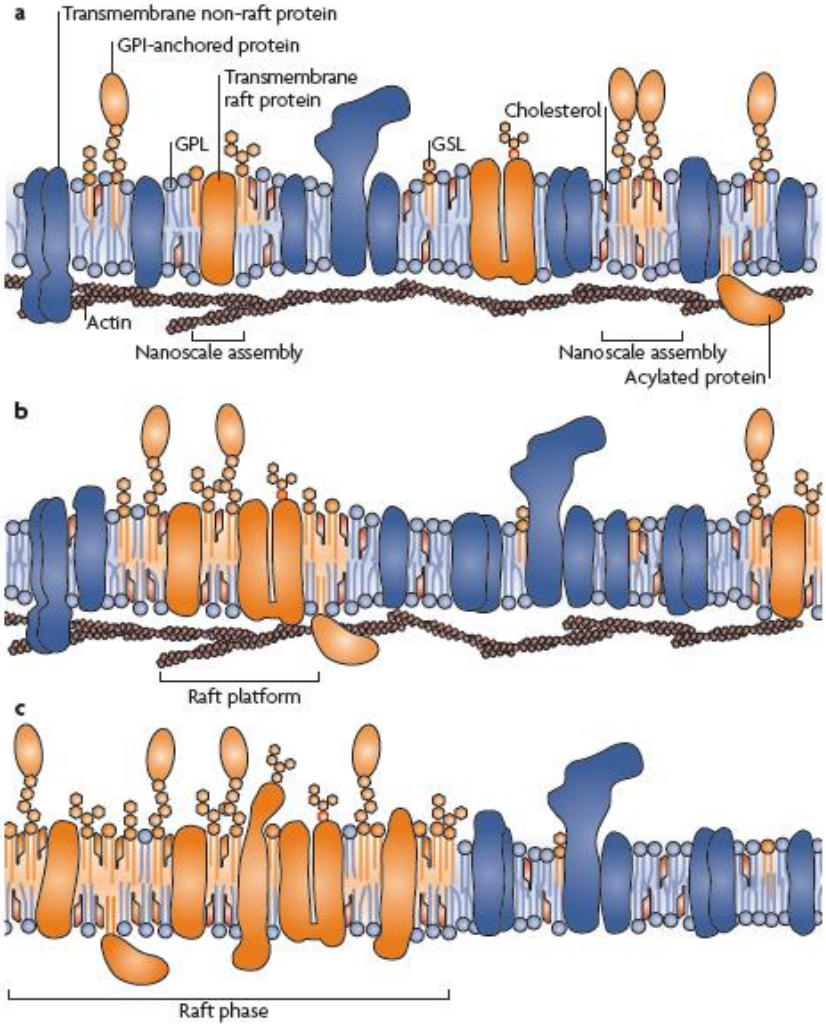

Figure 15. The hierarchy of raft-based heterogeneity in plasma membranes. **a.** Nanoscale assemblies of sphingolipids such as sphingomyelin and glycosphingolipids, sterols such as cholesterol, and proteins in the plasma membrane fluctuate in composition. Their constituents can be modulated by actin filaments. Transmembrane non-raft proteins are excluded from these assemblies. **b.** Activated nanoscale assemblies coalesce into raft platforms through lipid–lipid, lipid–protein and protein–protein oligomerizing interactions. These platforms are important for membrane trafficking and cell signaling. **c.** At equilibrium, raft platforms can be stabilized further to form raft phases (Simons and Gerl, 2010).

In accord to the mechanism of polarized membrane transport, the increase in raft lipids, ergosterol, and sphingolipids promotes a raft coalescence process induced by clustering of raft components that could lead to selective raft protein and lipid segregation in TGN membranes. The immiscibility of the two liquid phases in the membrane bilayer introduces an energetic penalty that promotes membrane bending because of increased thickness and order of the raft domain compared to the more disordered vicinity. This bulk sorting of proteins and lipids is fine-tuned by specific sorting, aided by accessory proteins that bind to raft cargoes. Protein machinery involved in bending and release would also be required to bud the membrane domain into a transport vesicle, leading to regulated protein and lipid sorting at the exit from the TGN. Thus, raft phase segregation principle is a means to form a selective carrier for lateral sorting of cell surface-destined constituents within the TGN (Simons and Gerl, 2010; Simons and Sampaio, 2011).

Summarizing Remarks

The lipid raft hypothesis has replaced the classical fluid mosaic model in which plasma membranes are viewed as a passive and equilibrated solvent for membrane proteins. The lipid raft hypothesis was originally proposed to explain the targeted membrane trafficking in polarized epithelial cells. Lipid rafts were thought as spontaneously-formed, stable, large (visible by light microscopy), uniform, and freely diffusing lipid domains in cellular membranes that are enriched in sphingolipids and cholesterol. These domains actively sort membrane components to facilitate various cellular processes.

The concept of lateral membrane organization behind the lipid raft hypothesis was long obscured by the extensive use of the simplistic operational definitions of lipid rafts that shifted the focus on lipid domain to detergent-resistant membranes. The development of new techniques that enable detection of membrane organization in less perturbed plasma membranes has revived the concept of lateral membrane organization.

Nevertheless, emerging evidence has called for amendments to the original hypothesis of lipid rafts. Currently, lipid rafts are defined as actively maintained, dynamic, small (nanometer-sized), heterogeneous, sterol- and sphingolipid-enriched, ordered assemblies of proteins and lipids. To mediate cellular processes, small and ephemeral lipid rafts at resting state can be stimulated to coalesce into larger, more stable lipid rafts by specific lipid–lipid, protein–lipid, and protein–protein interactions.

References

Ahmed, S.N., Brown, D.A. and London, E. 1997. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry* **36**, 10944-10953.

