MOLECULAR ORGANIZATION OF MEMBRANES: WHERE BIOLOGY MEETS BIOPHYSICS

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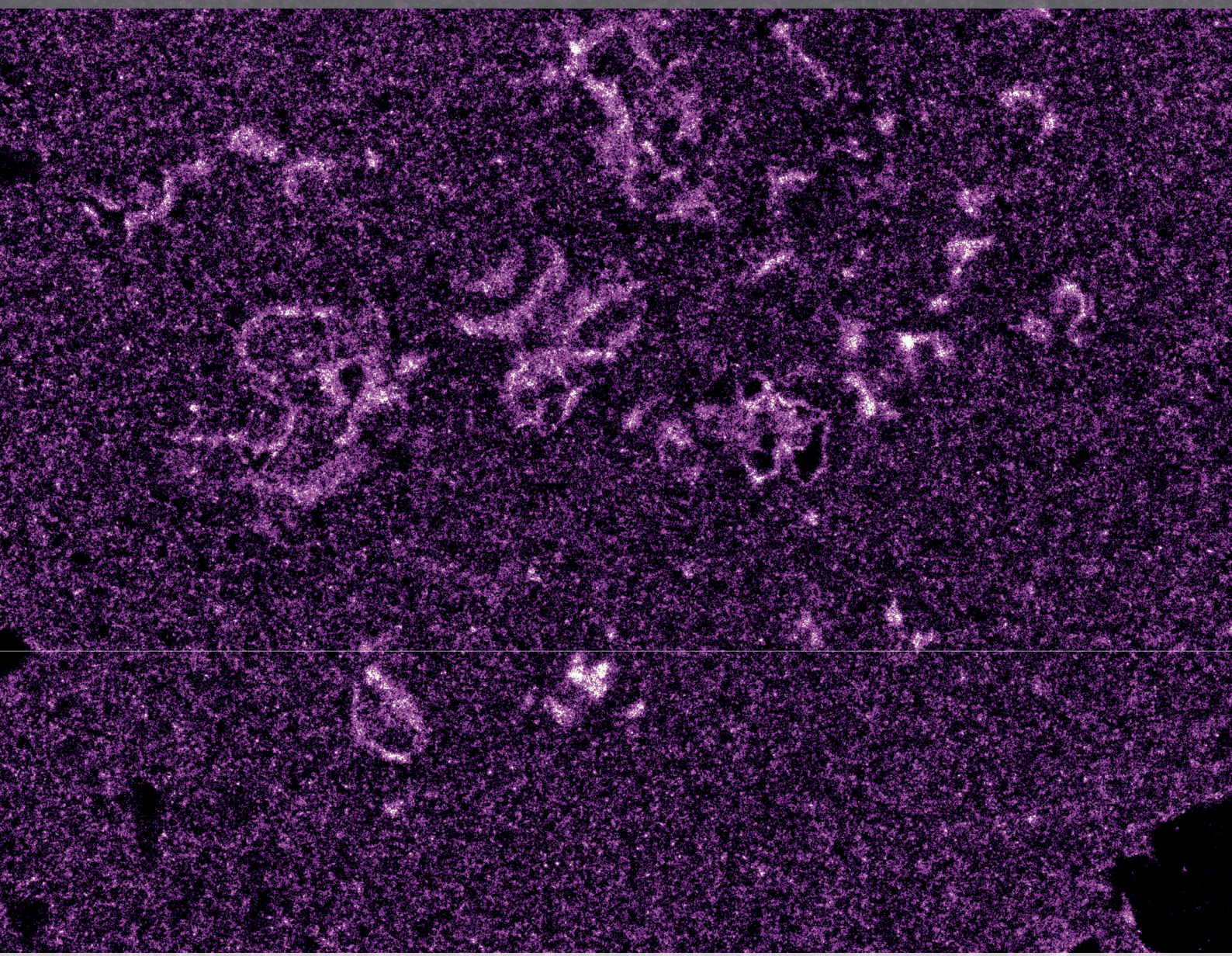

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MOLECULAR ORGANIZATION OF MEMBRANES: WHERE BIOLOGY MEETS BIOPHYSICS

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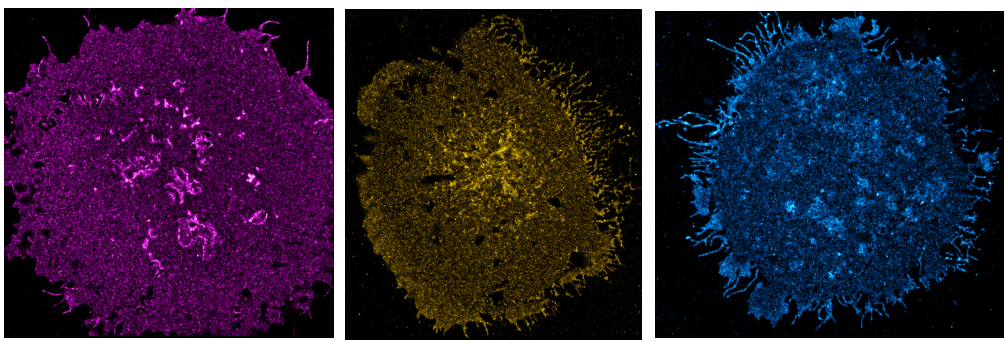

Super-resolution imaging of transmembrane adaptor protein (LAT) involved in signalling of human T lymphocytes. LAT proteins were genetically fused to the photoswitchable fluorescent protein PS-CFP2, expressed transiently in model Jurkat T cells and total internal reflection fluorescence (TIRF) imaging was performed 20 hours after transfection. Super-resolution image (PALM) was produced using Thunderstorm Plugin in ImageJ open-source software platform for light microscopists. Different colours were applied to the 16-bit greyscale image in postprocessing.

The images were acquired and processed in the laboratory of Dr. Marek Cebecauer by Tomas Chum.

Biological membranes protect cells and organelles from the surrounding environment, but serve also as organising platforms for physiological processes such as cell signalling. The hydrophobic core of membranes is composed of lipids and proteins influencing each other. Local membrane composition and properties define its molecular organisation and, in this way, regulate the function of all associated molecules. Therefore, studying interactions of components, biophysical properties and overall membrane dynamics provides essential information on its function in the context of cell activities. Such knowledge can contribute to biomedical fields such as pharmacology, immunology, neurobiology and many others.

The goal of the Research Topic entitled 'Molecular organisation of membranes: where biology meets biophysics' was to provide a comprehensive platform for publishing articles, reviews and opinions focused on membrane organisation and the forces behind its heterogeneous and dynamic structure. We collected 11 works which cover topics as diverse as general membrane organisation models, membrane trafficking and signalling regulation, biogenesis of caveolae, protein-lipid interactions and the importance of membrane-associated tetraspanins networks. The prevalent theme was the existence of membrane nanodomains. To this point, new emerging technologies are presented which own the power to bring a novel insight on how membrane nanodomains are formed and maintained and what is their function. We believe that the collection of works in this Research Topic brings forward some important questions which will stimulate further research in this difficult but exciting field.

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EDITORIAL
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Editorial: Molecular Organization of Membranes: Where Biology Meets Biophysics

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Keywords: nanodomains, tetraspanins, membrane properties, cell membrane, fluorescence microscopy, superresolution microscopy, membrane trafficking, caveolae

Editorial on the Research Topic

Molecular Organization of Membranes: Where Biology Meets Biophysics

Membranes delimit the shapes of cells and their internal compartments, form a passive barrier between interior and exterior, but function also as organizing platforms for cellular processes. These highly diverse structures are formed by a large number of lipid and protein species. It is now generally accepted that both lipids and proteins are heterogeneously distributed in cell membranes (Cebecauer et al., 2010; Holowka and Baird, 2015; Sezgin et al., 2017). Such non-homogeneous organization of membranes linked to cellular functions attracts attention of scientists from fields as diverse as physiology, cell biology and biophysics. Indeed, the presence of various nanodomains in membranes forms a unifying link between the articles of this Research Topic.

The Research Topic collects 11 articles from authors with a broad background and focuses mainly on three issues resonating in membrane biology and biophysics: (i) physical properties of membranes contributing to cell membrane organization in molecular assemblies and domains; (ii) emerging role of tetraspanins, an evolutionarily conserved superfamily of membrane structural proteins, as critical players in membrane organization and (iii) novel tools to study cell membranes.

Various models were suggested to describe molecular organization of membranes and their capacity to respond to various chemical and physical stimuli. Five most frequently discussed models are summarized in the work of de la Serna et al. which also reports on the original works supporting or stimulating these hypotheses. Models are assessed for their ability to deal with a highly complex nature of membranes. This complexity is comprehensively described in sections focused on membrane structure and properties. The authors conclude that it is difficult to sum all membrane properties in one universal model. Fujimoto and Parmryd emphasize the importance of membrane asymmetry, interleaflet coupling and pinning in formation of lipid domains and membrane organization. These aspects of plasma membrane were overlooked in the past. Interleaflet coupling and pinning were studied only in a handful of works using model systems. Further studies will help to adapt current models of membrane organization to this rather new perspective.

Existence of lipid domains was believed to depend on sphingolipids and cholesterol (Simons and Ikonen, 1997). Whereas both lipid species are essential for plasma membrane structure and function, heterogenous distributions of cholesterol in domains has not been observed in tested membranes (Frisz et al., 2013; Honigmann et al., 2014). On the contrary, segregation of sphingolipids and glycosphingolipids into distinct membrane domains was demonstrated using a broad spectrum of techniques, as summarized in the work of Kraft. Implications of sphingolipid clustering on plasma membrane organization in domains are discussed. In analogy to lipids,

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proteins can form supramolecular assemblies associated with membranes. For example, caveolins interact at the membrane to create caveolae, flask-shaped domains decorating the surface of cells. Caveolae are involved in cellular processes such as lipid homeostasis or mechanotransduction. Even though these domains are relatively stable and easy to visualize, there is currently no consensus regarding their biogenesis. Han et al. report on issues associated with studies of caveolae biogenesis and suggest more intense use of disease-associated mutants to better understand these important membrane structures.

Full assembly of caveolae can lead to endocytosis and downregulation of membrane molecules from the cellular surface. Lou et al. report on the reverse process, exocytosis, and its importance for signaling events on T cells. Their work summarizes current knowledge on the diversity of exosomes involved in delivery of a principal receptor (TCR) and its effectors to the plasma membrane of T cells. The idea is that spatial separation of signaling molecules in vesicles prevents uncontrolled activation in the absence of stimulus. The impact of physical separation on receptor function is also studied in the only original research article of this Research Topic. Using *in silico* simulations, Kerketta et al. demonstrate a potential regulatory role of differential nanodomain partitioning of the two receptors, ErbB2 and ErbB3. Heterodimerisation and phosphorylation of these receptors depends on their relative accumulation in nanodomains. Whether in vesicles or in nanodomains, both articles emphasize the importance of compartmentalization and physical segregation of signaling molecules and the competence of cellular membranes to facilitate such processes. These works also underline the dynamic character of molecular separation in domains or vesicles. After stimulation, membrane domains move laterally and exosomes vertically fuse with bulk membranes to set up a new distribution allowing acceleration or deceleration of signaling events.

The presence of individual molecules in nanoscopic structures such as membrane domains or vesicles poses a challenge for standard imaging techniques (Cebecauer et al., 2010; Owen et al., 2010). Currently developed super-resolution techniques overcome this problem and allow visualization of molecules in cells with nanoscopic precision. Even though these techniques are prevalently used for static imaging, dynamic events can be visualized at nanoscopic level as reported in the work of Sergé. How emerging techniques help to better understand membrane-associated processes at nanoscopic level is illustrated on studies investigating dynamic reorganization of focal adhesions, and immune and neuronal synapses. These techniques are still in their early years and many improvements are needed. Here, Mateos-Gil et al. provide detailed description how to investigate distribution of membrane molecules using efficient and non-invasive labeling and super-resolution imaging. By adapting metabolic labeling and click chemistry combined with dSTORM, they characterized the whole glycome on the surface of cultured cells at nanoscopic level (Letschert et al., 2014).

Super-resolution imaging and improved labeling procedures accelerate research focused on membrane organization in general but, with their commercial availability, detailed characterization of individual molecules is emerging in the literature. Tetraspanins were commonly studied by standard microscopy. More recently, new imaging techniques provided a more detailed insight into the tetraspanin web (Zuidscherwoude et al., 2015). Two articles in this Research Topic summarize accumulating evidence on critical roles of tetraspanins in broad spectrum of cellular processes taking place on membranes. Termini and Gillette focuses on crosstalk between tetraspanins and signaling receptors (e.g., EGFR) or integrins involved in cell adhesion. Tetraspanins were shown to modulate dimerization, clustering and endocytosis of receptors. This effect is controlled by post-translational modifications of tetraspanins (e.g., palmitoylation or glycosylation). Halova and Draber report on accumulating evidence of cooperation between tetraspanins and membrane adaptors of the TRAP family (e.g., LAT) in immune cells. Functions of these proteins are regulated by their lipid modification but also by their colocalization in membrane nanodomains. The importance of cholesterol for this phenomenon is noted but no direct data are available yet. This can be caused by difficulties associated with characterization of protein-lipid interactions in cells of higher eukaryotes.

Singh in his work offers an alternative approach for investigation of protein-lipid interactions. Due to their simpler lipid metabolism and large panel of available genetic and cell-biological tools, he argues that yeasts provide an elegant tool for such studies.

We hope that this Research Topic will stimulate further studies to confirm or revise presented opinions.

AUTHOR CONTRIBUTIONS

Both authors equally contributed to the drafting and revision of the editorial and approved it for publication.

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REVIEW
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There Is No Simple Model of the Plasma Membrane Organization

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Ever since technologies enabled the characterization of eukaryotic plasma membranes, heterogeneities in the distributions of its constituents were observed. Over the years this led to the proposal of various models describing the plasma membrane organization such as lipid shells, picket-and-fences, lipid rafts, or protein islands, as addressed in numerous publications and reviews. Instead of emphasizing on one model we in this review give a brief overview over current models and highlight how current experimental work in one or the other way do not support the existence of a single overarching model. Instead, we highlight the vast variety of membrane properties and components, their influences and impacts. We believe that highlighting such controversial discoveries will stimulate unbiased research on plasma membrane organization and functionality, leading to a better understanding of this essential cellular structure.

Keywords: plasma membrane, membrane organization models, nanodomains, heterogenous distribution, membrane physical properties

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Membranes are one of the key structures in cell biology. Besides being instrumental in compartmentalizing and protecting cells, their role as organizing centers for tasks such as metabolism or signaling is increasingly recognized. In fact, a majority of cellular processes are associated with membranes (Stryer, 1995). Membranes provide useful docks for correct localisation of proteins which is essential for their function (Miosge and Zamoyska, 2007; Grecco et al., 2011; Hung and Link, 2011). Importantly, in humans, mislocalization of membrane proteins leads to the loss-of-function and, frequently, can develop into diseases (Edwards et al., 2000; Matsuda et al., 2008; Hung and Link, 2011; Schaeffer et al., 2014). Nevertheless, the presence of proteins at a particular membrane is usually not sufficient for their function. Often, the nanoscopic localization, oligomerisation and/or clustering of membrane proteins can affect the efficiency of cellular processes (Cebecauer et al., 2010; Matthews, 2012; Nussinov, 2013; Garcia-Parajo et al., 2014). Membranes, the lipid environment and membrane properties in general, influence nanoscale organization and function of these molecules. It is, therefore, important to understand molecular details of membrane structure and mechanisms responsible for its dynamics organization.

Here, we review membrane properties, models of membrane organization and useful techniques for studies of membrane organization and dynamics, with a special focus on the plasma membrane of higher eukaryotes (mammals). Our specific aim is to re-emphasize currently omitted or underestimated biophysical principles and discuss their role in dynamic membrane organization. We attempt to provide a comprehensive description of membrane complexity and suggestions how to avoid interpretation of membrane-associated phenomena within the borders of a single theory. As a reader will see, we believe that there is no universal model of the plasma membrane dynamic

lateral organization. These more general issues will be discussed in the last section. First, let us start with the very basic structure of membranes.

BASIC STRUCTURE OF CELL MEMBRANES

A lipid bilayer forms the basis of all cellular membranes. It is a lamellar structure with a hydrophobic core and a polar headgroup region on both sides (**Figure 1A**). In cells, it is composed of hundreds, if not thousands, of different phospholipid species. These differ in their polar headgroup moiety but mainly in the length and saturation of acyl chains forming a hydrophobic core of a lipid bilayer. Other lipid and fatty acid species add to this complexity. Of those, sterols (cholesterol in mammals) are the most abundant in the plasma membrane and can represent up to 40% of total lipid (van Meer and de Kroon, 2011). Cholesterol has a special structure (**Figure 1A**) enabling strong impact on basic membrane properties such as viscosity or interleaflet coupling, as described multiple times in comprehensive articles (Ipsen et al., 1987; Mouritsen and Zuckermann, 2004; Maxfield and van Meer, 2010).