Allan, D. and Walklin, C.M. 1988. Endovesiculation of human erythrocytes exposed to sphingomyelinase C: a possible explanation for the enzyme-resistant pool of sphingomyelin. *Biochim Biophys Acta.* **938**, 403-410.

Aloia, R.C., Tian, H. and Jensen, F.C. 1993. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. *Proc Natl Acad Sci USA.* **90**, 5181-5185.

Anderson, R.G. 1998. The caveolae membrane system. *Annu Rev Biochem.* **67**, 199-225.

Anderson, R.G. and Jacobson, K. 2002. A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science* **296**, 1821-1825.

Babiychuk, E.B. and Draeger, A. 2006. Biochemical characterization of detergent-resistant membranes: a systematic approach. *Biochem J.* **397**, 407-416.

Bacia, K., Schuette, C.G., Kahya, N., Jahn, R., and Schwille, P. 2004. SNAREs prefer liquid-disordered over "raft" (liquid-ordered) domains when reconstituted into giant unilamellar vesicles. *J Biol Chem.* **279**, 37951-37955.

Bagnat, M., Keränen, S., Shevchenko, A., Shevchenko, A., and Simons, K. 2000. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proc Natl Acad Sci USA.* **97**, 3254-3259.

Baumgart, T., Hammond, A.T., Sengupta, P., Hess, S.T., Holowka, D.A., Baird, B.A., and Webb, W.W. 2007. Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc Natl Acad Sci USA.* **104**, 3165-3170.

Belcher, J.D., Hamilton, R.L., Brady, S.E., Hornick, C.A., Jaeckle, S., Schneider, W.J., and Havel, R.J. 1987. Isolation and characterization of three endosomal fractions from the liver of estradiol-treated rats. *Proc Natl Acad Sci USA.* **84**, 6785-6789.

Bickel, P.E., Scherer, P.E., Schnitzer, J.E., Oh, P., Lisanti, M.P., and Lodish, H.F. 1997. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. *J Biol Chem.* **272**, 13793-13802.

Boggs, J.M. 1987. Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function. *Biochim Biophys Acta.* **906**, 353-404.

Bretscher, M.S. 1973. Membrane structure: some general principles. *Science* **181**, 622-629.

Bretscher, M.S. and Raff, M.C. 1975. Mammalian plasma membranes. *Nature* **258**, 43-49.

Browman, D.T., Hoegg, M.B. and Robbins, S.M. 2007. The SPFH domain-containing proteins: more than lipid raft markers. *Trends Cell Biol.* **17**, 394-402.

Browman, D.T., Resek, M.E., Zajchowski, L.D., and Robbins, S.M. 2006. Erlin-1 and erlin-2 are novel members of the prohibitin family of proteins that define lipid-raft-like domains of the ER. *J Cell Sci.* **119**, 3149-3160.

Brown, D.A. and London, E. 1998. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol.* **14**, 111-136.

Brown, D.A. and Rose, J.K. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533-544.

Brown, R.E. and Thompson, T.E. 1987. Spontaneous transfer of ganglioside GM1 between phospholipid vesicles. *Biochemistry* **26**, 5454-5460.

Brügger, B., Glass, B., Haberkant, P., Leibrecht, I., Wieland, F.T., and Kräusslich, H.G. 2006. The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci USA.* **103**, 2641-2646.

Brügger, B., Sandhoff, R., Wegehingel, S., Gorgas, K., Malsam, J., Helms, J.B., Lehmann, W.D., Nickel, W., and Wieland, F.T. 2000. Evidence for segregation of sphingomyelin and cholesterol during formation of COPI-coated vesicles. *J Cell Biol.* **151**, 507-518.

Burack, W.R., Lee, K.H., Holdorf, A.D., Dustin, M.L., and Shaw, A.S. 2002. Cutting edge: quantitative imaging of raft accumulation in the immunological synapse. *J Immunol.* **169**, 2837-2841.

Castelletti, D., Alfalah, M., Heine, M., Hein, Z., Schmitte, R., Fracasso, G., Colombatti, M., and Naim, H.Y. 2008. Different glycoforms of prostate-specific membrane antigen are intracellularly transported through their association with distinct detergent-resistant membranes. *Biochem J.* **409**, 149-157.

Chang, W.J., Ying, Y.S., Rothberg, K.G., Hooper, N.M., Turner, A.J., Gambliel, H.A., De Gunzburg, J., Mumby, S.M., Gilman, A.G., and Anderson, R.G. 1994. Purification and characterization of smooth muscle cell caveolae. *J Cell Biol.* **126**, 127-138.

Colbeau, A., Nachbaur, J. and Vignais, P.M. 1971. Enzymic characterization and lipid composition of rat liver subcellular membranes. *Biochim Biophys Acta.* **249**, 462-92.

Crane, D.I. and Masters, C.J. 1986. The effect of clofibrate on the phospholipid composition of the peroxisomal membranes in mouse liver. *Biochim Biophys Acta.* **876**, 256-263.

Crane, J.M. and Tamm, L.K. 2004. Role of cholesterol in the formation and nature of lipid rafts in planar and spherical model membranes. *Biophys J.* **86**, 2965-2979.

Danielsen, E.M., Cowell, G.M., Norén, O., and Sjöström, H. 1984. Biosynthesis of microvillar proteins. *Biochem J.* **221**, 1-14.

Daum, G. 1985. Lipids of mitochondria. *Biochim Biophys Acta.* **822**, 1-42.

de Gassart, A., Geminard, C., Fevrier, B., Raposo, G., and Vidal, M. 2003. Lipid raft-associated protein sorting in exosomes. *Blood* **102**, 4336-4344.

de Laat, S.W., van der Saag, P.T., Elson, E.L., and Schlessinger, J. 1979. Lateral diffusion of membrane lipids and proteins is increased specifically in neurites of differentiating neuroblastoma cells. *Biochim Biophys Acta.* **558**, 247-250.

Delaunay, J.L., Breton, M., Trugnan, G., and Maurice, M. 2008. Differential solubilization of inner plasma membrane leaflet components by Lubrol WX and Triton X-100. *Biochim Biophys Acta.* **1778**, 105-112.

Dermine, J.F., Duclos, S., Garin, J., St-Louis, F., Rea, S., Parton, R.G., and Desjardins, M. 2001. Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. *J Biol Chem.* **276**, 18507-18512.

Devaux, P.F. and Morris, R. 2004. Transmembrane asymmetry and lateral domains in biological membranes. *Traffic* **5**, 241-246.

Diamond, J.M. 1977. Twenty-first Bowditch lecture. The epithelial junction: bridge, gate, and fence. *Physiologist* **20**, 10-18.

Dietrich, C., Volovyk, Z.N., Levi, M., Thompson, N.L., and Jacobson, K. 2001. Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc Natl Acad Sci USA.* **98**, 10642-10647.

Dietrich, C., Yang, B., Fujiwara, T., Kusumi, A., and Jacobson, K. 2002. Relationship of lipid rafts to transient confinement zones detected by single particle tracking. *Biophys J.* **82**, 274-284.

Douglass, A.D. and Vale, R.D. 2005. Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell* **121**, 937-950.

Drbal, K., Moertelmaier, M., Holzhauser, C., Muhammad, A., Fuertbauer, E., Howorka, S., Hinterberger, M., Stockinger, H., and Schütz, G.J. 2007. Single-molecule microscopy reveals heterogeneous dynamics of lipid raft components upon TCR engagement. *Int Immunol.* **19**, 675-684.

Edidin, M. 1997. Lipid microdomains in cell surface membranes. *Curr Opin Struct Biol.* **7**, 528-532.

Edidin, M. 2003. The state of lipid rafts: from model membranes to cells. *Annu Rev Biophys Biomol Struct.* **32**, 257-283.

Edidin, M., Kuo, S.C. and Sheetz, M.P. 1991. Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers. *Science* **254**, 1379-1382.

Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S., Belov, V.N., Hein, B., von Middendorff, C., Schönle, A., and Hell, S.W. 2009. Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* **457**, 1159-1162.

Engelman, D.M. 2005. Membranes are more mosaic than fluid. *Nature* **438**, 578-580.

Estep, T.N., Mountcastle, D.B., Biltonen, R.L., and Thompson, T.E. 1978. Studies on the anomalous thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine-cholesterol mixtures. *Biochemistry* **17**, 1984-1989.

Fastenberg, M.E., Shogomori, H., Xu, X., Brown, D.A., and London, E. 2003. Exclusion of a transmembrane-type peptide from ordered-lipid domains (rafts) detected by fluorescence quenching: extension of quenching analysis to account for the effects of domain size and domain boundaries. *Biochemistry* **42**, 12376-12390.

Fra, A.M., Williamson, E., Simons, K., and Parton, R.G. 1995. De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc Natl Acad Sci USA.* **92**, 8655-8659.

Fujita, A., Cheng, J., Hirakawa, M., Furukawa, K., Kusunoki, S., and Fujimoto, T. 2007. Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. *Mol Biol Cell.* **18**, 2112-2122.

Fujiwara, T., Ritchie, K., Murakoshi, H., Jacobson, K., and Kusumi, A. 2002. Phospholipids undergo hop diffusion in compartmentalized cell membrane. *J Cell Biol.* **157**, 1071-1081.

García-Sáez, A.J., Chiantia, S. and Schwille, P. 2007. Effect of line tension on the lateral organization of lipid membranes. *J Biol Chem.* **282**, 33537-33544.

Giocondi, M.C., Vié, V., Lesniewska, E., Goudonnet, J.P., and Le Grimellec, C. 2000. In situ imaging of detergent-resistant membranes by atomic force microscopy. *J Struct Biol.* **131**, 38-43.

Gkantiragas, I., Brügger, B., Stüven, E., Kaloyanova, D., Li, X.Y., Löhr, K., Lottspeich, F., Wieland, F.T, and Helms, J.B. 2001. Sphingomyelin-enriched microdomains at the Golgi complex. *Mol Biol Cell.* **12**, 1819-1833.

Glebov, O.O. and Nichols, B.J. 2004. Lipid raft proteins have a random distribution during localized activation of the T-cell receptor. *Nat Cell Biol.* **6**, 238-243.

Glebov, O.O., Bright, N.A. and Nichols, B.J. 2006. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol.* **8**, 46-54.

Goswami, D., Gowrishankar, K., Bilgrami, S., Ghosh, S., Raghupathy, R., Chadda, R., Vishwakarma, R., Rao, M., and Mayor, S. 2008. Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity. *Cell* **135**, 1085-1097.

Griffiths, G. and Simons, K. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science* **234**, 438-443.

Grzybek, M., Kubiak, J., Łach, A., Przybyło, M., and Sikorski, A.F. 2009. A raft-associated species of phosphatidylethanolamine interacts with cholesterol comparably to sphingomyelin. A Langmuir-Blodgett monolayer study. *PLoS One* **4**, e5053.

Hancock, J.F. 2006. Lipid rafts: contentious only from simplistic standpoints. *Nat Rev Mol Cell Biol.* **7**, 456-462.

Hansson, G.C., Simons, K. and van Meer, G. 1986. Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *EMBO J.* **5**, 483-489.

Hao, M., Mukherjee, S. and Maxfield, F.R. 2001. Cholesterol depletion induces large scale domain segregation in living cell membranes. *Proc Natl Acad Sci USA.* **98**, 13072-13077.

Harder, T., Scheiffele, P., Verkade, P., and Simons, K. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J Cell Biol.* **141**, 929-942.

Heerklotz, H. 2002. Triton promotes domain formation in lipid raft mixtures. *Biophys J.* **83**, 2693-2701.