Proteins constitute approximately half of the total plasma membrane mass (Dupuy and Engelman, 2008). We distinguish integral and peripheral membrane proteins depending on their anchorage into a lipid bilayer via transmembrane domain(s) or a lipid moiety, respectively (**Figure 1B**). In addition, some proteins may associate with the membrane via electrostatic interactions with lipid headgroups (**Figure 1B**; McLaughlin and Murray, 2005) or a variety of protein-protein or protein-glycan interactions (**Figure 1C**; Stryer, 1995). Such proteins are commonly termed as "membrane-associated." Extracellular parts of lipids and proteins are frequently glycosylated (**Figure 1C**). Indeed, glycans form a dense structure at the outer surface of the plasma membrane (Berrier and Yamada, 2007). This molecular complexity of membranes has probably evolved to serve as a selective barrier and organizing center with a high fidelity and robustness (Cebecauer et al., 2010). But what are those unique properties which were selected in the process of evolution to control critical cellular processes with such efficiency?

INTRINSIC PROPERTIES OF CELL MEMBRANES ESSENTIAL FOR THEIR FUNCTION

Early definitions, of which the "fluid mosaic model" of Singer and Nicolson (SN model Singer and Nicolson, 1971, 1972) is the best known, highlighted **fluidity** as one of the most critical membrane features. Indeed, fluidity of membranes provides important advantage over other cellular structures such as the cytoskeleton or ribonucleoproteins. It forms the basis for the highly dynamic

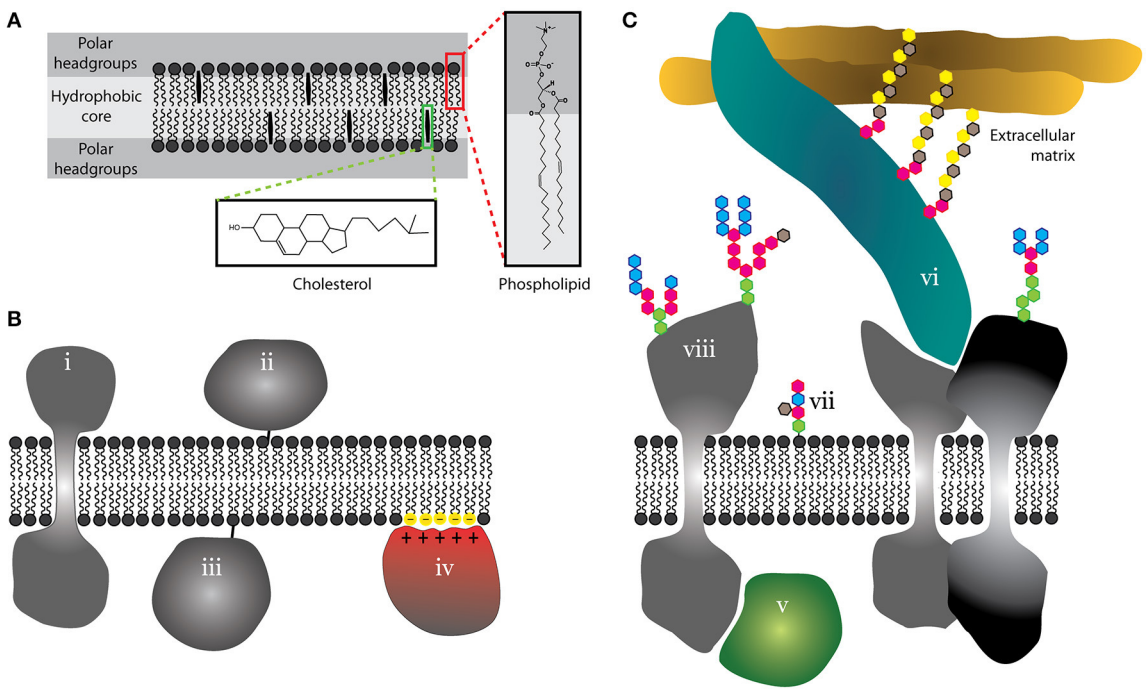

FIGURE 1 | Schematic illustration of the basic structure of lipid bilayer and proteo-lipidic membranes. (A) Cell membranes are lamellar structures with a hydrophobic core and a polar headgroup space. As examples, phospholipids and cholesterol are shown with almost atomistic detail (red and green boxes). **(B)** Membrane proteins can integrate into membranes (i), but can use lipid anchors (ii and iii) or peripherally associate with membranes via electrostatic interactions (iv). **(C)** Proteins can further associate with membranes via protein-protein interactions on the cytosolic side (v) or at the interface between the plasma membrane and the extracellular matrix (vi). Outer leaflet lipids (vii) and extracellular domains of proteins (viii) are often glycosylated.

character of membrane-associated (bio)chemical reactions and other cellular processes. Membrane fluidity enables the majority of molecules to diffuse freely over long distances and rotate or re-orientate to adopt optimal conformation. Membranes can be considered as two-dimensional solutions. This two-dimensional character also distinguishes membranes from other three-dimensional cellular solutes (e.g., the cytosol). The fundamental importance of fluidity is, for example, underlined by the fact that cells modify the saturation of their lipid acyl-chains to keep their membranes fluid when adapting to the environment, e.g., different temperatures (Fraenkel and Hopf, 1940; Buda et al., 1994).

Although membranes are fluid, they have higher **viscosity** (**Box 1**) than the cytosol (Luby-Phelps et al., 1993). This has a direct impact on the mobility of membrane molecules. Membrane viscosity can be modified by lipid composition or other factors, such as the presence of proteins or poorly mobile structures, and will thus vary over space and time.

Another property emphasized in the SN model is **continuity** of the plasma membrane (Singer and Nicolson, 1972). The plasma membrane fully covers the cell surface. Its continuity is especially important for membrane receptors or effector molecules which need rapidly to re-localize, e.g., when a cell is changing its direction of chemotactic mobility (Janetopoulos and Firtel, 2008). Continuity also supports intermolecular interactions or the formation of multi-molecular assemblies within or at the surface of membranes. In some cells, membrane continuity is limited to the apical or basal side due to the presence of tight junctions eliminating free mobility of membrane molecules (Balda and Matter, 2008). We will discuss viscosity and continuity, and their impact on the organization of membranes in more detail further in the text.

Almost all molecules can interact and influence each other in cellular membranes. As a consequence, coexistence of molecules in membranes has **cooperative** character. Cooperativity of molecules was already mentioned for fluid cellular membranes in the SN model (Singer and Nicolson, 1972) but seems to be recently overseen. This property has a dramatic impact on experiments, in which systemic perturbance of membranes (e.g., by chemical or genetic treatment) was employed to support specific models of membrane organization.

Lipid membranes undergo **interleaflet coupling**, meaning that acyl chains of lipids in one leaflet interdigitate into the space of the other leaflet (**Figure 2A**; Nickels et al., 2015). Theoretical predictions suggest that interleaflet coupling can coordinate the organization of molecules between the two leaflets (Schmidt et al., 1978; Duzgunes et al., 1988; Merkel et al., 1989; Kiessling et al., 2006; Raghupathy et al., 2015; Williamson and Olmsted, 2015). Yet, White and co-workers recently provided an alternative view (Mihailescu et al., 2011; Capponi et al., 2016). They do not negate the existence of strong coupling between the two leaflets of lipid bilayer but observed no direct complementarity between the opposite acyl chains (Capponi et al., 2016). Cholesterol, which was predicted to intensify interleaflet coupling in membrane lipid domains, was found to reduce the level of acyl-chain interdigitation (Mihailescu et al., 2011). These works indicate that we need more experimental data in order to better understand the effect of interleaflet coupling in lipid bilayers.

The plasma membrane of eukaryotic cells is **asymmetric** (**Figure 2B**) in terms of lipid and surface ion composition, as well as the presence of specific proteins (Rothman and Lenard, 1977; van Meer et al., 2008). The lipid asymmetry is maintained by flippases and other lipid translocating or transport proteins (Canagarajah et al., 2008; Devaux et al., 2008). Chemical asymmetry, a gradient of ions, drives a number of vital cellular processes (e.g., generation of chemical energy and metabolism). On the other hand, lipid asymmetry further adds to the diversity and complexity of cellular compartments, thereby helping to optimize cellular processes. For example, negatively charged lipid headgroups in the inner leaflet provide the binding surface for proteins with specific binding domains (**Figure 2C**; McLaughlin and Murray, 2005). This can cause protein relocalisation often

BOX 1 | MEMBRANE FLUIDITY, VISCOSITY AND MOBILITY

Viscosity is a macroscopic parameter describing the behavior of a large, rigid sphere in a Newtonian fluid. Its use for membranes is imperfect and should be treated with care (Valeur and Berberan-Santos, 2012; Olšinová et al., 2014). Membranes are nanoscopic structures with 2D character and highly heterogeneous composition in terms of size and chemistry. Due to a lack of a better parameter, we use the term "viscosity" to describe membrane properties such as membrane lateral compressibility and acyl chain ordering, which influence the mobility of membrane components. Other terms, e.g., "microviscosity" or "rigidity" were also used in literature to cover these properties in one word (Shinitzky and Inbar, 1976; Kowalska and Cierniewski, 1983; Gut et al., 1985; Sherbet, 1989).

The term "fluidity" is frequently used to replace "viscosity" for biological membranes or other highly heterogeneous materials (Valeur and Berberan-Santos, 2012). We use term "fluidity" in this work to distinguish membranes from other cellular structures which exhibit much higher stability (e.g., nucleoproteins), thereby limiting rapid, long-range mobility of associated compounds.

Efforts to measure viscosity of cellular membranes are associated with serious technical difficulties (Valeur and Berberan-Santos, 2012; Olšinová et al., 2014). Instead, measurements of rotational or lateral diffusion were successfully applied to characterize membrane viscosity. In cellular membranes, lateral diffusion is frequently substituted with the term "mobility." Mobility of membrane molecules can be influenced by many different factors, such as (i) membrane ordering or, in the other terms, how densely lipids and proteins are packed in the membrane (Kahya et al., 2003), (ii) lateral pressure of the membrane which is partially linked to ordering but also membrane hydration (polarity) and directly influences bilayer compressibility and elasticity (Marsh, 1996; Cantor, 1999), and (iii) macromolecular crowding (Saxton, 1987; Guigas and Weiss, 2015). Mobility of membrane components is further influenced by other intrinsic and extrinsic factors as described in the main text.

To illustrate dramatic differences in the mobility of molecules in synthetic and cellular environments, we provide a few values of diffusion coefficients in **Table 1**. These should be considered as a simple guideline due to differences in the precision with which these values were measured. We also provide the time scale a molecule requires to traverse the distance of 20 μm (longitudal size of HeLa cells) by random (Brownian) 2D motion. This should underline dramatic differences in the mobility of molecules in real space.

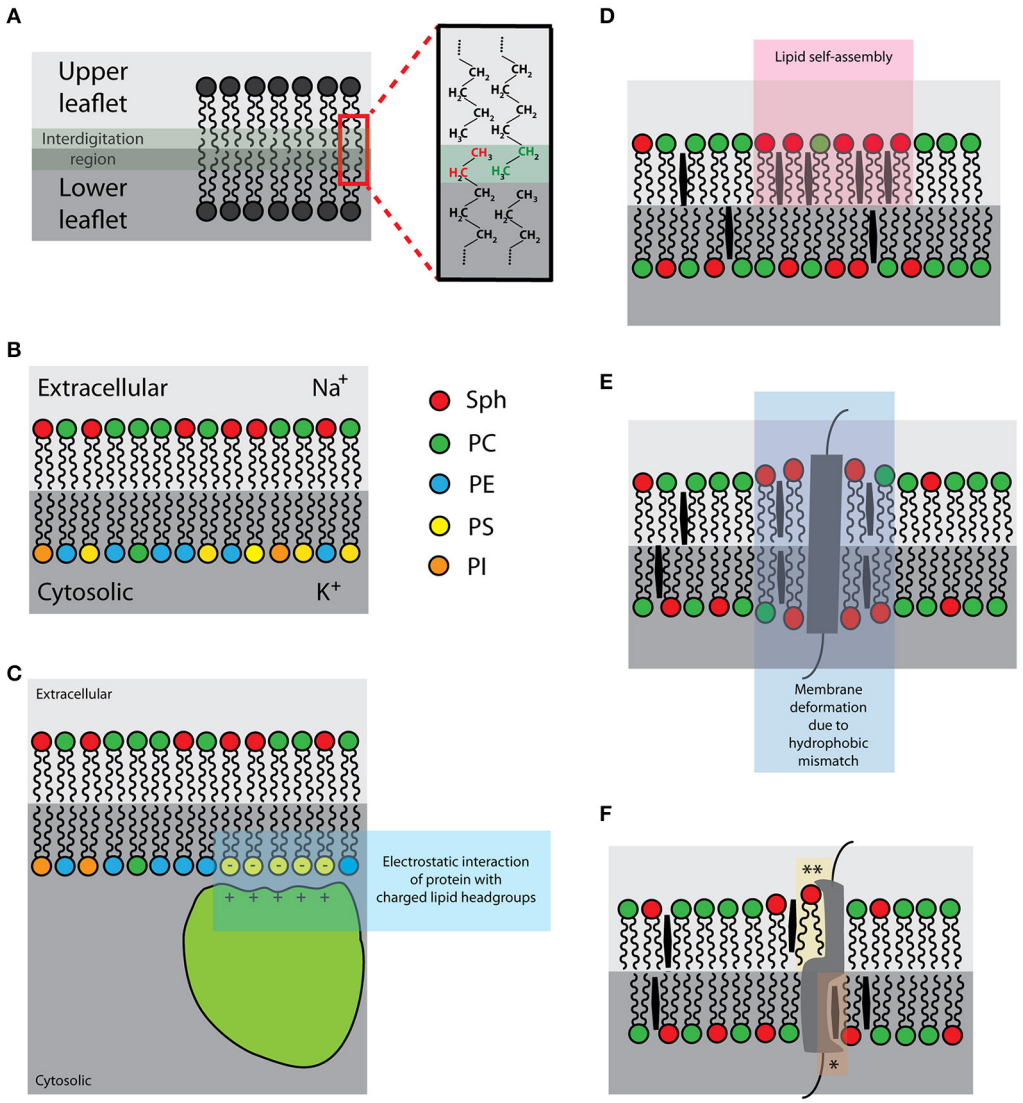

FIGURE 2 | Schematic illustrations of the selected intrinsic membrane properties: (A) Interleaflet coupling [interdigitating lipid acyl chains in green-gray; zoom: interdigitating ethyl groups of upper (green) and lower (red) leaflets]; **(B)** Asymmetric distribution of lipids and ions [right hand-side: color-coding of lipid species]; **(C)** Negatively charged lipids (yellow) of the plasma membrane inner leaflet [for the association of proteins with basic-rich domains (light green)]; **(D)** Lipid self-assemblies (pink); **(E)** Hydrophobic mismatch (purple); **(F)** Protein-lipid interactions [*sphingolipid- and **cholesterol-binding pockets].

leading to the initiation of signaling events (Yeung et al., 2008). In addition, chemical asymmetry and the presence of ions induces heterogeneous distribution of lipids, at least in simulations and in model systems (Vácha et al., 2009; Jurkiewicz et al., 2012). Whether this effect contributes to the organization of plasma membrane in living cells is experimentally difficult to test; an asymmetric membrane is indispensable for cell viability. At the same time, the formation of asymmetric model membranes *in vitro* is a rather delicate process and was successfully performed only in a few cases in past (Kiessling et al., 2006; Collins and Keller, 2008; Chiantia et al., 2011). Therefore, data demonstrating lateral (re)organization due to membrane asymmetry are still rare.

Even though lipids interact only weakly, preferential **self-assemblies** of certain lipid species or conformations (**Figure 2D**) were demonstrated in model lipid mixtures (Björkbom et al., 2010; Ivankin et al., 2010). Under certain circumstances, lipid self-assembling may extensively reduce miscibility of its molecules, i.e., generate physico-chemical heterogeneities. A well-known example of lipid self-assembly and segregation is the formation of separated lipid phases in vesicles composed of two or more lipid species with different melting points (Bagatolli and Gratton, 1999; Korlach et al., 1999; Bernardino de la Serna et al., 2004; Veatch and Keller, 2005). Importantly, lipids are prone to phase separation or miscibility transitions also in cell membrane-derived vesicles

and blebs, as well as artificial vesicles generated from lipids extracts and from native membranes (Bernardino de la Serna et al., 2004; Baumgart et al., 2007; Veatch et al., 2008). All these observations were achieved using equilibrated membranes; however, cells are non-equilibrium systems (Stryer, 1995). Indeed, no miscibility phase transitions were observed in living cells over a wide range of temperatures (Lee et al., 2015). Putative impact of lipid self-assembly and ordered lipid membranes on cell membranes is discussed in the section "Plasma membrane organization–general models and concepts."

Hydrophobic thickness of a lipid bilayer is defined mainly by the length and saturation of acyl chains and the presence of sterols. Bilayer lipids interact non-specifically and transiently with transmembrane domains of integral proteins (Marsh, 1993). Imparity of the hydrophobic thickness of the bilayer and the hydrophobic length of TMD (s) is called **hydrophobic mismatch** (**Figure 2E**). Hydrophobic mismatch was proposed to induce molecular aggregation/segregation in lipid bilayers, as described in the mattress model (Mouritsen and Bloom, 1984). For example, lipids with longer and more saturated acyl chains will preferentially reside in the annulus of helical TMD with long hydrophobic length. More about the mattress model is discussed in the section "Plasma membrane organization–general models and concepts."