Henderson, R.M., Edwardson, J.M., Geisse, N.A., and Saslowsky, D.E. 2004. Lipid rafts: feeling is believing. *News Physiol Sci.* **19**, 39-43.

Henning, R. and Stoffel, W. 1973. Glycosphingolipids in lysosomal membranes. *Hoppe Seylers Z Physiol Chem.* **354**, 760-770.

Hess, S.T., Gould, T.J., Gudheti, M.V., Maas, S.A., Mills, K.D., and Zimmerberg, J. 2007. Dynamic clustered distribution of hemagglutinin resolved at 40 nm in living cell membranes discriminates between raft theories. *Proc Natl Acad Sci USA.* **104**, 17370-17375.

Hess, S.T., Kumar, M., Verma, A., Farrington, J., Kenworthy, A., and Zimmerberg, J. 2005. Quantitative electron microscopy and fluorescence spectroscopy of the membrane distribution of influenza hemagglutinin. *J Cell Biol.* **169**, 965-976.

Hong-wei, S. and McConnell, H. 1975. Phase separations in phospholipid membranes. *Biochemistry* **14**, 847-854.

Horvath, A., Sütterlin, C., Manning-Krieg, U., Movva, N.R., and Riezman, H. 1994. Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. *EMBO J.* **13**, 3687-3695.

Hubbell, W.L. and McConnell, H.M. 1969. Orientation and motion of amphiphilic spin labels in membranes. *Proc Natl Acad Sci USA.* **64**, 20-27.

Hui, S.W. and Parsons, D.F. 1975. Direct observation of domains in wet lipid bilayers. *Science* **190**, 383-384.

Ingelmo-Torres, M., Gaus, K., Herms, A., González-Moreno, E., Kassan, A., Bosch, M., Grewal, T., Tebar, F., Enrich, C., and Pol, A. 2009. Triton X-100 promotes a cholesterol-dependent condensation of the plasma membrane. *Biochem J.* **420**, 373-381.

Ipsen, J.H., Karlström, G., Mouritsen, O.G., Wennerström, H., and Zuckermann, M.J. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim Biophys Acta.* **905**, 162-172.

Ipsen, J.H., Mouritsen, O.G. and Zuckermann, M.J. 1989. Theory of thermal anomalies in the specific heat of lipid bilayers containing cholesterol. *Biophys J.* **56**, 661-667.

Jacobson, K., Mouritsen, O.G. and Anderson, R.G. 2007. Lipid rafts: at a crossroad between cell biology and physics. *Nat Cell Biol.* **9**, 7-14.

Jakop, U., Fuchs, B., Süss, R., Wibbelt, G., Braun, B., Müller, K., and Schiller, J. 2009. The solubilisation of boar sperm membranes by different detergents - a microscopic, MALDI-TOF MS, (31)P NMR and PAGE study on membrane lysis, extraction efficiency, lipid and protein composition. *Lipids Health Dis.* **8**, 49.

Kaiser, H.J., Lingwood, D., Levental, I., Sampaio, J.L., Kalvodova, L., Rajendran, L., and Simons, K. 2009. Order of lipid phases in model and plasma membranes. *Proc Natl Acad Sci USA.* **106**, 16645-6650.

Kalvodova, L., Kahya, N., Schwille, P., Ehehalt, R., Verkade, P., Drechsel, D., and Simons, K. 2005. Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro. **J Biol Chem. 280**, 36815-36823.

Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L., Dawidowicz, E.A., McIntyre, D.E., Salzman, E.A., and Klausner, R.D. 1982. Lipid domains in membranes. *Ann N Y Acad Sci.* **401**, 61-75.

Keller, P. and Simons, K. 1998. Cholesterol is required for surface transport of influenza virus hemagglutinin. *J Cell Biol.* **140**, 1357-1367.

Kenworthy, A.K. and Edidin, M. 1998. Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of <100 A using imaging fluorescence resonance energy transfer. *J Cell Biol.* **142**, 69-84.

Kenworthy, A.K., Petranova, N. and Edidin, M. 2000. High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. *Mol Biol Cell.* **11**, 1645-1655.

Klausner, R.D. and Wolf, D.E. 1980. Selectivity of fluorescent lipid analogues for lipid domains. *Biochemistry* **19**, 6199-6203.

Klausner, R.D., Kleinfeld, A.M., Hoover, R.L., and Karnovsky, M.J. 1980. Lipid domains in membranes. Evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. *J Biol Chem.* **255**, 1286-1295.

Klemm, R.W., Ejsing, C.S., Surma, M.A., Kaiser, H.J., Gerl, M.J., Sampaio, J.L., de Robillard, Q., Ferguson, C., Proszynski, T.J., Shevchenko, A., and Simons, K. 2009. Segregation of sphingolipids and sterols during formation of secretory vesicles at the trans-Golgi network. *J Cell Biol.* **185**, 601-612.

Korey, S.R. and Gonatas, J. 1963. Separation of human brain gangliosides. *Life Sci.* **5**, 296-302.

Korlach, J., Schwille, P., Webb, W.W., and Feigenson, G.W. 1999. Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy. *Proc Natl Acad Sci USA.* **96**, 8461-8466.

Kurzchalia, T.V. and Parton, R.G. 1999. Membrane microdomains and caveolae. *Curr Opin Cell Biol.* **11**, 424-431.

Kurzchalia, T.V., Dupree, P., Parton, R.G., Kellner, R., Virta, H., Lehnert, M., and Simons, K. 1992. VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. *J Cell Biol.* **118**, 1003-1014.

Kusumi, A. and Sako, Y. 1996. Cell surface organization by the membrane skeleton. *Curr Opin Cell Biol.* **8**, 566-574.

Kusumi, A., Koyama-Honda, I. and Suzuki, K. 2004. Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic* **5**, 213-230.

Kusumi, A., Sako, Y. and Yamamoto, M. 1993. Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. *Biophys J.* **65**, 2021-2040.

Lang, D.M., Lommel, S., Jung, M., Ankerhold, R., Petrausch, B., Laessing, U., Wiechers, M.F., Plattner, H., and Stuermer, C.A. 1998. Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. *J Neurobiol.* **37**, 502-523.

Lasserre, R., Guo, X.J., Conchonaud, F., Hamon, Y., Hawchar, O., Bernard, A.M., Soudja, S.M., Lenne, P.F., Rigneault, H., Olive, D., Bismuth, G., Nunès, J.A., Payrastre, B., Marguet, D., and He, H.T. 2008. Raft nanodomains contribute to Akt/PKB plasma membrane recruitment and activation. *Nat Chem Biol.* **4**, 538-547.

Lenne, P.F., Wawrezinieck, L., Conchonaud, F., Wurtz, O., Boned, A., Guo, X.J., Rigneault, H., He, H.T., and Marguet, D. 2006. Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. *EMBO J.* **25**, 3245-3256.

Leventis, R. and Silvius, J.R. 2001. Use of cyclodextrins to monitor transbilayer movement and differential lipid affinities of cholesterol. *Biophys J.* **81**, 2257-2267.

Li, X.M., Momsen, M.M., Smaby, J.M., Brockman, H.L., and Brown, R.E. 2001. Cholesterol decreases the interfacial elasticity and detergent solubility of sphingomyelins. *Biochemistry* **40**, 5954-5963.

Lingwood, D. and Simons, K. 2010. Lipid rafts as a membrane-organizing principle. *Science* **327**, 46-50.

Lingwood, D., Ries, J., Schwille, P., and Simons, K. 2008. Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc Natl Acad Sci USA.* **105**, 10005-10010.

Mabrey, S., Mateo, P.L. and Sturtevant, J.M. 1978. High-sensitivity scanning calorimetric study of mixtures of cholesterol with dimyristoyl- and dipalmitoylphosphatidylcholines. *Biochemistry* **17**, 2464-2468.

Matlin, K.S. and Simons, K. 1984. Sorting of an apical plasma membrane glycoprotein occurs before it reaches the cell surface in cultured epithelial cells. *J Cell Biol.* **99**, 2131-2139.

Mayor, S. and Maxfield, F.R. 1995. Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. *Mol Biol Cell.* **6**, 929-944.

Mayor, S. and Rao, M. 2004. Rafts: scale-dependent, active lipid organization at the cell surface. *Traffic* **5**, 231-240.

Mayor, S. and Riezman, H. 2004. Sorting GPI-anchored proteins. *Nat Rev Mol Cell Biol.* **5**, 110-120.

Mayor, S., Rothberg, K.G. and Maxfield, F.R. 1994. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science* **264**, 1948-1951.

Mayor, S., Sabharanjak, S. and Maxfield, F.R. 1998. Cholesterol-dependent retention of GPI-anchored proteins in endosomes. *EMBO J.* **17**, 4626-4638.

McConnell, H.M. and Radhakrishnan, A. 2003. Condensed complexes of cholesterol and phospholipids. *Biochim Biophys Acta.* **1610**, 159-173.

Megha and London, E. 2004. Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. *J Biol Chem.* **279**, 9997-10004.

Mielenz, D., Vettermann, C., Hampel, M., Lang, C., Avramidou, A., Karas, M., and Jäck, H.M. 2005. Lipid rafts associate with intracellular B cell receptors and exhibit a B cell stage-specific protein composition. *J Immunol.* **174**, 3508-3517.

Milhiet, P.E., Giocondi, M.C., Baghdadi, O., Ronzon, F., Roux, B., and Le Grimellec, C. 2002. Spontaneous insertion and partitioning of alkaline phosphatase into model lipid rafts. *EMBO Rep.* **3**, 485-490.

Mineo, C., Gill, G.N. and Anderson, R.G. 1999. Regulated migration of epidermal growth factor receptor from caveolae. *J Biol Chem.* **274**, 30636-30643.

Mouritsen, O.G. and Bloom, M. 1993. Models of lipid-protein interactions in membranes. *Annu Rev Biophys Biomol Struct.* **22**, 145-171.

Munro, S. 2003. Lipid rafts: elusive or illusive? *Cell* **115**, 377-388.

Murata, M., Peränen, J., Schreiner, R., Wieland, F., Kurzchalia, T.V., and Simons, K. 1995. VIP21/caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci USA.* **92**, 10339-10343.

Neumann-Giesen, C., Falkenbach, B., Beicht, P., Claasen, S., Lüers, G., Stuermer, C.A., Herzog, V., and Tikkanen, R. 2004. Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. *Biochem J.* **378**, 509-518.

Nichols, B.J., Kenworthy, A.K., Polishchuk, R.S., Lodge, R., Roberts, T.H., Hirschberg, K., Phair, R.D., and Lippincott-Schwartz, J. 2001. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J Cell Biol.* **153**, 529-541.

Nichols, G.E., Shiraishi, T., Allietta, M., Tillack, T.W., and Young, W.W. Jr. 1987. Polarity of the Forssman glycolipid in MDCK epithelial cells. *Biochim Biophys Acta.* **930**, 154-166.

Orci, L., Montesano, R., Meda, P., Malaisse-Lagae, F., Brown, D., Perrelet, A., and Vassalli, P. 1981. Heterogeneous distribution of filipin--cholesterol complexes across the cisternae of the Golgi apparatus. *Proc Natl Acad Sci USA.* **78**, 293-297.

Owicki, J.C. and McConnell, H.M. 1980. Lateral diffusion in inhomogeneous membranes. Model membranes containing cholesterol. *Biophys J.* **30**, 383-397.

Palade, C.E. 1953. Fine structure of blood capillaries. *J. Applied Phys.* **24**, 1424.

Parton, R.G. 1996. Caveolae and caveolins. *Curr Opin Cell Biol.* **8**, 542-548.