Lipids can also interact with proteins in a more specific manner (Haberkant et al., 2008; Fantini and Barrantes, 2013; Yeagle, 2014). Several proteins carry lipid-binding domains (Ernst et al., 2010; Contreras et al., 2011; Fantini and Barrantes, 2013) to which lipids bind with a higher affinity compared to the lipids of the first shell interacting with transmembrane domains non-specifically. Such **protein-lipid interactions** (**Figure 2F**) can be highly specific in a way that lipid headgroup, acyl chain length and its saturation determine the affinity of such interactions (Contreras et al., 2012). Specific protein-lipid interactions have been shown to modulate protein stability and its function (Uittenbogaard and Smart, 2000; Hanson et al., 2008; Contreras et al., 2012) or are directly involved in transport of lipids between subcellular compartments (Kwon et al., 2009). But what is their impact on the lateral organization of plasma membrane is to date unclear.

The abovementioned intrinsic properties can be ascribed to any proteo-lipid membranes, independent of whether these are artificial or cellular structures. But what is so specific about membranes of living cells? Can "clever" use of these intrinsic properties, their local amplification, reduction and/or combination lead to such limitless concert of events such as metabolism and signal transduction? Or is there a need for extrinsic factors to support those basal membrane properties?

EXTRINSIC FACTORS INFLUENCING THE PLASMA MEMBRANE ORGANISATION

The plasma membrane is built to interact with surrounding structures such as cortical actin, the extracellular matrix or a variety of ligand molecules. These form the basis of extrinsic factors which can shape the plasma membrane.

We assigned **protein-protein interactions** to the section of extrinsic factors, given the fact that extra-membranous (extracellular and cytosolic) domains are the predominant structures involved in persistent associations of proteins. Further, since these interactions often involve non-membranous protein scaffolds, we believe that protein-protein interactions have, to some extent, extrinsic character.

In contrast to lipids, proteins can interact with high affinity and thus form relatively stable structures (**Figure 3A**) within a sea of lipid molecules. Indeed, the interaction of proteins is a common process associated, for example, with leukocyte signaling or cellular adhesion, both taking place at the surface of cells (Douglass and Vale, 2005; Rossier et al., 2012). Supramolecular complexes of proteins can be relatively large and can further interact with other cellular components such as the cytoskeleton, thereby forming protein networks which can have local or systemic impact on membranes (see below).

More recently, a concept of **protein islands** was presented based on the fact that proteins were detected in distinct domains interspaced with protein-free areas, when membrane patches were imaged by electron microscopy (Wilson et al., 2000; Lillemeier et al., 2006). Heterogeneous distribution of proteins in entities reminiscent of such "protein islands" were often found by super-resolution fluorescence imaging of the cellular plasma membrane (for example Sieber et al., 2007; Lillemeier et al., 2010; Letschert et al., 2014; Saka et al., 2014; see also **Figure 4**). However, it is not yet clear whether such entities are created and stabilized by protein-protein interactions or other mechanisms are involved. The impact of the underlying actin cytoskeleton on protein islands was reported in the past (Wilson et al., 2001; Lillemeier et al., 2006).

Certain cytosolic proteins can **interact with headgroups of selected lipid species** (e.g., negatively charged phosphatidylserines and phosphoinositols) via electrostatic interactions (**Figure 2C**; McLaughlin and Murray, 2005). In cells, binding of proteins to charged headgroups of inner leaflet lipids is well-documented to control important cellular processes, e.g., phagocytosis (Botelho et al., 2000). In addition, peripheral protein interactions at the inner leaflet of the plasma membrane modulate localization and mobility of charged lipids, as well as some other, probably associated, molecules (Golebiewska et al., 2008, 2011; Yeung et al., 2008). But whether such peripheral interactions can modulate the mobility of other membrane components (e.g., at the outer leaflet) and have a more general impact on the plasma membrane organization awaits its direct proof.

Due to membrane plasticity and flexibity, certain lipids with non-conical shape, e.g., lysophospholipids or phosphatidylethanolamines found also in cell membranes, can deform the planar structure of lipid bilayers by bending (**Figure 3B**), thereby changing its **curvature** (Šachl et al., 2011). Similar to protein-protein interactions, curvature is not a typical extrinsic factor. But in cells, highly curved membranes are prevalently generated by curvature-forming proteins (e.g., BAR-domain containing proteins) or cytoskeleton-induced mechanical forces (McMahon and Gallop, 2005; Mattila and Lappalainen, 2008). Both these processes are externally regulated

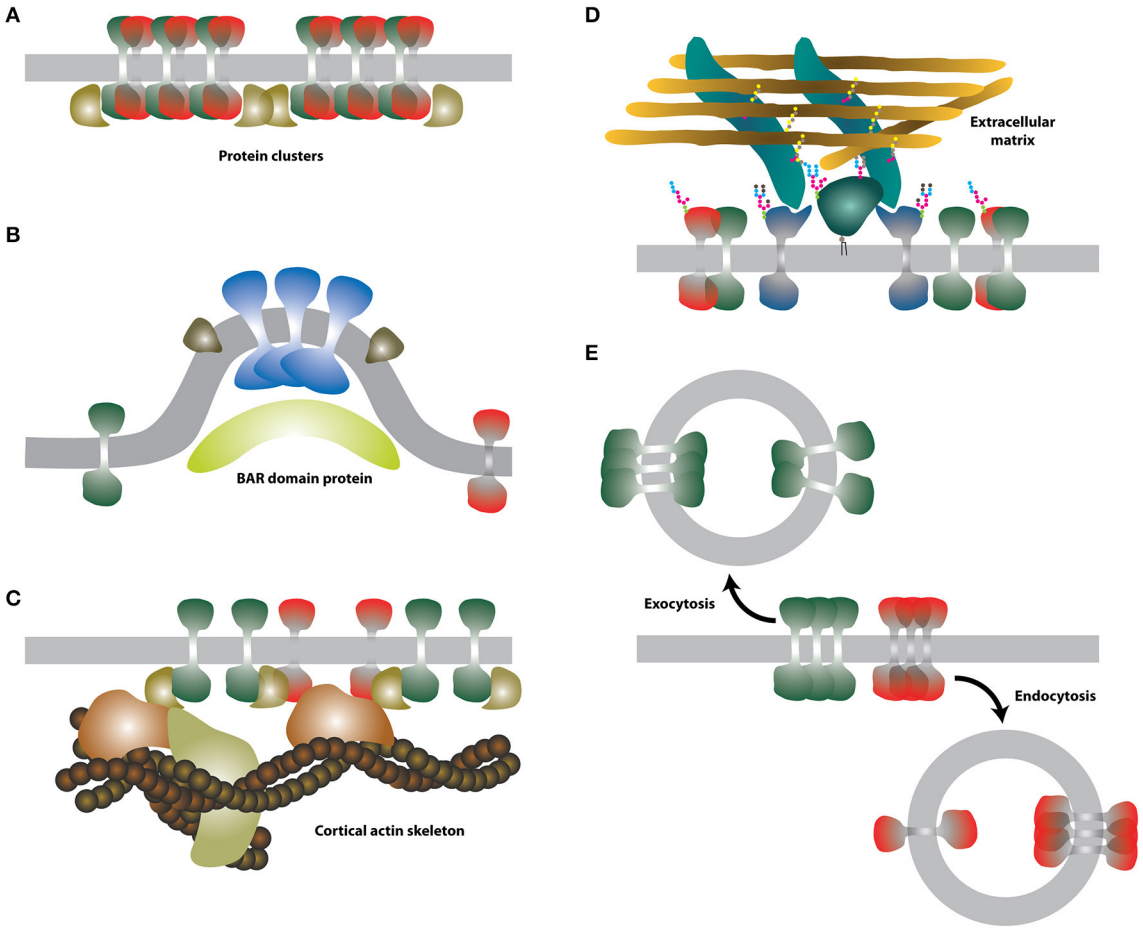

FIGURE 3 | Schematic illustrations of the selected extrinsic membrane properties. (A) Protein-protein interactions. Putative membrane proteins (green and red) forming heterodimers can assemble into larger clusters and be stabilized by further interaction with cytosolic proteins (dark yellow). **(B)** Membrane curvature. Certain proteins (blue and green-brown) may prefer curved membranes. Curved membranes can be stabilized e.g., by BAR proteins (light yellow). **(C)** Intracellular cortical actin skeleton. Actin-binding proteins (dark yellow and orange) can associate with integral membrane proteins and form larger assemblies with reduced mobility. **(D)** Extracellular glycocalyx. Interaction of certain membrane proteins (dark blue and dark green) with the extracellular matrix may lead to the formation of larger assemblies with reduced mobility. **(E)** Endo-/exocytosis. Membrane lipids and proteins are rapidly endocytosed (red) or exocytosed (green).

and require energy and/or cofactors (Mima et al., 2008; Frolov et al., 2010). For example, processes of endo/exocytosis are initiated by protein-induced membrane bending and ATP/GTP hydrolysis (Vilmart-Seuwen et al., 1986; Hansen and Nichols, 2009; Stachowiak et al., 2013). The plasma membrane has the capacity to form specialized extensions with high curvature to accomplish some of its specific functions, e.g., the formation of microvilli in polarized cells for the efficient uptake of nutrients (Crawley et al., 2014), or of membrane nanotubes for the inter-cellular communication (Onfelt et al., 2004). In theory, curvature can modulate the distribution of membrane molecules (Bozic et al., 2006; Wu and Liang, 2014). Indeed, some proteins accumulate in curved or filamentous membranes in cells, but the mechanisms responsible for such diversity are probably based on targeted delivery of molecules to these specific structures and partial impermeability of the basal region of such membrane extensions, e.g., of cilia (Trimble and Grinstein, 2015). In model membranes, specific proteins undergo curvature-driven sorting while others do not (Hatzakis et al., 2009; Aimon et al., 2014; Quemeneur et al., 2014). Specific intermolecular interactions between lipids and proteins were suggested to be responsible for such selectivity (Callan-Jones et al., 2011), nonetheless it is still unclear whether curvature-based protein and lipid sorting can occur in highly dynamic membranes of cells.

The **cortical actin** (CA) skeleton (**Figure 3C**) helps to keep and modulate the shape of living cells (Murase et al., 2004). In addition, it is involved in the regulation of membrane trafficking and signaling (for example Suzuki et al., 2007; Jaqaman et al., 2011; Gowrishankar et al., 2012; Johnson et al., 2012). The impact of the CA on the organization of the plasma membrane is well described and forms the basis of a key model discussed in the following section.

Similarly, the **glycocalyx** (**Figure 3D**), a part of the extracellular matrix (ECM) in vertebrates, forms a dense structure at the surface of eukaryotic cells (Stryer, 1995). Glycosaminoglycans and associated glycoproteins and

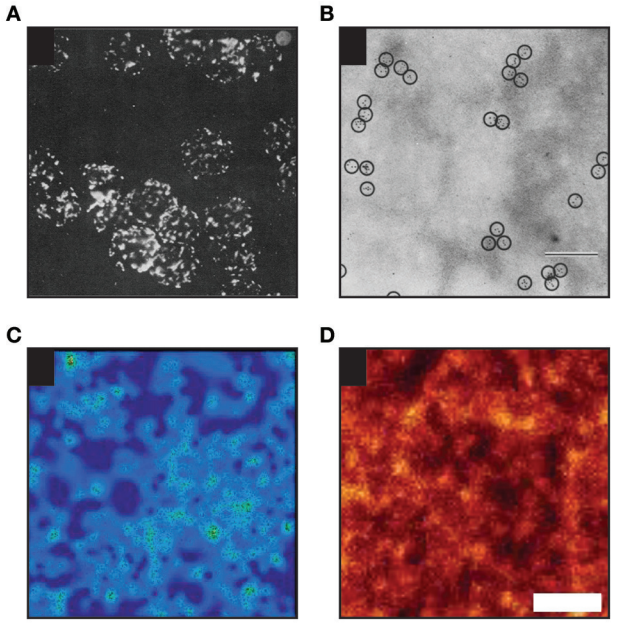

FIGURE 4 | Examples of heterogenous distribution of proteins in the plasma membrane of mammalian cells. (A) Heterogenous distribution of MHC molecules as observed on murine lymphoid cells in 1967 by Cerottini and Brunner using epifluorescence microscopy (Adapted by permission from John Wiley and Sons Ltd; (Cerottini and Brunner, 1967). **(B)** MHC clustering on murine red blood cells as detected in 1971 by Nicolson and colleagues by electron microscopy (EM; scale bar: 200 nm; Adapted by permission from RUPress: ©1971 Nicolson et al., 1971). **(C)** Distribution of individual TCR molecules on activated primary human T cells analyzed by dSTORM (Adapted by permission from Macmillan Publishers Ltd: Nature Methods (Rubin-Delanchy et al., 2015), copyright 2015). Showing 3 × 3 μm area. **(D)** Distribution of proteins in membrane sheets derived from a neuroendocrine cell line as revealed by STED microscopy [Adapted by permission from Macmillan Publishers Ltd: Nature Communications (Saka et al., 2014), copyright 2015]. Scale bar: 500 nm.

proteoglycans of the glycocalyx were shown to modulate signaling by direct association with surface receptors (Bass et al., 2007; Morgan et al., 2007) or by binding of ligands (Hynes, 2009). In this way, the glycocalyx and the extracellular matrix regulate the shape of multicellular organisms. The glycocalyx was also predicted to influence the general organization of the plasma membrane (Jacobson et al., 1987). Indeed, glycosylated extracellular domains were shown to modulate the organization (Anderson and Fambrough, 1983) and mobility (Wier and Edidin, 1986; Zhang et al., 1991; Hartel et al., 2015) of membrane proteins. The molecular mechanism is still unknown and, to our knowledge, has not been studied in detail.

Cells keep their membranes "healthy" by a rapid turnover of its components. This is achieved mainly by vesicular transport—**endo-/exocytosis** (**Figure 3E**)—but also by a less well understood protein-mediated lipid transport mechanism(s) (Lev, 2012). Each exo-/endocytic event delivers or removes a material equivalent to a surface area of ≈ 30.000 nm^2 (estimated for the average diameter of exo-/endocytic vesicles to be ∼100 nm). Therefore, every such event can transiently, but dramatically change the local membrane composition and, thereby, organization. Whereas no preferred sites of exo-/endocytosis were reported under resting conditions (Schmoranzer et al., 2000), stimulation of cells can result in more localized vesicular transport and fusion/fission hotspots (Stinchcombe et al., 2001; Gaffield et al., 2009). This can further accelerate changes in the plasma membrane.

Alternatively, membrane components (specifically lipids) can be delivered to the plasma membrane by **specific lipid transporters** (Raychaudhuri et al., 2006; Voelker, 2009; Tarling et al., 2013). These may travel through the cytosol by diffusion or, more probably, such events can take place at **membrane contact sites** between the endoplasmic reticulum (ER) and plasma membrane (see Figure 5 in Fernández-Busnadiego et al., 2015). These sites are responsible for the synthesis, transport of lipids between the ER and plasma membrane (e.g., by Osh sterol transporters Raychaudhuri et al., 2006) and regulation of lipid metabolism in the plasma membrane (Stefan et al., 2011).

Vesicular and protein-mediated transport are the two main mechanisms responsible for a rapid turnover of membrane molecules (estimated to exchange almost all of its components within 1 h), but other routes such as free diffusion of small molecules (e.g., glucose, ions Cortizo et al., 1990) or infection of cells by viruses and other pathogens (Mazzon and Mercer, 2014) can further modulate membrane composition and organization.

Active transport of protons and ions, together with chemical asymmetry, generates an electrostatic potential across the plasma membrane of living cells (Hodgkin and Huxley, 1952). In addition to the function of the **membrane potential** in metabolism and transport of essential molecules in and out of cells, it has an impact on properties of model and cell membranes (O'Shea et al., 1984; Grossmann et al., 2007; Herman et al., 2015). Even in the absence of ions, asymmetric distribution of lipids in the bilayer can generate a transmembrane potential (Gurtovenko and Vattulainen, 2007). As a consequence, it is technically challenging to uncouple membrane potential and asymmetry. Of note, the available theoretical and experimental evidence related to the electrostatic potential and the organization of cell membranes was recently reviewed (Malinsky et al., 2016).

On their own, extrinsic factors do not have the capability to control all plasma membrane processes. Hence, more holistic hypotheses combining intrinsic and extrinsic factors are needed. In the following section, we will briefly describe a more general concept and five most popular models. A reader will find more detailed descriptions of these models and some alternative views in recently published reviews (e.g., Lingwood and Simons, 2010; Owen et al., 2010; Klammt and Lillemeier, 2012; Klotzsch and Schütz, 2013; Nicolson, 2014; Rao and Mayor, 2014; Mouritsen and Bagatolli, 2015; Sevcsik and Schütz, 2016).