Parton, R.G. 2003. Caveolae--from ultrastructure to molecular mechanisms. *Nat Rev Mol Cell Biol.* **4**, 162-167.

Pascher, I. 1976. Molecular arrangements in sphingolipids. Conformation and hydrogen bonding of ceramide and their implication on membrane stability and permeability. *Biochim Biophys Acta.* **455**, 433-451.

Patton, J.L. and Lester, R.L. 1991. The phosphoinositol sphingolipids of Saccharomyces cerevisiae are highly localized in the plasma membrane. *J Bacteriol.* **173**, 3101-3108.

Pizzo, P., Giurisato, E., Tassi, M., Benedetti, A., Pozzan, T., and Viola, A. 2002. Lipid rafts and T cell receptor signaling: a critical re-evaluation. *Eur J Immunol.* **32**, 3082-3091.

Plowman, S.J., Muncke, C., Parton, R.G., and Hancock, J.F. 2005. H-ras, K-ras, and inner plasma membrane raft proteins operate in nanoclusters with differential dependence on the actin cytoskeleton. *Proc Natl Acad Sci USA.* **102**, 15500-15505.

Pomorski, T. and Menon, A.K. 2006. Lipid flippases and their biological functions. *Cell Mol Life Sci.* **63**, 2908-2921.

Pralle, A., Keller, P., Florin, E.L., Simons, K., and Hörber, J.K. 2000. Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J Cell Biol.* **148**, 997-1008.

Prusiner, S.B., Scott, M.R., DeArmond, S.J., and Cohen, F.E. 1998. Prion protein biology. *Cell* **93**, 337-348.

Pyenta, P.S., Holowka, D. and Baird, B. 2001. Cross-correlation analysis of inner-leaflet-anchored green fluorescent protein co-redistributed with IgE receptors and outer leaflet lipid raft components. *Biophys J.* **80**, 2120-2132.

Radeva, G. and Sharom, F.J. 2004. Isolation and characterization of lipid rafts with different properties from RBL-2H3 (rat basophilic leukaemia) cells. *Biochem J.* **380**, 219-230.

Recktenwald, D.J. and McConnell, H.M. 1981. Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol. *Biochemistry* **20**, 4505-4510.

Reuter, A., Binkle, U., Stuermer, C.A., and Plattner, H. 2004. PrPc and reggies/flotillins are contained in and released via lipid-rich vesicles in Jurkat T cells. *Cell Mol Life Sci.* **61**, 2092-2099.

Rietveld, A. and Simons, K. 1998. The differential miscibility of lipids as the basis for the formation of functional membrane rafts. *Biochim Biophys Acta.* **1376**, 467-479.

Rindler, M.J., Ivanov, I.E., Plesken, H., and Sabatini, D.D. 1985. Polarized delivery of viral glycoproteins to the apical and basolateral plasma membranes of Madin-Darby canine kidney cells infected with temperature-sensitive viruses. *J Cell Biol.* **100**, 136-151.

Rinia, H.A., Snel, M.M., van der Eerden, J.P., and de Kruijff, B. 2001. Visualizing detergent resistant domains in model membranes with atomic force microscopy. *FEBS Lett.* **501**, 92-96.

Rodriguez-Boulan, E. and Nelson, W.J. 1989. Morphogenesis of the polarized epithelial cell phenotype. *Science* **245**, 718-725.

Roelofsen, B., van Meer, G. and Op den Kamp, J.A. 1981. The lipids of red cell membranes. Compositional, structural and functional aspects. *Scand J Clin Lab Invest Suppl.* **156**, 111-115.

Römer, W., Berland, L., Chambon, V., Gaus, K., Windschiegl, B., Tenza, D., Aly, M.R., Fraisier, V., Florent, J.C., Perrais, D., Lamaze, C., Raposo, G., Steinem, C., Sens, P., Bassereau, P., and Johannes, L. 2007. Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature* **450**, 670-675.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.S., Glenney, J.R., and Anderson, R.G. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell* **68**, 673-682.

Rothberg, K.G., Ying, Y.S., Kolhouse, J.F., Kamen, B.A., and Anderson, R.G. 1990. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. *J Cell Biol.* **110**, 637-649.

Rothman, J.E. and Lenard, J. 1977. Membrane asymmetry. *Science* **195**, 743-753.

Ryan, T.A., Myers, J., Holowka, D., Baird, B., and Webb, W.W. 1988. Molecular crowding on the cell surface. *Science* **239**, 61-64.

Sabharanjak, S., Sharma, P., Parton, R.G., and Mayor, S. 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell.* **2**, 411-423.

Sako, Y. and Kusumi, A. 1994. Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. *J Cell Biol.* **125**, 1251-1264.

Samsonov, A.V., Mihalyov, I. and Cohen, F.S. 2001. Characterization of cholesterol-sphingomyelin domains and their dynamics in bilayer membranes. *Biophys J.* **81**, 1486-1500.

Sankaram, M.B. and Thompson, T.E. 1990. Interaction of cholesterol with various glycerophospholipids and sphingomyelin. *Biochemistry* **29**, 10670-10675.

Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M.P. 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J Cell Biol.* **122**, 789-807.

Sarnataro, D., Campana, V., Paladino, S., Stornaiuolo, M., Nitsch, L., and Zurzolo, C. 2004. PrP(C) association with lipid rafts in the early secretory pathway stabilizes its cellular conformation. *Mol Biol Cell.* **15**, 4031-4042.

Saslowsky, D.E., Lawrence, J., Ren, X., Brown, D.A., Henderson, R.M., and Edwardson, J.M. 2002. Placental alkaline phosphatase is efficiently targeted to rafts in supported lipid bilayers. *J Biol Chem.* **277**, 26966-26970.

Schnitzer, J.E., McIntosh, D.P., Dvorak, A.M., Liu, J., and Oh, P. 1995. Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science* **269**, 1435-1439.