PLASMA MEMBRANE ORGANISATION–GENERAL MODELS AND CONCEPTS

Let us begin this section with a brief inspection of the mobility of membrane components. This will indicate how simple concepts

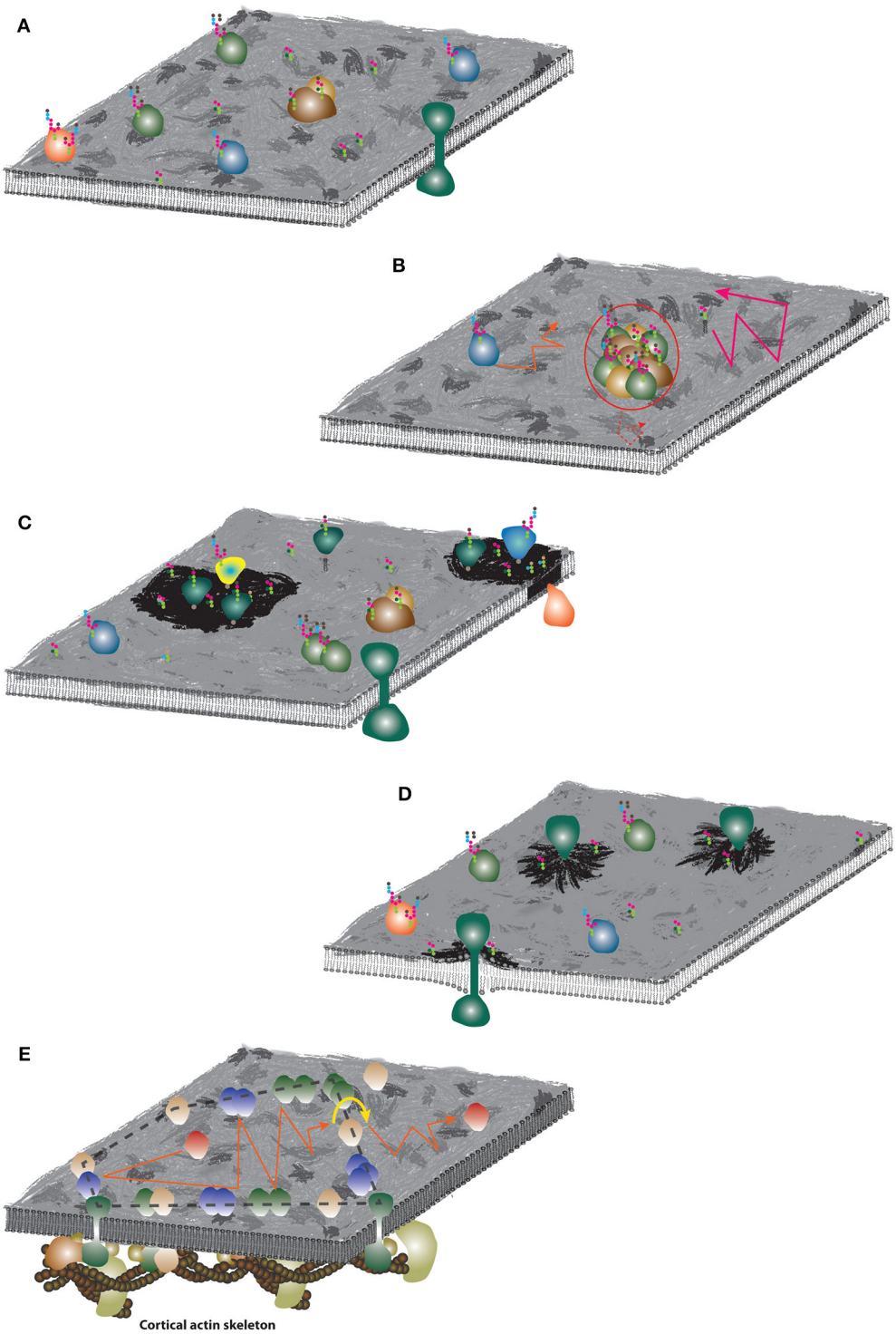

FIGURE 5 | Schematic illustrations of the plasma membrane organization models. (A) Fluid mosaic model. The membrane surface was artistically decorated to indicate non-homogenous distribution of molecules. Colored objects represent various species of membrane proteins, strings of colored hexagons illustrate glycosylation of proteins and lipids. **(B)** Hydrodynamic model. Similar mobility of lipids and proteins are indicated by orange and pink trajectories. Large assemblies (red circle) with significantly larger radius can exhibit slower diffusion (dashed red trajectory). **(C)** Lipid membrane domains. Dark membrane patches indicate lipid self-assemblies and different lipid (and protein) composition. **(D)** Mattress model. Dark membrane patches indicate accumulation of lipid species due to increased hydrophobic length of protein TMDs. **(E)** Picket-and-fence model. Accumulation of proteins around the underlying CA skeleton and formation of fences (dashed black line) which may restrict "free" diffusion of non-associated proteins (red) to a limited area (red trajectory). For long-distance mobility, proteins have to "hop" over the fence (yellow arrow-line) which limits their long-range diffusion coefficient.

highlighting intrinsic properties, namely viscosity and continuity can, to some extent, explain certain puzzles related to the plasma membrane organization and function. Measurements of lateral diffusion of membrane components over the last few decades uncovered much slower molecular mobility of molecules in cell membranes compared to their model counterparts (Wier and Edidin, 1986; Jacobson et al., 1987; Lippincott-Schwartz et al., 2001). On average, lipid tracers (e.g., DiI or BODIPY-DPPE) diffuse about four times faster in model membranes than in the plasma membrane of living cells (**Box 1**; **Table 1**). This difference can be explained by the compositional complexity of the plasma membrane. The large proportion of lipids with long and saturated acyl chains and cholesterol (van Meer et al., 2008) cause a higher rigidity (Sezgin et al., 2015) and, thereby, viscosity of membranes (Kucik et al., 1999). In addition, the presence of integral membrane proteins further increases the local viscosity in their immediate environment, which reduces the mobility of membrane constituents in general (Peters and Cherry, 1982; Chazotte and Hackenbrock, 1988; Frick et al., 2007; Saxton, 2008; Niemelä et al., 2010). A plethora of lipid-lipid and lipid-protein interactions, heterogeneities in general, can further contribute to this reduction in mobility.

Therefore, intrinsic properties, particularly viscosity, can be responsible for the reduced long-range diffusion rates measured for lipids in cell membranes. Since membranes are continuous, all of its lipid components should be influenced similarly and equally throughout the whole area. For lipids which do not comply with this statement, localization and mobility is regulated by other factors such as proteins interacting with charged lipid headgroups, endocytosis,... etc. This simple concept works for lipids. But the extremely slow mobility of many plasma membrane proteins—one-to-two orders of magnitude lower compared to model membranes–calls for a more elaborate explanation.

*Fluid Mosaic Model (SN Model; **Figure 5A**).* The SN model in a large detail summarizes the understanding of the plasma membrane composition, structure and thermodynamics 45 years ago (Singer and Nicolson, 1972). The emphasis is placed on the fluidity of the membrane and coexistence of lipids and proteins in this essential cellular structures. We have already described crucial issues of this model in the previous sections. Here, we would like to underline that the word "mosaic" in the SN model was primarily used to accent a mixed character of cell membranes where diverse lipids and proteins coexist in a single lamellar structure. Later, this was frequently misinterpreted as homogeneous or random distribution of molecules. But **heterogeneity** of cell membranes was observed and reported as early as in 1960s (**Figures 4A,B**; Cerottini and Brunner, 1967; Aoki et al., 1969; Kourilsky et al., 1971; Nicolson et al., 1971). Indeed, Nicolson described putative mechanisms responsible for clustering of proteins (or formation of domains) in his pillar work already in 1979 (Nicolson, 1979 and Figure 4 therein). These assumptions are still valid almost 40 years later (Nicolson, 2014).

*Hydrodynamic Model (**Figure 5B**).* The mobility of transmembrane proteins and their aggregates in cell membranes can be defined by the hydrodynamic model (Saffman and Delbrück, 1975). This model hypothesizes that molecular diffusion rates depend mainly on membrane viscosity and thickness, and only weakly on the size of proteins and aggregates. This model was later updated many times (e.g., for arbitrary viscosity of membranes and solutes

TABLE 1 | Examples of diffusion coefficients and their translation to the times needed to traverse a distance of 20 μm (e.g., HeLa cell).

Molecule and environment	Diffusion coefficient ($\mu m^2/s$)	Time to traverse 20 μm (Brownian diffusion; seconds)#	Reference(s)*
Small molecule (fluorescein) in water	430	0.2	Culbertson et al., 2002
Protein (GFP) in water	90	>1	Swaminathan et al., 1997
Small molecule (fluorescein) in cytoplasm	30	>3	Luby-Phelps et al., 1986
Protein (GFP) in cytoplasm (CHO cell)	30	>3	Swaminathan et al., 1997
Protein (GFP) in cytoplasm (bacterium)	8	12.5	Elowitz et al., 1999
Lipid tracer in fluid model membranes (DOPC; free-standing membrane)	5–15	1.6–20	Ramadurai et al., 2009
Lipid tracer in membrane blebs (cell membrane without cortical actin)	1–10	10–100	Tank et al., 1982
Lipid-anchored protein@ in fluid model membrane	5	20	Kahya et al., 2005
Integral membrane protein in fluid model membrane	2–5	20–50	Ramadurai et al., 2009
Lipid tracer in cell membrane	0.5–4	25–200	Tank et al., 1982
Lipid-anchored protein@ in cell membrane	0.1–1	100–1000	Zhang et al., 1991
Lipid-anchored protein@ in cell membrane blebs (without CA skeleton)	0.3–0.6	170–330	Zhang et al., 1991
Integral membrane protein in cell membrane blebs (CA skeleton-free)	0.01–0.5	200–10000	Tank et al., 1982
Integral membrane protein in cell membrane$	0.001–0.1	1000–100000	Tank et al., 1982

#The time to traverse the distance x was calculated as $\tau \approx x^2/4D$, where D denotes the diffusion coefficient.

$ Some membrane proteins can exhibit only small mobile fraction or have even slower D.

@GPI-anchored proteins were tested in cited works.

**Original articles listed only.*

(Hughes et al., 1981) or asymmetric membranes Evans and Sackmann, 1988), and experimentally confirmed in model membranes (e.g., Ramadurai et al., 2009). However, it applies only for freely moving molecules absent of interactions with objects which do not co-diffuse as a single entity. The model is further limited by the density of objects in the membrane and their lipid environment. First, the presence of slowly moving obstacles and molecular crowding can strongly influence the mobility of membrane components (Saxton, 2008; Guigas and Weiss, 2015). Second, lipids in the vicinity of TMDs of integral membrane proteins (annular lipids or lipid shells) exhibit reduced lateral diffusion (Meier et al., 1987; Anderson and Jacobson, 2002). This is probably caused by the fact that TMDs form relatively large and rigid structure in the bilayer (Meier et al., 1987; Niemelä et al., 2010) but also due to the rough surface of TMDs. Therefore, the complexity of cell membranes evidently does not allow the application of hydrodynamic model or its variants as a general model of the plasma membrane organization. Nevertheless, it can provide a useful alternative to more complex models for local changes (nanoscale; see below).

*Self-Assemblies of Lipids and Ordered Lipid Domains (**Figure 5C**).* Observation of protein clusters (DePierre and Karnovsky, 1973), lipid segregation (Shimshick and McConnell, 1973a,b; Klausner et al., 1980) and heterogeneous distribution of certain lipids and proteins between apical and basal membranes of polarized cells (van Meer and Simons, 1982) led to the suggestions that lipids and their self-assemblies can determine the fate of newly synthesized or recycled membrane molecules (Karnovsky et al., 1982; Simons and van Meer, 1988). This concept was modified by Simons and Ikonen (Simons and Ikonen, 1997) who proposed "lipid rafts" as the plasma membrane platforms of high molecular order enriched in cholesterol and sphingolipids, in which proteins involved in signaling can selectively interact with effector molecules. In parallel, biochemical analyses revealed inefficient solubilisation of some, but not all, membrane proteins and lipids in mild detergents, forming the basis of detergent resistant membranes (DRMs). Throughout the years, the ordered lipid character of "model lipid rafts" was emphasized and suggested to correspond to domains present in the plasma membrane of cells. All these terms, lipid rafts, DRMs and ordered lipid domains, were used inconsistently and frequently led to misinterpretations which were highlighted in recent reviews (Cebecauer et al., 2009; Owen et al., 2010; Kraft, 2013; Sevcsik and Schütz, 2016). In addition, the data supporting spontaneous formation of lipid domains in living cells are rather controversial and inconclusive (e.g., Eggeling et al., 2009; Brameshuber et al., 2010; Owen et al., 2012; Honigmann et al., 2014; Sevcsik et al., 2015). On the other hand, an undisputable capacity of certain lipids (e.g., gangliosides) to self-aggregate (Fujita et al., 2007; Chen et al., 2008), anomalous diffusion and/or distribution of lipids in highly complex mixtures (Kusumi et al., 2005; Eggeling et al., 2009; He and Marguet, 2011; Jeon et al., 2012) and spontaneous formation of fluid nanoclusters (van Zanten et al., 2010; Amaro et al., 2016) were demonstrated *in silico*, in model membranes as well as in living cells. Such fluctuations can potentially contribute to the overall heterogeneity of the plasma membrane and the peculiar mobility of certain lipids and proteins therein. Yet, the direct observation of such anomaly remains challenging due the required spatial and temporal resolution to disclose molecular-scale objects at sub-millisecond rates, albeit recent advances in super-resolution optical microscopy and ultrafast single-molecule tracking indicate remedies to this limitation.

*Mattress Model (**Figure 5D**).* As mentioned above, lipids in the vicinity of TMDs exhibit abnormal behavior (Lee, 2004; Niemelä et al., 2010), particularly in cell membranes with a large variety of lipid species and TMDs. The average membrane hydrophobic thickness increases between the ER, Golgi apparatus and plasma membrane (Mitra et al., 2004). During protein translation, proteins with long TMDs are incorporated into the relatively thin membrane of the ER, causing hydrophobic mismatch. Lipids with longer and saturated acyl chains can form metastable shells surrounding such TMDs, thereby generating heterogeneity in the membrane of the ER. At a larger scale, hydrophobic mismatch was proposed to induce the formation of lipid/protein domains also in the plasma membrane (Mouritsen and Bloom, 1993; Anderson and Jacobson, 2002; Kaiser et al., 2011). Significant impact of hydrophobic mismatch is well-documented for the sorting of proteins in cell membranes (Munro, 1995; Sharpe et al., 2010; Chum et al., 2016). But whether similar "sorting" of lipids and proteins due to hydrophobic mismatch contributes to the nanoscale organization of the plasma membrane in living cells has so far not been experimentally proven, mainly due to aforementioned limitations on spatial and temporal resolution of potential direct observation methods.

*Cortical Actin Skeleton (**Figure 5E**).* Membrane-proximal positioning of the CA skeleton and its direct association with the plasma membrane via actin-binding proteins or complexes makes it the first-hand structure to influence the mobility of plasma membrane molecules and their lateral organization. Indeed, the actin skeleton was demonstrated to affect membrane molecules in numerous works employing a variety of experimental approaches (e.g., Golan and Veatch, 1980; Sheetz et al., 1980; Tank et al., 1982; Fujiwara et al., 2002; Ritchie et al., 2003; Murase et al., 2004; Mueller et al., 2011; Andrade et al., 2015). The effect of the CA skeleton is to date the most accepted model for membrane organization, independent of whether we speak about indirect sterical hindrance (picket-and-fence model; (Koppel et al., 1981; Jacobson et al., 1984; Sako and Kusumi, 1995; Machta et al., 2011) or direct interactions of proteins with the CA skeleton (Saxton, 1990; Sheetz et al., 2006; Mueller et al., 2011; Rao and Mayor, 2014). Its undisputable impact was described in more detail in current reviews (Kusumi et al., 2010; Rao and Mayor, 2014). On the other hand, the CA skeleton provides a

good explanation for many, but probably not all membrane-associated phenomena (see below).

THERE IS NO UNIVERSAL MODEL OF THE PLASMA MEMBRANE LATERAL ORGANISATION

Models listed in the previous sections, better or worse, contribute to the overall understanding how cells potentially organize molecules in their plasma membrane. Some of these models passed through their glorious periods, in which almost any article assumed the applicability of this one particular mechanism for the function and/or organization of the studied membrane molecule(s). A handful of recent experimental work (e.g., Kenworthy et al., 2004; Frisz et al., 2013; Honigmann et al., 2014; Letschert et al., 2014; Sevcsik et al., 2015; Wilson et al., 2015) and reviews (Kraft, 2013; Sevcsik and Schütz, 2016) argue against these universal theories. Improvements in technology for observing membrane studies have more and more reduced the affection for such a single, universal theory. A dynamic and complex plasma membrane is the environment where all molecules play in concert to achieve the optimal physiological output.

As a metaphor, one can think of human society. Similar to cell membranes, it is highly complex and dynamic, with activities difficult to investigate. As an example one can consider clustering. "Clustering" occurs in human society at the nanoscale (e.g., families), mesoscale (e.g., clubs, classes or other small interest groups), or macroscale (e.g., villages, cities, states). The formation of such "clusters" depends on intrinsic properties like affection or animosity, the local or global economic situation, but also the health and mobility of the individuals. As an analogy for extrinsic parameters we may consider the environmental situation (sunshine/rain, drought/flooding), local factors (alpine landscape vs. influence of the sea), but also the interaction with other "clusters." As we know from experience, social systems may develop rather stable phenotypes at the macroscale (e.g., the current western society), which are still characterized by high dynamics at the nano—or mesoscale. On the contrary, there are periods in history, in which no stable situation was reached for many years. Our point is, that it is virtually impossible to predict the behavior of a large society from simple models, even if the intrinsic and extrinsic parameters are well-known at high detail. Or, if we return to the topic of cell membranes: currently, it seems impossible to explain the plasma membrane organization based on individual models described in the previous section. Hence, future challenges will include the clever combination of this principle models into more holistic meta-models to increase their predictive power. Or, in the other words, we believe there is no simple, universal mechanism underlying the organization of the plasma membrane of mammalian cells.

Why we believe this is so? And what are the consequences?

Starting with the first question, one has to look at the sections with the lists of intrinsic and extrinsic factors influencing the behavior of molecules in the plasma membrane. Both, intrinsic and extrinsic factors are highly interconnected and can occur at the same time or, more probably, in rapid, sequential events. If intrinsic properties should be considered as rather general factors, to which all molecules must adapt, extrinsic factors may have more specific effects. Tuning of intrinsic properties (e.g., fluidity or viscosity) requires significant changes in molecular composition. This can rapidly occur locally (at the nanoscale) and transiently (sub-second), but would require substantial costs of energy to induce large-scale and more stable changes. On the contrary, extrinsic factors (e.g., the CA skeleton or glycocalyx) can affect larger surface areas for longer periods of time with higher efficiency. It is, therefore, probably a combination of these factors which regulates behavior of molecules in the plasma membrane at a full spectrum of spatial and temporal scales.

This brings us to the second question about the consequences of the non-existence of omnipotent, universal model applicable to all plasma membrane components and events. First, when interpreting data acquired during the analysis of cell membranes, one should not ignore intrinsic membrane properties. Even though less visible (detectable), these form the basis of membrane organization and function. Extrinsic factors are important but may be consequential. In order to fully understand membrane-associated processes and avoid undesirable borders of a single theory, a careful analysis of sequential events, which may lead to the observed effect, needs to be performed (**Box 2**).

Another concern with the interpretation of membrane-focused data is the systemic use of chemical and genetic tools as a proof of one or the other model of the plasma membrane organization. Specific side-effects of some of these treatments (e.g., detergents, methyl-β-cyclodextrin, cytochalasin D or temperature changes) have been described in past (Ailenberg and Silverman, 2003; Lichtenberg et al., 2005; Magee et al., 2005; Shvartsman et al., 2006; Zidovetzki and Levitan, 2007). Due to the fluidity and cooperativity, systemic treatment (both, chemical and genetic) will often influence the behavior of many (if not all) molecules present in or associated with the membrane, instead of only specific ones. In addition, the procedure of observing the system may potentially introduce artifacts, for example labels or intense light sources employed in fluorescence microscopy (Sezgin et al., 2012; Magidson and Khodjakov, 2013). Therefore, employment of treatments or observation techniques requires cautious interpretation and experiments performed with extensive number of controls. Leaving space for alternative interpretations and emphasis on possible side-effects should be a good practice in this kind of works.

In summary, we provide here a comprehensive list of membrane features and peripheral structures which were previously demonstrated or proposed to control lateral mobility and organization of the plasma membrane in mammalian cells. We also offer alternative views how to interpret results measured on the plasma membrane of living cells. We re-emphasize the impact of the intrinsic membrane properties which were discovered and characterized more than 20 years

BOX 2 | SEQUENTIAL EVENTS INFLUENCING THE PLASMA MEMBRANE ORGANISATION

Imaging techniques are excellent tools to monitor changes of the plasma membrane organization. High details can be explored using current advanced techniques (Eggeling, 2015). But all methods suffer from the fact that the preeminent feature (e.g., CA skeleton reorganization) can hide one or more less well detectable events (e.g., changes in local viscosity) accompanying an observed process. In some cases, these undetectable fluctuations may be the determining factors or triggers of a transformation process.

To illustrate the consequences of the abovementioned limitation(s), let's imagine a putative membrane-associated process: A ligand binds to its receptor which is followed by receptor oligomerisation or nanoscale clustering. Such increased protein density causes increased membrane viscosity which, in turn, reduces mobility of molecules in the vicinity of a cluster (Peters and Cherry, 1982; Niemelä et al., 2010). As a consequence, an actin-binding protein can collide with a receptor cluster, enhance low-affinity interactions by crosslinking cluster components and trigger CA skeleton reorganization. It is the last event which will stabilize the overall structure and, at the same time, it is the best detectable feature of this imaginary process. But the interpretation that the CA skeleton is responsible for the observed changes is only part of the story. In this case, the ability to detect small-scale viscosity fluctuations would help to better understand such process. Unfortunately, at present, such tools are not available for living cells.

ago but were sometimes overlooked in more recent works. We finish with the hope that development of novel improved observation techniques such as fast single-molecule tracking (Ritchie et al., 2005; Ortega-Arroyo and Kukura, 2012), TOCSSL (Brameshuber et al., 2010), STED-FCS (Eggeling et al., 2009; Mueller et al., 2013; Eggeling, 2015), iMSD or related image correlation techniques (Hebert et al., 2005; Digman et al., 2009; Di Rienzo et al., 2013), will be rewarded with a more precise information about players responsible for the uniqueness of the plasma membrane. In case the improvements will be still insufficient, we should probably overpass the barrier (obstacle) between researchers studying mammalian cells and those focused on yeasts and plants. These organisms own membranes which behave much friendlier on temporal scale compared to the plasma membrane of mammalian cells. Such membranes are highly heterogeneous and can be imaged with the use of existing methods (Malínská et al., 2003; Spira et al., 2012). Cell cycle regulation and RNA interference were also discovered in yeast and plants.

AUTHOR CONTRIBUTIONS

JBdlS, GS, CE, and MC defined the topic and wrote the manuscript.

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REVIEW
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Interleaflet Coupling, Pinning, and Leaflet Asymmetry—Major Players in Plasma Membrane Nanodomain Formation

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The plasma membrane has a highly asymmetric distribution of lipids and contains dynamic nanodomains many of which are liquid entities surrounded by a second, slightly different, liquid environment. Contributing to the dynamics is a continuous repartitioning of components between the two types of liquids and transient links between lipids and proteins, both to extracellular matrix and cytoplasmic components, that temporarily pin membrane constituents. This make plasma membrane nanodomains exceptionally challenging to study and much of what is known about membrane domains has been deduced from studies on model membranes at equilibrium. However, living cells are by definition not at equilibrium and lipids are distributed asymmetrically with inositol phospholipids, phosphatidylethanolamines and phosphatidylserines confined mostly to the inner leaflet and glyco- and sphingolipids to the outer leaflet. Moreover, each phospholipid group encompasses a wealth of species with different acyl chain combinations whose lateral distribution is heterogeneous. It is becoming increasingly clear that asymmetry and pinning play important roles in plasma membrane nanodomain formation and coupling between the two lipid monolayers. How asymmetry, pinning, and interdigitation contribute to the plasma membrane organization is only beginning to be unraveled and here we discuss their roles and interdependence.

Keywords: membrane nanodomains, molecular pinning, liquid ordered domains, interleaflet coupling, membrane asymmetry, lipid interdigitation, phospholipid distribution

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MEMBRANE ASYMMETRY

Already in the early 1970's it was known that the human erythrocyte membrane displays leaflet asymmetry in the phospholipid composition (Bretscher, 1972; Verkleij et al., 1973) with most phosphatidylcholine (PC) and sphingomyelin (SM) present in the outer leaflet, whereas phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) are in the inner leaflet (**Figure 1**). This architecture has often been regarded as a prototype of the plasma membrane of mammalian cells.

In recent years methods to produce asymmetric phospholipid membranes have been developed and together with sophisticated molecular simulation techniques, they have provided compelling evidence that lipids in one leaflet of the membrane can influence molecular diffusion and domain formation of lipids in the other leaflet without protein intervention, i.e., interleaflet coupling. In

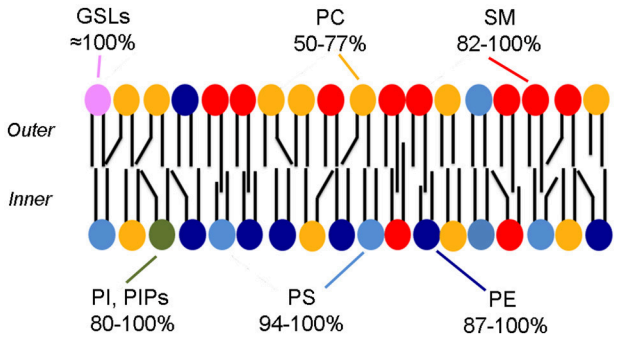

FIGURE 1 | Phospholipid asymmetry in the erythrocyte membrane. The colors indicate the asymmetric distribution of phospholipids. The range in percentages indicates the amounts present in the preferred leaflet [all but the GSLs (Lingwood, 2011) from Table 1 from Zachowski (1993)]. Note that most phospholipids are also likely to be present in the less favored leaflet, albeit in a small amounts. Cholesterol is also a major component of the membrane but is not shown.

these studies, model membranes with perfect phospholipid distribution asymmetry are often used, for example with PC and/or SM in one of the leaflets and PS, PE, and/or PI confined to the opposite leaflet. However, in natural membranes, no phospholipid species is likely to distribute exclusively to only one leaflet and also small "imperfections" in asymmetry might have a significant impact in some membrane properties as we discuss below.

To better understand the properties of natural membranes, it is important to establish the degree of asymmetric phospholipid distribution. We will therefore summarize what is known about the distribution of individual lipids in the plasma membrane leaflets of mammalian cells. Here we have to emphasize two points. The first is the divergence between cell types: the most well-studied human erythrocyte membrane and the plasma membrane of other cells. The second is the degree of asymmetry: an asymmetric distribution of a particular lipid means that the distribution between the two leaflets is not 50:50. It could be 60:40, but it could also be nearly 100:0, and the distribution or any change thereof is likely to influence membrane properties. Using the lipid distribution data from natural membranes, we should be able to generate realistic models to explain the role of the asymmetric lipid distribution in cellular processes taking place in membranes.

LIPID ASYMMETRY IN THE PLASMA MEMBRANE

Phosphatidylcholine

PC in the human erythrocyte membrane is predominantly found in the outer leaflet, and the proportion of PC in the outer leaflet was estimated to be 76–78% (Verkleij et al., 1973; van Meer et al., 1981). This level of asymmetry does not appear to exist in other cell types or even in erythrocytes of other species. For example, the proportion of PC in the outer leaflet of mouse, rat, and monkey erythrocyte membranes was reported to be 50% (Rawyler et al., 1985), 62–63% (Renooij et al., 1976; Crain and Zilversmit, 1980), and 67% (Van der Schaft et al., 1987). The PC distribution was examined by biochemical methods, utilizing covalent binding of membrane-impermeable reagents (Gordesky and Marinetti, 1973; Whiteley and Berg, 1974), enzymatic digestion (Verkleij et al., 1973), or use of phospholipid exchange proteins (Barsukov et al., 1976), but these methods are not appropriate for accurate measurement of asymmetry (Op den Kamp, 1979; Etemadi, 1980; Zachowski, 1993). Nevertheless, the divergent results from erythrocytes of different species were obtained by similar methods, suggesting that the PC distribution in non-human erythrocytes may not show such extreme asymmetry across the plasma membrane leaflet as that found in human erythrocytes.

More recently, freeze-fracture replica labeling EM utilizing metabolic labeling with a clickable choline analog indicated that PC exists in equivalent amounts in both leaflets of the plasma membrane in cells other than erythrocytes (Iyoshi et al., 2014). In contrast, studies applying an anti-PC antibody to freeze-fracture replicas showed predominant labeling in the outer leaflet, but the capture ratio was extremely low (Fujimoto et al., 1996; Murate et al., 2015), at least partially because the antibody preferentially detected only some PC subpopulations (Nam et al., 1990).

Although the exact ratio of PC in the two leaflets is not clear, the bulk of the data suggests that more than 20% of PC could exist in the inner leaflet of the erythrocyte membrane. In the plasma membrane of other cell types, the proportion of PC in the inner leaflet may be even higher.

Aminophospholipids

Both PS and PE were initially thought to be confined to the inner leaflet of the human erythrocyte membrane, and a similar asymmetry was assumed to be a general property of all plasma membranes. Lack of cell surface binding of PS-specific annexin V (Koopman et al., 1994) and PE-specific Ro09-0198 (Emoto et al., 1996) in normal interphase cells further strengthened the assumption. However, the result with annexin V and Ro09-0198 only suggested that the level of PS and PE in the outer leaflet is below a threshold level, and cannot necessarily be equated with their complete absence. Actually, biochemical studies indicated that a sizable fraction of PS and PE exists in the outer leaflet, and moreover, that the proportion in the outer leaflet is highly variable among different cell types, 0–44% (PS) and 0–73% (PE) (it is generally higher for PE than PS; see tables in Devaux, 1991; Zachowski, 1993 for summary of results). Although the methods used in those studies may not be accurate or quantitative (Op den Kamp, 1979; Etemadi, 1980; Zachowski, 1993), it is difficult to explain the divergent results by the methodological insufficiency alone. We presume that non-negligible amounts of PS and PE distribute to the outer leaflet of the plasma membrane in most cells.

Phosphatidylinositol and Phosphoinositides

PI is also assumed to exist largely in the inner leaflet of the plasma membrane, but biochemical studies indicated that PI may also be present in the outer leaflet in the human erythrocyte

membrane as well as in the outer plasma membrane leaflet of several nucleated cell types (Rawyler et al., 1985; Bütikofer et al., 1990; Gascard et al., 1991). The lack of a PI specific probe makes it difficult to confirm the above result microscopically, but several phosphoinositides could be labeled at the cell surface by applying membrane-impermeable probes (Gascard et al., 1991; Kale et al., 2010). This suggests that inositol phospholipids are present in the outer leaflet of the plasma membrane as physiological constituents.

Sphingomyelin

The proportion of SM in the outer leaflet of the human erythrocyte membrane was reported to be 80–85% (Verkleij et al., 1973) and 79% (van Meer et al., 1981). In contrast to PC, erythrocytes of other species also showed a similar or even higher fraction of SM in the outer leaflet (Renooij et al., 1976; Crain and Zilversmit, 1980; Rawyler et al., 1985; Van der Schaft et al., 1987). Also in other cell types, the proportion of SM in the outer leaflet was generally shown to be higher than that of PC (see tables in Devaux, 1991; Zachowski, 1993 for summary of results). Nevertheless, it is notable that a significant proportion of SM, e.g., 10–20%, is found in the inner leaflet when biochemical methods are used. In addition, freeze-fracture replica labeling EM also showed labeling of the inner leaflet with an SM-binding toxin, lysenin (Murate et al., 2015). Quantitativity and specificity of the lysenin labeling in freeze-fracture replica needs to be rigorously tested, but the result is consistent with the presence of SM in the inner leaflet of the plasma membrane.

Glycosphingolipids

Glycosphingolipids (GSLs) are generally believed to exist only in the outer leaflet. Glucosylceramide and galactosylceramide start their synthesis at the cytoplasmic side of the ER and the Golgi, and are then flipped to the luminal side for further glycosylations. Complex GSLs like gangliosides are not likely to flip back to the cytoplasmic leaflet because of their bulky hydrophilic headgroup, but short carbohydrate chain GSLs can translocate across the membrane (Buton et al., 2002). Therefore, the presence of a small amount of GSL in the inner leaflet of the plasma membrane cannot be excluded.

Cholesterol

Despite the development of new methodologies (Frisz et al., 2013; Solanko et al., 2015), neither the lateral distribution of cholesterol nor its distribution across the two plasma membrane leaflets is well-characterized (Marquardt et al., 2015). A polyene antibiotic, filipin, has been used frequently to visualize endogenous cholesterol distribution both by fluorescence microscopy and EM. Filipin has been reported to probe also GM1 (Arthur et al., 2011), but given that 5–7 mol% cholesterol required for filipin visualization in membranes (Behnke et al., 1984) and the scarcity of GM1 this should not be a problem in cell staining. However, membrane deformation caused by filipin-cholesterol complex formation does not indicate in which leaflet of the membrane cholesterol exists. As an alternative, analogues with various fluorescent tags have been used, but none of them give satisfactory results, because the presence of large tags significantly changes the molecular property of cholesterol, in particular the quick flip-flop between the two leaflets (Klymchenko and Kreder, 2014; Sezgin et al., 2016).

Dehydroergosterol, that is very similar to cholesterol in the molecular structure, is considered to be the best fluorescent analog. Its optical properties are not favorable for imaging, but a study using dehydroergosterol showed its enrichment in the inner leaflet (Mondal et al., 2009), which is in accordance with theoretical considerations (Giang and Schick, 2014; Falkovich et al., 2016). In contrast, preferential distribution of cholesterol to the outer leaflet of the human erythrocyte membrane was shown by biochemical analysis of a phospholipid monolayer obtained by freeze-fracture (Fisher, 1976). It is not clear whether the disparity between the two studies is derived from difference between the cell types examined, methodology, or both.

Acyl Chains

The acyl chain composition of phospholipids is thought to be a critical factor to determine properties of individual membrane leaflets, but our knowledge of this aspect is even less complete than that of the head groups. Classical work using thin layer chromatography and phospholipase hydrolysis suggested that the proportion of saturated and unsaturated acyl chains is highly variable between different phospholipid species and also between different tissues (Yabuuchi and O'Brien, 1968; Wood and Harlow, 1969). Tandem mass spectrometric analysis confirmed the tissue-to-tissue variation, but it also indicated that PS, PE, and PI tend to have a higher proportion of polyunsaturated acyl chains than PC, which is enriched with mono- and di-unsaturated acyl chains (Hicks et al., 2006).