Schnitzer, J.E., Oh, P., Pinney, E., and Allard, J. 1994. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol.* **127**, 1217-1232.

Schroeder, R., London, E. and Brown, D. 1994. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc Natl Acad Sci USA.* **91**, 12130-12134.

Schroeder, R.J., Ahmed, S.N., Zhu, Y., London, E., and Brown, D.A. 1998. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. *J Biol Chem.* **273**, 1150-1157.

Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., and Simons, K. 2003. Resistance of cell membranes to different detergents. *Proc Natl Acad Sci USA.* **100**, 5795-5800.

Schulte, T., Paschke, K.A., Laessing, U., Lottspeich, F., and Stuermer, C.A. 1997. Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. *Development* **124**, 577-587.

Schütz, G.J., Kada, G., Pastushenko, V.P., and Schindler, H. 2000. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. *EMBO J.* **19**, 892-901.

Sengupta, P., Jovanovic-Talisman, T., Skoko, D., Renz, M., Veatch, S.L., and Lippincott-Schwartz, J. 2011. Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. *Nat Methods.* **8**, 969-975.

Sharma, P., Varma, R., Sarasij, R.C., Ira, Gousset, K., Krishnamoorthy, G., Rao, M., and Mayor, S. 2004. Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* **116**, 577-589.

Shaw, A.S. 2006. Lipid rafts: now you see them, now you don't. *Nat Immunol.* **7**, 1139-1142.

Sheets, E.D., Lee, G.M., Simson, R., and Jacobson, K. 1997. Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. *Biochemistry* **36**, 12449-12458.

Sheetz, M.P. and Singer, S.J. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc Natl Acad Sci USA.* **71**, 4457-4461.

Shimshick, E.J. and McConnell, H.M. 1973. Lateral phase separation in phospholipid membranes. *Biochemistry* **12**, 2351-2360.

Shvartsman, D.E., Kotler, M., Tall, R.D., Roth, M.G., and Henis, Y.I. 2003. Differently anchored influenza hemagglutinin mutants display distinct interaction dynamics with mutual rafts. *J Cell Biol.* **163**, 879-888.

Silvius, J.R. 2003. Role of cholesterol in lipid raft formation: lessons from lipid model systems. *Biochim Biophys Acta.* **1610**, 174-183.

Simionescu, N., Lupu, F. and Simionescu, M. 1983. Rings of membrane sterols surround the openings of vesicles and fenestrae, in capillary endothelium. *J Cell Biol.* **97**, 1592-1600.

Simons, K. and Fuller, S.D. 1985. Cell surface polarity in epithelia. *Annu Rev Cell Biol.* **1**, 243-288.

Simons, K. and Gerl, M.J. 2010. Revitalizing membrane rafts: new tools and insights. *Nat Rev Mol Cell Biol.* **11**, 688-699.

Simons, K. and Ikonen, E. 1997. Functional rafts in cell membranes. *Nature* **387**, 569-572.

Simons, K. and Sampaio, J.L. 2011. Membrane organization and lipid rafts. *Cold Spring Harb Perspect Biol.* **3**, a004697.

Simons, K. and van Meer, G. 1988. Lipid sorting in epithelial cells. *Biochemistry* **27**, 6197-6202.

Simons, K. and Vaz, W.L. 2004. Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct.* **33**, 269-295.

Simson, R., Sheets, E.D. and Jacobson, K. 1995. Detection of temporary lateral confinement of membrane proteins using single-particle tracking analysis. *Biophys J.* **69**, 989-993.

Singer, S.J. and Nicolson, G.L. 1972. The fluid mosaic model of the structure of cell membranes. *Science* **175**, 720-731.

Skrzypek, M., Lester, R.L. and Dickson, R.C. 1997. Suppressor gene analysis reveals an essential role for sphingolipids in transport of glycosylphosphatidylinositol-anchored proteins in Saccharomyces cerevisiae. *J Bacteriol.* **179**, 1513-1520.

Steck, T.L. and Dawson, G. 1974. Topographical distribution of complex carbohydrates in the erythrocyte membrane. *J Biol Chem.* **249**, 2135-2142.

Stefanová, I., Horejsí, V., Ansotegui, I.J., Knapp, W., and Stockinger, H. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* **254**, 1016-1019.

Stoffel, W. 1971. Sphingolipids. *Annu Rev Biochem.* **40**, 57-82.

Stuermer, C.A., Lang, D.M., Kirsch, F., Wiechers, M., Deininger, S.O., and Plattner, H. 2001. Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. *Mol Biol Cell.* **12**, 3031-3045.

Subczynski, W.K. and Kusumi, A. 2003. Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy. *Biochim Biophys Acta.* **1610**, 231-243.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M.A., Keen, J.H., and McGraw, T.E. 1999. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci USA.* **96**, 6775-6780.

Suzuki, K.G., Fujiwara, T.K., Edidin, M., and Kusumi, A. 2007b. Dynamic recruitment of phospholipase C gamma at transiently immobilized GPI-anchored receptor clusters induces IP3-Ca2+ signaling: single-molecule tracking study 2. *J Cell Biol.* **177**, 731-742.

Suzuki, K.G., Fujiwara, T.K., Sanematsu, F., Iino, R., Edidin, M., and Kusumi, A. 2007a. GPI-anchored receptor clusters transiently recruit Lyn and G alpha for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J Cell Biol.* **177**, 717-730.

Takeuchi, M., Miyamoto, H., Sako, Y., Komizu, H., and Kusumi, A. 1998. Structure of the erythrocyte membrane skeleton as observed by atomic force microscopy. *Biophys J.* **74**, 2171-2183.

Thewalt, J.L. and Bloom, M. 1992. Phosphatidylcholine: cholesterol phase diagrams. *Biophys J.* **63**, 1176-1181.