It is largely unknown how phospholipids with different acyl chain compositions distribute two-dimensionally in the plasma membrane. Notably, PC of particular acyl chain compositions were shown to have heterogeneous distributions along the neuronal axon both when assessed by imaging mass spectroscopy (Yang et al., 2012) and by use of a unique monoclonal antibody (Kuge et al., 2014). But with these methods, it is not easy to distinguish acyl chains in one leaflet of a membrane from another. Freeze-fracture may be one of a few possible ways to separate the two leaflets, but has not yet been used for analysis of the acyl chain composition.

Ordered Membrane Nanodomains in the Cytoplasmic Plasma Membrane Leaflet

Currently the co-existence of two liquid phases, liquid ordered (lo)- and liquid disordered (ld), is the best explanation for domains of differential lipid packing observed in the plasma membrane although the perfect proof to characterize them as phases is almost as challenging to obtain as the absolute evidence for their dismissal. This neither means that proteins or lipids could not also show local enrichment unrelated to differential lipid packing, that plasma membrane domains need to be in the micron range nor that cellular membranes are at equilibrium (Ackerman and Feigenson, 2015; Mouritsen and Bagatolli, 2015).

An outstanding question in the membrane nanodomain field is whether lo-domains can form in the lipid mixes present in the inner leaflet of the plasma membrane. From model membrane

studies it is clear that the lipid mixes mimicking the outer plasma membrane leaflet (PC, SL, and cholesterol), can form co-existing ld- and lo-domains (Ahmed et al., 1997; de Almeida et al., 2003; Veatch and Keller, 2005). The main phospholipids in the inner leaflet (PE, PS, and PC) can only form ld-phase even in the presence of cholesterol (Wang and Silvius, 2001; Kiessling et al., 2006). In fact, when the acyl chains of PE, PS, and PC are mostly unsaturated, as they are *in vivo*, lipid mixes with a high PE content do not even form a bilayer but form a hexagonal or cubic phase (Boni and Hui, 1983) and it was recently proposed that the high bending free energy of PE is what attracts cholesterol to the inner leaflet (Giang and Schick, 2014). Despite the failure of symmetric PE/PC/PS lipid mixes to sometimes form bilayers, they do make up one half of the cellular plasma membrane highlighting that effects of asymmetric lipid compositions cannot be predicted from studies of symmetrical model membranes. Moreover, the difference in the membrane order of lo- and ld-domains in most model membrane studies is far greater than that possible in the plasma membrane considering its lipid composition, making predictions of probe, lipid, and protein partitioning in the plasma membrane from such studies of limited use.

In model membranes lo-domains can be characterized biochemically by their insolubility in non-ionic detergents, Triton X-100 (TX) being the most widely used, that when used at 4°C produces detergent resistant membranes (DRMs) that float in sucrose density gradients. The amount of TX-DRMs reflects the fraction of lo-domains in the membranes (Ahmed et al., 1997) and the lipids retrieved in TX-DRMs can form lo-phase (Schroeder et al., 1994). In cells, the relationship between DRMs and lo-domains is more tenuous (Lichtenberg et al., 2005; Ashrafzadeh and Parmryd, 2015; Sevcsik and Schütz, 2016). TX-DRMs from T cells are gigantic rather than nanosize (Magee and Parmryd, 2003) and proteins normally resident in organelles other than the plasma membrane appear in T cell TX-DRMs (von Haller et al., 2001). However, TX-DRMs do suggest that lo-domains in the two plasma membrane leaflets are coupled since they are enriched in both sphingomyelin likely to originate from the outer leaflet and saturated glycerophospholipids probably originating from the inner leaflet relative to both total cell and plasma membrane lipids (Fridriksson et al., 1999). Moreover, TX-DRMs contain acylated proteins that in intact cells are anchored to the inner plasma membrane leaflet indicative of, but not evidence for, inner leaflet lo-domains (Melkonian et al., 1999).

Several other lines of investigation indicate that lo-domains could exist in the inner plasma membrane leaflet and highlight that leaflet asymmetry changes the behavior of monolayers. In molecular dynamics (MD) simulations of asymmetric bilayers, lo-domains in one leaflet can induce registered ordered domains in an opposing leaflet with a composition that on its own would not allow lo-domain formation (Perlmutter and Sachs, 2011; Polley et al., 2014). MD simulations and theoretical considerations have also suggested the opposite possibility that membrane fluidisation in one leaflet may cause a decrease in the order of or even prevent phase separation in the opposing leaflet (Wagner et al., 2007; Sun et al., 2015). In asymmetric supported bilayers, lo-domains on the supported side can induce lo-domains in the free side with inner leaflet lipid mixes that only form ld-phase on their own (Kiessling et al., 2006), a process that was later shown to require acyl chain diversity among the inner leaflet lipids (Wan et al., 2008). In black membranes it has also been demonstrated that ld and lo phase separation in one leaflet can induce phase separation in an opposing ld-phase lipid mix and moreover that ld and lo phase separation can be suppressed by ld-phase lipid mixes (Collins and Keller, 2008). Also in asymmetric vesicles, produced by lipid exchange, registered lo-domains can be induced in a ld lipid mix by an opposing ld and lo phase separated bilayer (Lin and London, 2015) and, conversely, a gel phase becomes less ordered than that in symmetric bilayers when facing ld-phase (Heberle et al., 2016). However, direct evidence of the existence of inner plasma membrane leaflet lo-domains is still missing.

THE REGISTRATION OF MEMBRANE NANODOMAINS IN THE TWO LEAFLETS

The aggregation of registered domains containing non-transmembrane signaling molecules could facilitate signal transduction across the plasma membrane by separating deactivating molecules from their substrates (Simons and Toomre, 2000; Magee et al., 2005; Mongrand et al., 2010), but whether domains in cell plasma membranes are registered is largely unknown. When ld- and lo-domains co-exist in both leaflets of a bilayer, they can adopt two strategies to minimize the energy cost of the domain thickness mismatch. Domains could either be registered to minimize the contact area between the two phases, or anti-registered to minimize the difference in thickness over the bilayer (**Figure 2**). Note that complete anti-registration is only possible when each domain type occupies 50% of the total membrane distributed at any fractional division between the two leaflets. Since the proportion of plasma membrane lo- and ld-domains in live cells is not fixed (Mahammad et al., 2010; Owen et al., 2012; Dinic et al., 2013; Golfetto et al., 2015), domain registration is the more likely scenario. Moreover, the likely similarity in lipid composition of plasma membrane ld- and lo-phases favors their registration (Fowler et al., 2016).

Contributing factors to domain registration could be transmembrane proteins, lipid interdigitation, membrane curvature, line tension, cholesterol flip-flop, and electrostatic interactions (Nickels et al., 2015) of which chain interdigitation

FIGURE 2 | Domain registration across the bilayer. Interleaflet interactions favor domains in the two leaflets to be registered **(A)**, whereas hydrophobic mismatch favors domain antiregistration **(B)**, which serves to even the membrane thickness.

has been invoked as the major contributing factor (May, 2009) but also has been totally dismissed (Collins, 2008). A recent experimental determination of the strength of the coupling parameter that causes domain registration matches a theoretical predictions with values around 0.01 k_BT/nm^2 (Putzel et al., 2011; Blosser et al., 2015), but considerably higher values have also been suggested from theoretical considerations (May, 2009). Intriguingly, MD simulations of polymers have shown that forced splitting of registered lo-domains make them move toward one another long before they are in contact, i.e., they sense each others presence through the ld-phase (Pantano et al., 2011). This suggests that coupling between lipids is substantial and in symmetric bilayers the energy involved in the coupling has been estimated to be around 100 cal/mol of phospholipid (Zhang et al., 2007).

Recently, it has been argued both that line tension alone (Galimzyanov et al., 2015) or the competition between line tension and leaflet coupling determines domain registration (Williamson and Olmsted, 2015). However, line tension in the plasma membrane is likely to be small due to the wide range of lipid species available to smoothen any hydrophobic mismatch—a diversity that would also result in a high similarity in the composition of co-existing ld- and lo-domains. Thus the lipid diversity and as well as the asymmetric lipid distribution need to be incorporated into both theoretical and model membrane studies. In their absence the results are less physiologically relevant.

Registration of lo-domains in asymmetric bilayers has been described in MD simulations, supported bilayers, black membranes and vesicles (Collins and Keller, 2008; Wan et al., 2008; Cheng and London, 2011; Chiantia et al., 2011; Perlmutter and Sachs, 2011; Polley et al., 2014), indicating that registration is the favored organization also in cells. Of note is that domain coupling in supported bilayers is dependent on distancing the membrane from the support (Garg et al., 2007).

We pioneered cell studies on the registration of plasma membrane nanodomains between leaflets by using a probe that exclusively report on the membrane order of the outer plasma membrane leaflet. We found that decreasing the level of actin filaments attached to inner plasma leaflet lipids resulted in a lower fraction of lo-domains in the outer leaflet (Dinic et al., 2013). The implication of our results is that a direct effect on one plasma membrane leaflet is communicated to the other without the involvement of transmembrane proteins.

SIZE OF DOMAINS

When interleaflet coupling is studied in liposomes and supported lipid bilayers, domains of 1 μm or larger are usually examined, but such large domains are not likely to exist in the plasma membrane of most mammalian cells (Lingwood and Simons, 2010; Ashrafzadeh and Parmryd, 2015). Actually, studies using single particle tracking indicated that a transient domain as small as 10 nm can transmit signals to the cytoplasm to induce physiological reactions (Suzuki et al., 2007a,b). Therefore, in order to study physiological importance of domain registration in the plasma membrane, domains in the small size range need to be examined.

This is a challenging task, however, considering the methods currently available. For example, GFP-tagged lipid-binding proteins are commonly used as probes to define the lipid distribution by fluorescence microscopy. But when one probe molecule binds to the head group of a target lipid, which should be in the range of ∼1 nm in diameter, binding of another probe molecule to adjacent target lipids is precluded due to steric hindrance. This limitation is not derived from the limitation in spatial resolution of microscopes, but from the large size of probes compared to lipid head groups, so that use of super-resolution microscopes is not helpful (for other potential problems of the method, see Takatori et al., 2014). Interferometric detection of scattering circumvents several of the problems of particle tracking and has a lateral resolution in the low nm scale (Lindfors et al., 2004), but has not yet been applied to cells. Importantly tracking methods can, if cell topography is accounted for (Adler et al., 2010), detect anomalous diffusion caused by plasma membrane domain co-existence but do not reveal what is causing domain formation; for example differential lipid packing, protein concentration or charge aggregation.

In electron microscopy, colloidal gold particles of larger than 5–10 nm in diameter are often used as markers and one such gold particle may overlay many lipid molecules (Fujita et al., 2007; Zhou et al., 2014). Therefore, a cluster of colloidal gold labels indicates enrichment of the target lipid in the area, but does not necessarily indicate clustering of lipid molecules (although clustering of lipids is likely to occur more frequently when their local density is higher).

It is even more difficult is to examine whether domains in the two leaflets are registered. Use of complementary freeze-fracture replicas has enabled the examination of matching areas in the two leaflets (Hagiwara et al., 2005), but even with this method, the registration of small domains is difficult to study without a technical breakthrough.

CROSSING THE MID-LINE—A PRIVILEGE OF ASYMMETRIC LIPIDS WITH ONE LONG ACYL CHAIN

The lipid raft hypothesis states that small tightly packed lipid domains float in a sea of less tightly packed lipids and proposes that lipid rafts in the two leaflets are coupled by the interdigitation of long saturated acyl chains on glycosphingolipids in the outer leaflet extending into the inner leaflet (Simons and Ikonen, 1997). The proposed type of interdigitation was different from the initial use of the word for long acyl chains spanning the entire hydrophobic core of the bilayer made up of lipids with considerably shorter acyl groups (Boggs and Koshy, 1994; Schram and Thompson, 1995). Instead the now accepted meaning of interdigitation, long acyl chains crossing the midplane of the bilayer and penetrating a small distance into the opposing leaflet (**Figures 1**, **3**), was highlighted. This type of interdigitation is for instance

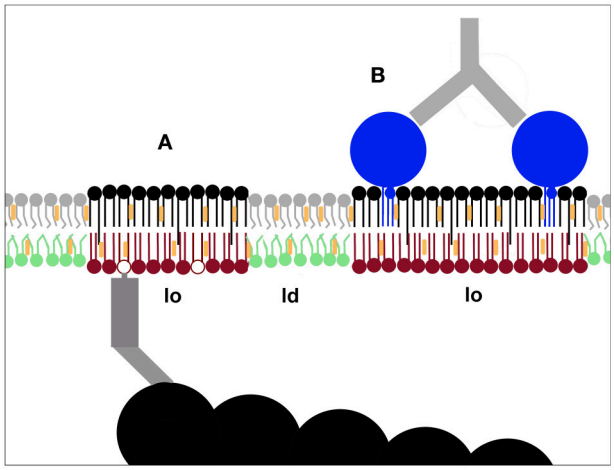

FIGURE 3 | Pinning as a mechanism to nucleate membrane nanodomains. Immobilising plasma membrane components can induce lo-domain formation. This has been demonstrated both for inner leaflet phoshoinositides linked to actin filaments **(A)** and outer leaflet components like GPI-anchored proteins **(B)** (Dinic et al., 2013). The creation of new lo-domains is likely to play an important role in the molecular sorting required for cellular processes such as signaling and membrane trafficking.

observed for long acyl chain (C24) glycosphingolipids when they are minor components in a biologically relevant (C16-C18) matrix (Mehlhorn et al., 1988; Morrow et al., 1995). Although the ideas brought together in the lipid raft hypothesis were not new, it had an enormous impact in promoting membrane research. Much of the polarized discussion in the field has been caused by the use of questionable methodology (Klotzsch and Schütz, 2013; Ashrafzadeh and Parmryd, 2015), but the existence of plasma membrane nanodomains with different lipid packing was suggested from studies that predate the lipid raft hypothesis by decades (Morrisett et al., 1975; Karnovsky et al., 1982). Although frequently misinterpreted, the 1972 fluid mosaic model does not rule out, but rather insists, that short-range order in the plasma membrane exists (Singer and Nicolson, 1972), an important feature that was recently clarified by one of the model founders (Nicolson, 2014).

Sphingolipids (SLs) can have a big difference in chain length between the sphingosine chain, that tends to be C18, and the amide-linked acyl chains, that differ from C16 to C24 in naturally occurring SLs, creating many asymmetric SL species. Unsurprisingly, MD simulations of symmetric bilayers have shown that the greater the length mismatch between the matrix lipid acyl chains and the greater the length mismatch between the acyl and the sphingosine chains, the more interdigitation is observed (Niemelä et al., 2006). Bending of long acyl chains toward their own leaflet is also a possibility and has recently been observed in both MD simulations of SLs and model membranes studies of free fatty acids in glycerophospholipid bilayers (Paz Ramos et al., 2016; Róg et al., 2016). However, bending toward the water interface can be greatly reduced by cholesterol revealed by a model membrane study on methyl distributions (Mihailescu et al., 2011). Interestingly, simulations show that coupling is stronger if there is more cholesterol in the outer than in the inner leaflet (Róg et al., 2016).

A recent simulation study on symmetrical membranes made up of asymmetric PCs with differences in their acyl chain lengths suggested that lipid complementarity in chain length could act to assure uniform acyl chain packing (Capponi et al., 2016). This is along the lines of previous lipid complementarity studies indicating that, when present at matching concentrations, short acyl chains are found opposite long ones to create a smooth membrane (Zhang et al., 2004, 2005; Stevens, 2005). That such an organization should occur in biological membranes, with diverse and asymmetric lipid compositions, is less clear, especially since domains of different order and thus presumably thickness are found in the plasma membrane (Gaus et al., 2003; Dinic et al., 2013).

That asymmetric lipids from both leaflets can affect coupling in asymmetric membranes was first demonstrated in GUVs where SM C24:0 was shown to reduce the diffusion of POPC far more than that of DOPC (Chiantia and London, 2012). A recent MD simulation study, with asymmetric membranes with compositions closely mimicking cell plasma membranes, supported this finding with another lipid class and found a stronger coupling firstly in asymmetric vs. symmetric membranes and secondly the strongest coupling when the inner leaflet contained PS 18:0/18:1 and the outer leaflet contained SM 24:0 (Róg et al., 2016). That PS 18:0/18:1 plays an important biological role was implied in a recent study revealing that PS is required for retaining cholesterol in the inner leaflet in cells and that PS 18:0/18:1 uniquely has the capacity to protect cholesterol in model symmetrical membranes from oxidation (Maekawa and Fairn, 2015). Intriguingly, GPI-anchor protein clustering was also shown to be dependent on inner leaflet PS but with long acyl chains, rather than a specific acyl chain combination, being the requirement (Raghupathy et al., 2015). This was interpreted as enabling interaction with the long acyl chains required for the GPI-anchored proteins to cluster allowing cross-talk of specific lipids across the bilayer midplane. In light of the study discussed above (Maekawa and Fairn, 2015), we speculate that the PS may have been required to maintain the cholesterol level in the inner leaflet at a level sufficient for the formation of lo-domains since clustered GPI-anchored proteins are found in such (Dinic et al., 2013) and cholesterol previously has been identified as important for their clustering (Sharma et al., 2004).

IMPERFECTION IN LIPID ASYMMETRY AND DOMAIN REGISTRATION

As discussed above, lipids have asymmetric distributions in the plasma membrane, but the asymmetry is not complete and non-negligible amounts of most lipids are likely to exist in the less-favored leaflet. Such imperfection in asymmetry, especially that of SM, could be of considerable importance for domain registration. In model membranes interleaflet coupling can occur with no (or a minimal amount of) SM in the inner leaflet (Wan et al., 2008; Visco et al., 2014; Lin and London, 2015).

However, if a small but significant amount of SM exists the inner leaflet, as suggested by biochemical and histochemical studies (Devaux, 1991; Zachowski, 1993; Murate et al., 2015), that SM could potentially contribute to lo-domain formation, which could make coupling with the outer leaflet lo-domains very efficient (Halling et al., 2008; Lönnfors et al., 2011). Naturally this SM-SM interaction mechanism does not exclude participation of other phospholipids in lo-domain formation and interleaflet coupling. Rather, the SM-based coupling may function as an initial seed to induce further changes by lateral interaction with other phospholipids, such as PS or inositol phospholipids.

PINNING

It has been shown repeatedly that the behavior of outer leaflet lipids and GPI-anchored proteins is influenced by actin dynamics (Fujiwara et al., 2002; Kwik et al., 2003; Goswami et al., 2008; Fujita et al., 2009; Mueller et al., 2011), but how actin in the cytoplasm can exert effects on the outer leaflet of the plasma membrane has been a longstanding enigma. Cell topography and the problematic interpretation of three dimensional data in 2D could explain at least some of this behavior (Adler et al., 2010), but pinning is also important for the communication between actin filaments and the outer leaflet.

Pinning, i.e., when the mobility of membrane components is either reduced or absent, alters the mixing entropy, which can alter the phase behavior and/or membrane organization (Putzel and Schick, 2009; Arumugam and Bassereau, 2015). Protein pinning can transmit the rearrangement of proteins from one leaflet to the other. This was first studied in erythrocyte ghosts where it was shown that the enclosure of antibodies to spectrin, a protein associated with the inner leaflet, caused the rearrangement of glycolipids and glycoproteins in the outer leaflet (Nicolson and Painter, 1973). Analogously, binding of lectins to the outer leaflet caused the rearrangement of spectrin (Ji and Nicolson, 1974). Lectins are also known to affect the physical properties of the plasma membrane (Evans and Leung, 1984). For lipids, pinning can nucleate either an ld-phase as shown in phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2]-containing giant unilamellar vesicles (GUVs) where actin polymerisation induced phase-separation (Liu and Fletcher, 2006), an lo-phase as shown in GUVs by GM1 cross-linking (Hammond et al., 2005) or upon adhesion (Gordon et al., 2008).

Simulations on critical point fluctuations have indicated that pinning of lipids to actin filaments could prevent large-scale phase separation (Ehrig et al., 2011; Machta et al., 2011) and earlier simulations suggested that membrane protein obstacles or pinning could have the same effect (Yethiraj and Weisshaar, 2007; Fan et al., 2010; Gómez et al., 2010), phenomena that were recently observed in model membranes (Honigmann et al., 2014; Arumugam et al., 2015). Critical point fluctuations may however be superfluous for phase separation since it can be achieved by coupling of mechanical forces like actin polymerisation to membrane composition (Sens and Turner, 2011). That pinning does prevent large-scale phase separation in the plasma membrane is supported by the observation that visible large-scale phase separation can be seen in giant plasma membrane vesicles (GPMVs) that do not contain cortical actin (Sengupta et al., 2008; Levental et al., 2011). Furthermore, membrane blebs in live cells, where the membrane connection to actin filaments has been lost (Charras et al., 2005), are structures in the μm range with a lower laurdan generalized polarization-value than that found in the bulk plasma membrane of the same cells, indicative of disordered lipid arrangement (Dinic et al., 2013). Since lo-domains form where actin filaments are pinned to the plasma membrane, blebs may contain ld-phase only and represent an example of microscopically visible domains in live cells alongside the remaining intermittently actin filament pinned, and from a lipid packing perspective, more heterogeneous plasma membrane of live cells.

In asymmetric model membrane studies the general assumption is that an outer-side-like lipid mix could induce domain formation in a cytoplasmic-side-like lipid mix that cannot phase separate on its own (Kiessling et al., 2006; Collins and Keller, 2008; Lin and London, 2015) but the opposite is less frequently considered. However, in cells phosphoinositides in the inner plasma membrane leaflet can be pinned to intracellular actin filaments and cause the formation of lo-domains in the outer plasma membrane leaflet (**Figure 3A**), demonstrating that there is an inside out communication for the lipid packing of the two leaflets (Dinic et al., 2013). Lo-domains can also form when outer leaflet components like GM1 and GPI-anchored proteins are patched (**Figure 3B**), demonstrating that the physical state of the plasma membrane is also influenced by the cell's extracellular environment (Dinic et al., 2013). That immobilization by pinning can cause the formation of lo-domains in live cells was thus demonstrated in 2013 (Dinic et al., 2013). Despite this a later prominent study using simulations and GPMVs concluded by speculating that pinning induced lo-domain formation might be possible in live cells, claiming it as a novel and original idea, reversing the usual process of observation following speculation (Raghupathy et al., 2015).

A link between GM1, pinning and interdigitation has recently been found in both simulations and model membrane studies (Spillane et al., 2014; Sun et al., 2015). It seems likely that the lo-domain formation observed in the plasma membrane after cross-linking outer leaflet components, described above, causes the pinned molecules to stretch and hence make contact with the inner leaflet lipids, both nucleating and stabilizing the domains. Pinning may also result in membrane curvature that affects both leaflets (Deverall et al., 2008). Although we are well-aware of both the importance and prevalence of membrane curvature in cells (Adler et al., 2010; Parmryd and Onfelt, 2013), we have chosen to limit the discussion in this review to other mechanisms of importance for membrane nanodomain formation.

PI(4,5)P2 is known to interact with many actin-binding proteins, regulating their activity and localization (Saarikangas et al., 2010) and may be the phosphoinositide that links changes in actin dynamics to the proportion of plasma membrane lo-domains (Dinic et al., 2013). Interestingly, although PI(4,5)P2 often contains a polyunsaturated acyl chain (McLaughlin et al., 2002), which might not be expected to

be found in lo-domains, PI(4,5)P2 is nevertheless enriched in plasma membrane lo-domains (Parmryd et al., 2003). A likely explanation for this apparent paradox is that the expectations arise from the behavior of lipids in well-separated ld- and lo-domains in model membranes that have an order difference impossible to achieve in the plasma membrane.

PS is also a candidate to mediate the actin-outer leaflet lipid coupling, as it is known to bind numerous cytoplasmic proteins (for a review, see Stace and Ktistakis, 2006), including actin-binding proteins (Cohen et al., 1986; Muguruma et al., 1995; Makuch et al., 1997). Actually, the mobility of PS in the plasma membrane has been shown to increase upon actin depolymerization (Kay et al., 2012; Zhou et al., 2014), indicating that PS may be constitutively bound to the actin meshwork or its movements confined by topographical features of the membrane (Adler et al., 2010). Binding between PS and actin-linking proteins is thought to occur electrostatically to the PS head group and thus should be independent of the acyl chain composition (Wood and Harlow, 1969; Hicks et al., 2006), but it is likely to be enhanced if PS is clustered.

Interestingly, the acyl chain composition of the different phospholipid species, especially PS, varies between tissues (Hicks et al., 2006). Both the efficiency of domain registration and interleaflet coupling may differ with acyl chain composition: with a stronger coupling, the inner leaflet should become more ordered and hence similar to the outer leaflet. How the acyl chain composition is regulated is not clear, but Land's cycle, remodeling inner leaflet phospholipids (Hishikawa et al., 2014), may be involved. The regulatory mechanism and the possible correlation between the acyl chain composition and interleaflet coupling warrant further studies.

OUTLOOK

Compared with amino acids and nitrogenous bases the lipid diversity is enormous and intriguing. Similarly, compared to proteins and nucleic acids, our knowledge of membranes is limited. Tools are now being developed to for instance unravel the roles of individual lipid species in cellular processes, to study the effect of leaflet asymmetry and to perform detailed examination of the lipid distribution within leaflets—all outstanding questions which require close collaborations of scientists from physical, chemical, and biological disciplines. We predict that within the next decade exciting interdisciplinary breakthroughs in the membrane biology field will unravel mechanisms of fundamental biological processes and that we will approach a consensus regarding the nature of plasma membrane nanodomains.

AUTHOR CONTRIBUTIONS

IP conceived the idea. IP and TF wrote the review.

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REVIEW
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Sphingolipid Organization in the Plasma Membrane and the Mechanisms That Influence It

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Sphingolipids are structural components in the plasma membranes of eukaryotic cells. Their metabolism produces bioactive signaling molecules that modulate fundamental cellular processes. The segregation of sphingolipids into distinct membrane domains is likely essential for cellular function. This review presents the early studies of sphingolipid distribution in the plasma membranes of mammalian cells that shaped the most popular current model of plasma membrane organization. The results of traditional imaging studies of sphingolipid distribution in stimulated and resting cells are described. These data are compared with recent results obtained with advanced imaging techniques, including super-resolution fluorescence detection and high-resolution secondary ion mass spectrometry (SIMS). Emphasis is placed on the new insight into the sphingolipid organization within the plasma membrane that has resulted from the direct imaging of stable isotope-labeled lipids in actual cell membranes with high-resolution SIMS. Super-resolution fluorescence techniques have recently revealed the biophysical behaviors of sphingolipids and the unhindered diffusion of cholesterol analogs in the membranes of living cells are ultimately in contrast to the prevailing hypothetical model of plasma membrane organization. High-resolution SIMS studies also conflicted with the prevailing hypothesis, showing sphingolipids are concentrated in micrometer-scale membrane domains, but cholesterol is evenly distributed within the plasma membrane. Reductions in cellular cholesterol decreased the number of sphingolipid domains in the plasma membrane, whereas disruption of the cytoskeleton eliminated them. In addition, hemagglutinin, a transmembrane protein that is thought to be a putative raft marker, did not cluster within sphingolipid-enriched regions in the plasma membrane. Thus, sphingolipid distribution in the plasma membrane is dependent on the cytoskeleton, but not on favorable interactions with cholesterol or hemagglutinin. The alternate views of plasma membrane organization suggested by these findings are discussed.

Keywords: sphingolipid distribution, plasma membrane organization, lipid domains, secondary ion mass spectrometry, SIMS, imaging

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INTRODUCTION

The plasma membranes of mammalian cells contain many different lipid species, but the distribution of sphingolipids within the plasma membrane and the mechanisms responsible for this organization are of particular interest. Sphingolipids function as structural components in cellular membranes, and they are metabolized to signaling molecules that modulate diverse cellular

processes, ranging from apoptosis (Herr et al., 1997; Carpinteiro et al., 2008; Yabu et al., 2015) to cytoskeletal reorganization (Bartke and Hannun, 2009; Milhas et al., 2010; Gandy et al., 2013; Adada et al., 2014). Regulation of sphingolipid metabolite signaling likely involves segregating the parent sphingolipid molecules within distinct plasma membrane domains, but the distributions of various sphingolipids within the plasma membrane are not well established. At present, the different subspecies within the sphingolipid family are known to vary in terms of their chemical properties, expression patterns, specific protein binding partners, and consequently, specialized functions (Hannun and Bell, 1989; Mutoh et al., 1995; Snook et al., 2006; Yu et al., 2011; Contreras et al., 2012; Fantini and Yahi, 2015; Prasanna et al., 2016). These divergent properties and functions may suggest that each sphingolipid subspecies is compartmentalized within a different region of the plasma membrane. Nonetheless, most studies have focused on just a few types of sphingolipid-enriched plasma membrane domains: lipid rafts and ceramide-rich domains.

The lipid raft is likely the most intensely studied sphingolipid domain that hypothetically exists in the plasma membrane. Lipid rafts are defined as small (<200 nm) and dynamic plasma membrane domains that are enriched with cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI)-anchored proteins (Pike, 2006; Lingwood and Simons, 2010; Nyholm, 2015; Levental and Veatch, 2016). Favorable interactions between the cholesterol and sphingolipids are widely thought to drive lipid raft formation, producing higher ordering within this domain than in the surrounding membrane (Simons and Ikonen, 1997; Rietveld and Simons, 1998). GPI-anchored proteins and some transmembrane proteins are postulated to have an affinity for the distinct chemical and physical environment within the lipid raft, which hypothetically promotes their association with these domains and interactions between the proteins within them (Simons and Ikonen, 1997; Lingwood and Simons, 2010; Levental and Veatch, 2016). Protein-protein interactions are proposed to stabilize the small and dynamic rafts, leading to the formation of larger structures (Harder and Simons, 1999; Nyholm, 2015; Simons, 2016). Lipid rafts are hypothesized to mediate many important cellular processes, including protein trafficking, signal transduction, and virus budding (Scheiffele et al., 1999; Nguyen and Hildreth, 2000; Simons and Toomre, 2000; Schuck and Simons, 2004; Ono and Freed, 2005; Luo et al., 2008; Takahashi and Suzuki, 2011). The postulated higher ordering of the sphingolipids, cholesterol, and proteins within lipid rafts was thought to make these putative domains insoluble in cold ionic detergents (Schroeder et al., 1994; Ahmed et al., 1997; Cremesti et al., 2002; Zajchowski and Robbins, 2002). Consequently, detergent extraction was once widely used to study lipid rafts. Detergent-resistant membranes isolated from cells later proved to be artificial structures that were not present *in vivo* (Lichtenberg et al., 2005). This increased the importance of imaging putative raft components, such as sphingolipids and GPI-anchored proteins, within intact cell membranes.

Ceramide-rich domains in the plasma membrane have also been the subject of many studies. These domains are produced by the hydrolysis of sphingomyelin to ceramide by sphingomyelinase in response to stimuli (i.e., multivalent binding to membrane receptors; Cremesti et al., 2001; Bollinger et al., 2005). Like lipid rafts, ceramide-rich domains are postulated to exhibit high ordering that enhances the recruitment of GPI-anchored proteins, which modulates their interactions with other membrane proteins (Cremesti et al., 2002; Bollinger et al., 2005). However, ceramide-rich domains are large enough to be detected with light microscopy, and they putatively lack cholesterol enrichment (Cremesti et al., 2002; Bollinger et al., 2005). In this review, ceramide-rich domains are defined solely according to their enrichment with ceramide, irrespective of their cholesterol or protein content.

The following sections describe the sphingolipid distributions that have been imaged in resting cells with a variety of techniques, and how these organizations are affected by various stimuli. Due to space limitations, this review focuses on reports that contextualize the development of current models of plasma membrane organization, and the results that that have led some to question or even reject the raft hypothesis (Shaw, 2006; Kenworthy, 2008; Kraft, 2013; Sevcsik and Schütz, 2016; Wüstner et al., 2016). Emphasis is placed on the findings acquired with a new approach for chemically mapping isotope-labeled lipids in the plasma membrane with high-resolution, which were reported by the author and collaborators. Finally, the implications of these findings on models of sphingolipid organization in the plasma membrane are discussed.

METHODS TO IMAGE SPHINGOLIPID DISTRIBUTION IN THE PLASMA MEMBRANES OF MAMMALIAN CELLS

In order to visualize the sphingolipids within the plasma membrane, they must be functionalized with a label that can be detected with an imaging technique. A variety of lipid probes and detection methods have been employed, each having distinct advantages and disadvantages. One of the most common strategies to date is to use an affinity tag, such as an antibody or toxin, to label the sphingolipid species of interest. Noteworthy, non-toxic recombinant versions of toxin molecules that retain their sphingolipid-binding properties have been developed to permit live-cell imaging without adversely affecting cell viability (Kishimoto et al., 2016). The affinity tag is usually conjugated to a fluorophore or heavy metal particle that can be visualized with fluorescence or immunoelectron microscopy, respectively. Alternatively, the affinity tag is labeled with a second affinity tag (i.e., a polyclonal antibody) that has been functionalized to permit detection. This approach is attractive because it enables attaching any desired detection probe to endogenous lipids on the cell surface. The main limitation is that only a fraction of the lipid molecules of interest can typically be labeled and detected with an affinity label. This low detection efficiency is primarily due to three factors. First, affinity labels often cannot access the entire cell surface due to their relatively large size; second, lipids that are already bound to endogenous proteins cannot be detected; third, affinity label binding often depends on the specific orientation and/or clustering of the target lipid (Mahfoud

et al., 2010; Mizuno et al., 2011; Kishimoto et al., 2016). Another disadvantage is that some anti-glycosphingolipid antibodies and the popular affinity label for GM1, cholera toxin subunit B, may also bind to glycoproteins, which compromises their ability to report the distribution of the target glycosphingolipid (Tonegawa and Hakomori, 1977; Blank et al., 2007; Day and Kenworthy, 2012; Wands et al., 2015).