Thompson, T.E. and Tillack, T.W. 1985. Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. *Annu Rev Biophys Biophys Chem.* **14**, 361-386.

Tomishige, M., Sako, Y. and Kusumi, A. 1998. Regulation mechanism of the lateral diffusion of band 3 in erythrocyte membranes by the membrane skeleton. *J Cell Biol.* **142**, 989-1000.

Tsuji, A. and Ohnishi, S. 1986. Restriction of the lateral motion of band 3 in the erythrocyte membrane by the cytoskeletal network: dependence on spectrin association state. *Biochemistry* **25**, 6133-6139.

Tsuji, A., Kawasaki, K., Ohnishi, S., Merkle, H., and Kusumi, A. 1988. Regulation of band 3 mobilities in erythrocyte ghost membranes by protein association and cytoskeletal meshwork. *Biochemistry* **27**, 7447-7452.

van Meer, G, Voelker, D.R. and Feigenson, G.W. 2008. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Bio* **9**, 112-124.

van Meer, G. 1989. Lipid traffic in animal cells. *Annu Rev Cell Biol.* **5**, 247-275.

van Meer, G. and de Kroon, A.I.P.M. 2011. Lipid map of the mammalian cell. *J Cell Sci.* **124**, 5-8.

van Meer, G. and Simons, K. 1982. Viruses budding from either the apical or the basolateral plasma membrane domain of MDCK cells have unique phospholipid compositions. *EMBO J.* **1**, 847-852.

van Meer, G. and Simons, K. 1986. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* **5**, 1455-1464.

van Meer, G. and Simons, K. 1988. Lipid polarity and sorting in epithelial cells. *J Cell Biochem.* **36**, 51-58.

van Meer, G., Stelzer, E.H., Wijnaendts-van-Resandt, R.W., and Simons, K. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J Cell Biol.* **105**, 1623-1635.

van Zanten, T.S., Cambi, A., Koopman, M., Joosten, B., Figdor, C.G., and Garcia-Parajo, M.F. 2009. Hotspots of GPI-anchored proteins and integrin nanoclusters function as nucleation sites for cell adhesion. *Proc Natl Acad Sci USA.* **106**, 18557-18562.

Varma, R. and Mayor, S. 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* **394**, 798-801.

Veatch, S.L., Cicuta, P., Sengupta, P., Honerkamp-Smith, A., Holowka, D., and Baird B. 2008. Critical fluctuations in plasma membrane vesicles. *ACS Chem Biol.* **3**, 287-293.

Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S.J., Smart, E.J., Anderson, R.G., Taraboulos, A., and Prusiner, S.B. 1996. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc Natl Acad Sci USA.* **93**, 14945-14949.

Waheed, A.A. and Freed, E.O. 2009. Lipids and membrane microdomains in HIV-1 replication. *Virus Res.* **143**, 162-176.

Walter, P., Gilmore, R. and Blobel, G. 1984. Protein translocation across the endoplasmic reticulum. *Cell* **38**, 5-8.

Wang, T.Y. and Silvius, J.R. 2001. Cholesterol does not induce segregation of liquid-ordered domains in bilayers modeling the inner leaflet of the plasma membrane. *Biophys J.* **81**, 2762-2773.

Wang, T.Y. and Silvius, J.R. 2003. Sphingolipid partitioning into ordered domains in cholesterol-free and cholesterol-containing lipid bilayers. *Biophys J.***84**, 367-378.

Wang, T.Y., Leventis, R. and Silvius, J.R. 2001. Partitioning of lipidated peptide sequences into liquid-ordered lipid domains in model and biological membranes. *Biochemistry* **40**, 13031-13040.

Weinstein, D.B., Marsh, J.B., Glick, M.C., and Warren, L. 1970. Membranes of animal cells. VI. The glycolipids of the L cell and its surface membrane. *J Biol Chem.* **245**, 3928-3937.

Williamson, R., Thompson, A.J., Abu, M., Hye, A., Usardi, A., Lynham, S., Anderton, B.H., and Hanger, D.P. 2010. Isolation of detergent resistant microdomains from cultured neurons: detergent dependent alterations in protein composition. *BMC Neurosci.* **11**, 120.

Wu, C., Butz, S., Ying, Y., and Anderson, R.G. 1997. Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane. *J Biol Chem.* **272**, 3554-3559.

Wu, M., Holowka, D., Craighead, H.G., and Baird, B. 2004. Visualization of plasma membrane compartmentalization with patterned lipid bilayers. *Proc Natl Acad Sci USA.* **101**, 13798-13803.

Xu, X. and London, E. 2000. The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. *Biochemistry* **39**, 843-839.

Yeagle, P.L. 1985. Cholesterol and the cell membrane. *Biochim Biophys Acta.* **822**, 267-287.

Zacharias, D.A., Violin, J.D., Newton, A.C., and Tsien, R.Y. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* **296**, 913-916.

Zambrano, F., Fleischer, S. and Fleischer, B. 1975. Lipid composition of the Golgi apparatus of rat kidney and liver in comparison with other subcellular organelles. *Biochim Biophys Acta.* **380**, 357-369.

Zech, T., Ejsing, C.S., Gaus, K., de Wet, B., Shevchenko, A., Simons, K., and Harder, T. 2009. Accumulation of raft lipids in T-cell plasma membrane domains engaged in TCR signalling. *EMBO J.* **28**, 466-476.

Zidovetzki, R. and Levitan, I. 2007. Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta.* **1768**, 1311-1324.

Zurzolo, C., van Meer, G. and Mayor, S. 2003. The order of rafts. Conference on microdomains, lipid rafts and caveolae. *EMBO Rep.* **4**, 1117-1121.

Zwaal, R.F., Bevers, E.M., Comfurius, P., Rosing, J., Tilly, R.H., and Verhallen, P.F. 1989. Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled red cells; mechanisms and physiological significance. *Mol Cell Biochem.* **91**, 23-31.