The direct imaging of fluorophore-labeled sphingolipid analogs incorporated into the membranes of living cells has been gaining popularity. These fluorescent sphingolipid analogs are advantageous because they afford more flexibility in terms of fluorophore selection, and they can be employed for live cell imaging. Fluorescent sphingolipid precursors that permit observing the lipid distribution that results from biosynthesis and trafficking have also been developed (Peters et al., 2007; Kim et al., 2013). The main drawback to this approach is that the relatively large and chemically distinct fluorophore may alter the interactions between the labeled sphingolipid and other membrane components, which can change the lipid distribution in the membrane (Devaux et al., 2002; Maier et al., 2002; Shaw et al., 2006).

The sphingolipid distribution in the plasma membranes of intact cells has also been imaged with a high-resolution secondary ion mass spectrometry (SIMS) technique. High-resolution SIMS performed on a commercial instrument, the Cameca NanoSIMS 50, enables visualizing the distributions of metabolically incorporated stable isotope-labeled lipids in the plasma membranes of intact cells with better than 100 nm lateral resolution (Klitzing et al., 2013; Kraft and Klitzing, 2014). The principles of SIMS performed with a Cameca NanoSIMS 50 instrument have been previously described in detail (Boxer et al., 2009; Kraft and Klitzing, 2014). Therefore, the following description emphasizes the aspects of the technique that affect its application to imaging the lipid distribution in the plasma membranes of intact cells.

During NanoSIMS analysis, a cesium primary ion beam with a diameter of ∼70 nm is raster scanned across the surface of the cell. The molecules within the beam's focal area are fragmented into small pieces, and the charged particles, which are called secondary ions, are ejected from the surface (top 5–10 nm) of the sample. This shallow depth of secondary ion ejection minimizes the detection of secondary ions from intracellular membranes, thereby restricting the analysis to the plasma membrane. The high-yielding monoatomic and diatomic secondary ions are collected by a mass spectrometer that can discriminate between ions that have the same nominal mass but different isotopic or elemental compositions (i.e., $^{13}C^{14}N^-$ at 27.0059 and $^{12}C^{15}N^-$ at 26.9996 amu). The intensities of the secondary ions detected at each pixel reveal the elemental and isotopic composition at the surface of the sample. Because elemental composition cannot be used to distinguish between lipid species, the sphingolipids must be labeled with distinct stable isotopes to allow their identification with a NanoSIMS instrument. This is achieved by metabolic labeling with isotope-labeled lipid precursors (Klitzing et al., 2013).

The strengths and weaknesses of high-resolution SIMS are complementary to those of imaging affinity tagged or fluorophore-labeled lipids with fluorescence microscopy. The strengths are that the stable isotope labels do not change the labeled lipid's chemical structure or molecular interactions, so its intracellular trafficking and distribution are not perturbed. Additionally, because distinct stable isotopes can be selectively and metabolically incorporated into the majority of the cellular sphingolipids, most sphingolipid molecules within the plasma membrane can be detected. The primary disadvantage is that this technique is performed under ultrahigh vacuum (UHV), so the cells must be dehydrated prior to analysis. However, previous studies demonstrate that chemical fixation techniques that crosslink the proteins with glutaraldehyde and the lipids with osmium tetroxide (OsO_4) preserve the laminar structure of biological membranes and prevent lipid reorganization during sample dehydration and subsequent analysis (Stoechenius et al., 1960; Frisz et al., 2013b). Consequently, the NanoSIMS images acquired from chemically fixed cells represent snapshots of the lipid organizations that were present in the moments prior to fixation.

The following sections summarize some of the results that have been acquired with the aforementioned approaches. Studies that used fluorescence or immunoelectron microscopy to detect sphingolipid-specific affinity tags to probe the involvement of a specific type of sphingolipid domain in cell response to external stimuli (i.e., involvement of lipid rafts or ceramide-rich domains in receptor clustering) are presented first. Next, studies that employed affinity-labeled sphingolipids and fluorescent sphingolipid analogs to visualize the distributions of specific sphingolipid subspecies in the plasma membranes of unstimulated cells are described. This includes a brief account of the insights into plasma membrane organization that were acquired with super-resolution fluorescence techniques. Then the sphingolipid distributions that have been imaged in the plasma membranes of intact mammalian cells with high-resolution SIMS are summarized. Finally, the implications of these experimental results on our view of plasma membrane organization are discussed.

GLYCOSPHINGOLIPID REDISTRIBUTION INDUCED BY ANTIGEN CROSSLINKING

Antibody binding to proteins on the surfaces of lymphocytes was first reported to induce the crosslinked proteins to form clusters that eventually segregate into a large patch, or "cap" at one end of the cell in 1971 (Taylor et al., 1971). Subsequent reports showed this capping is inhibited by drug treatments that impair microtubules (De Petris, 1974), and it can be induced on any motile mammalian cell by crosslinking its surface antigens with multivalent ligands, such as antibodies (Bretscher, 1984). An early hypothesis for the crosslinking-induced capping of membrane proteins postulated that cell surface proteins are associated with cytoskeletal components that actively cluster the crosslinked membrane proteins in response to multivalent binding interactions (de Petris, 1977). This hypothesis predicts that crosslinking the glycosphingolipids that reside in the outer leaflet of the plasma membrane would

not induce capping because these glycosphingolipids are not in direct contact with the cytoskeletal components in the cytoplasm. This prediction motivated the earliest efforts to characterize sphingolipid distribution in the plasma membrane in response to antigen capping. In 1975, Revesz and Greaves tested the prediction by labeling the GM1 in the plasma membranes of immune cells with cholera toxin, crosslinking the toxin with horse anti-cholera serum, and then labeling with fluorescent anti-horse secondary antibodies for visualization. They found the fluorescently labeled and crosslinked GM1 redistributed into multimicrometer-scale caps on the surfaces of the immune cells (Revesz and Greaves, 1975). The same year, Craig and Cuatrecasas reported that solely the binding of fluorescently labeled cholera toxin to GM1 was sufficient to induce the formation of large GM1 clusters on the surfaces of rat lymphocytes (Craig and Cuatrecasas, 1975). Like the capping of proteinaceous antigens, GM1 capping was inhibited by metabolic poisons and drugs that inhibit microtubules and microfilaments (Craig and Cuatrecasas, 1975; Revesz and Greaves, 1975). The sensitivity to microtubule and microfilament inhibitors implied that GM1 capping was mediated by cytoskeletal components. This unexpected implication instigated concerns that cholera toxin crosslinks both GM1 and glycosylated membrane proteins, and the observed capping was orchestrated by the cytoskeletal components associated with the crosslinked membrane glycoproteins.

Subsequent studies confirmed that capping could be induced by crosslinking glycosphingolipids with multivalent ligands other than cholera toxin. Exogenous Forssman glycolipid, a neutral glycosphingolipid consisting of five monosaccharides, inserted into mouse thymocytes could be capped by labeling it with a monoclonal primary antibody and then crosslinking with secondary antibodies (Stern and Bretscher, 1979). This capping was inhibited by chemically fixing the cells prior to crosslinking with the secondary antibody, and consistent with prior reports, by treatment with metabolic poisons or inhibitors of microfilaments and microtubules (Stern and Bretscher, 1979). Antibody crosslinking of the Forssman glycosphingolipid and globoside, a neutral glycosphingolipid with four monosaccharides, induced their aggregation in the membranes of erythrocytes (Tillack et al., 1983). However, anti-glycosphingolipid antibodies were reported to have an affinity for glycoproteins (Tonegawa and Hakomori, 1977), so these findings did not dissuade concerns that the observed capping was actually induced by the crosslinking of cell surface glycoproteins.

Spiegel and coworkers performed similar studies using gangliosides functionalized with non-native haptens (i.e., fluorophores) or biotin that could be crosslinked with antibodies or avidin, respectively, to ensure that the glycosphingolipid crosslinker had no affinity for endogenous proteins. The crosslinking of these exogenously incorporated gangliosides in the membranes of lynphocytes induced the formation of large patches and caps (Spiegel et al., 1979, 1984; Spiegel and Wilchek, 1981). Interestingly, anti-rhodamine antibodies elicited the co-aggregation of both rhodamine-labeled gangliosides and Lucifer yellow-labeled gangliosides on lymphocytes that contained both labeled gangliosides. However, anti-rhodamine antibodies did not induce the capping of Lucifer yellow-labeled gangliosides on lymphocytes that lacked rhodamine-labeled gangliosides (Spiegel et al., 1984). These experiments clearly demonstrate that capping can be induced by the crosslinking of glycosphingolipids, and also suggest that different gangliosides interact with one another within the plasma membrane.

The finding that metabolic poisons and inhibitors of cytoskeletal components impede glycosphingolipid capping (Craig and Cuatrecasas, 1975; Revesz and Greaves, 1975; Stern and Bretscher, 1979) implies that this capping involves energy-dependent cytoskeletal reorganization. But how could glycosphingolipid capping be mediated by cytoskeletal reorganization if the crosslinked glycosphingolipids on the cell surface do not contact the cytoplasm where cytoskeletal proteins reside? One hypothesis proposed that the glycosphingolipids selectively bind to membrane proteins that are associated with cytoskeletal components, and ligand binding induces cytoskeletal reorganization that actively clusters the crosslinked glycosphingolipids (Craig and Cuatrecasas, 1975; Bourguignon and Singer, 1977; Kellie et al., 1983). By the early 1980s, several reported observations indirectly supported this hypothetical model for glycosphingolipid capping. They included the detection of glycosphingolipids in isolated membrane protein complexes (Ji, 1974; Lingwood et al., 1980), the association of GM1 with cytoskeletons produced by detergent treatment (Sahyoun et al., 1981; Streuli et al., 1981; Hagmann and Fishman, 1982), and the accumulation of cytoskeletal proteins under the patches of crosslinked glycosphingolipids in intact cells (Kellie et al., 1983). An alternative hypothetical mechanism for ganglioside capping proposed that gangliosides self-associate with one another in resting cells, and crosslinking pulls these tiny ganglioside clusters together, forming larger lipid patches (Spiegel et al., 1984; Thomas et al., 1994). This hypothesis is consistent with the finding that GM1 crosslinking induced the co-capping of both GM1 and GM3 (Spiegel et al., 1984). However, this hypothetical mechanism for crosslinking-induced ganglioside capping did not predict a role for cytoskeletal components, or consequently, the impairment of ganglioside capping by metabolic poisons and inhibitors of cytoskeletal components.

The idea that lipid self-association drives the formation of distinct lipid domains that mediate capping and subsequent signal transduction further developed into the lipid raft hypothesis. This hypothesis states that attractive forces between sphingolipid and cholesterol molecules within the plasma membrane give rise to ordered cholesterol- and sphingolipid-enriched domains that are called lipid rafts (Simons and Ikonen, 1997). GPI-anchored proteins are hypothesized to have an affinity for, and thus concentrate within lipid rafts, thereby promoting their interactions with other raft-associated signaling proteins (Simons and Ikonen, 1997). The presence of lipid rafts at the site of antigen patching was inferred from the co-patching of crosslinked receptors and gangliosides, which are purportedly integral lipid raft components, on the surfaces of immune cells (Stauffer and Meyer, 1997; Harder et al., 1998). Clusters of GPI-anchored receptors and gangliosides were not detected on cells without crosslinking (Mayor et al., 1994; Mayor

and Maxfield, 1995; Fujimoto, 1996). Therefore, GPI-anchored proteins were hypothesized to reside in tiny lipid rafts that nucleate into structures that can be detected with conventional fluorescence microscopy when crosslinked (Harder et al., 1998). Actin accumulated under the crosslinked antigen patches, so these larger protein clusters were hypothesized to represent the coalescence of lipid rafts into larger domains that were stabilized by the actin cytoskeleton and its associated proteins (Ash et al., 1977; Bourguignon and Singer, 1977; Kellie et al., 1983; Pierini et al., 1996; Harder and Simons, 1999). The hypothesis that lipid raft clustering is responsible for the patching of crosslinked antigens was bolstered by the early finding that the co-clustering of crosslinked GPI-anchored proteins and GM1 was reduced by cholesterol depletion, which ostensibly eliminates lipid rafts (Harder et al., 1998; Harder and Simons, 1999).

The hypothetical role of lipid rafts in antigen patching stimulated new efforts to image the glycosphingolipid reorganization induced by antigen crosslinking. Based on the assumptions that GM1 and other gangliosides are markers for lipid rafts, and favorable cholesterol-sphingolipid interactions drive lipid raft formation, many studies focused on imaging GM1 proximity to crosslinked antigens and the effects of cholesterol depletion. These studies confirmed that antigen crosslinking induces local elevations in the fluorescence signals from both the crosslinked GPI-anchored protein and toxin-crosslinked GM1, and this co-clustering is inhibited by cholesterol depletion (Stauffer and Meyer, 1997; Harder et al., 1998; Huby et al., 1999; Janes et al., 1999; Grassmé et al., 2001a; Mitchell et al., 2002). Subsequent reports also confirmed that cytoskeletal elements accumulate under the site of antigen patching (Rodgers and Zavzavadjian, 2001; Delaguillaumie et al., 2004; Wilson et al., 2004).

Though many reports verified the signals from GM1 and the clustered membrane proteins were colocalized at the resolution of conventional fluorescence microscopy, other reports challenged the interpretation of this co-localization as evidence for antigen clustering in rafts. Fluorescence resonance energy transfer (FRET) studies indicated a lack of true co-localization between GPI-anchored proteins and cholera toxin-labeled GM1 (Kenworthy et al., 2000; Glebov and Nichols, 2004). The energy transfer between the antibody-labeled GPI-anchored proteins and cholera toxin B-labeled GM1 correlated with their surface densities, and were not selectively colocalized, which is inconsistent with GPI-anchored protein recruitment to lipid rafts (Kenworthy et al., 2000; Glebov and Nichols, 2004). The local increases in fluorescence from GPI-anchored proteins and cholera toxin-labeled GM1 observed after antigen crosslinking could instead be attributed to a local excess of cell membrane. Consistent with this conclusion, another report clearly showed numerous membrane folds and protrusions were present at the site where the fluorescence signals from the GPI-anchored proteins and cholera toxin-labeled GM1 were elevated on a Jurkat cell (Glebov and Nichols, 2004). An immunoelectron microscopy study also challenged the finding that crosslinked GPI-anchored proteins co-cluster with GM1. This work revealed a lack of GM1 enrichment in patches of crosslinked putative raft proteins, namely the GPI-anchored protein Thy-1 and the IgE receptor (Wilson et al., 2004), which argues that these crosslinked antigens do not reside in lipid rafts. Consequently, the observed patching of Thy-1 and IgE receptor could not have been mediated by either lipid rafts or the favorable cholesterol-sphingolipid interactions that hypothetically drive raft formation.

Recent reports that cholesterol depletion perturbs cytoskeletal organization (Ramprasad et al., 2007; Sun et al., 2007; Qi et al., 2009; Norman et al., 2010; Chubinskiy-Nadezhdin et al., 2013; Dick et al., 2013) may suggest that cholesterol depletion inhibits antigen patching by preventing the cytoskeletal proteins from actively clustering the crosslinked antigens. But, as mentioned above, if the cytoskeleton, and not lipid rafts, mediates the clustering of crosslinked antigens, the finding that crosslinking induces glycosphingolipid capping implies the glycosphingolipids in the outer leaflet of the plasma membrane are indirectly associated with cytoskeletal proteins. Studies of the trafficking of GD3, a disialoganglioside ganglioside, during CD95/Fas-mediated apoptosis seem to support this possibility. CD95/Fas-mediated apoptosis is initiated by the binding of either the Fas ligand or an antagonistic Fas antibody to CD95, a member of the TNF-receptor superfamily that is also called Fas (Wajant, 2014). This binding induces the recruitment of Fas-associated death domain (FADD) to the CD95 death domain. Next, procaspase-8 is recruited to FADD's death effector domain, forming the death-inducing signaling complex (DISC) that elicits apoptosis (Algeciras-Schimnich et al., 2002; Wajant, 2014). Interest in GD3 involvement in CD95/Fas-mediated apoptosis began with the discovery that the crosslinking of CD95 on lymphoid and myeloid cells induces GD3 production, and this ganglioside is required for apoptosis (De Maria et al., 1997). Immunoelectron and immunofluorescence imaging of GD3 and organelle markers in hepatocytes treated with tumor necrosis factor-α (TNF-α) revealed that GD3 moved from the plasma membrane to mitochondria prior to mitochondrial membrane depolarization and apoptosis (Garcıa-Ruiz et al., 2002). Malorni and coworkers identified multiple cytoskeletal proteins that GD3 may associate with during its transit to mitochondria in lymphoid cells treated with anti-CD95 antibodies. GD3 association with ezrin was suggested by the co-localization between ezrin and GD3 observed with immunofluorescence microscopy, and by the presence of GD3 in immunoprecipitates obtained with anti-ezrin monoclonal antibodies (Giammarioli et al., 2001). Another study by Malorni and coworkers provided strong evidence that GD3 also associates with tubulin (Sorice et al., 2009). This evidence includes the elevated FRET efficiency between GD3 and β-tubulin that was detected after Fas ligation, immunoelectron images showing immunogold-labeled GD3 on microtubules, and the presence of GD3 in immunoprecipitates obtained with anti-tubulin antibodies (Sorice et al., 2009). Furthermore, an *in silico* docking analysis predicted GD3 has a high affinity for a pore on polymerized tubulin, indicating selective GD3-tubulin interactions (Sorice et al., 2009). A subsequent FRET study revealed that GD3 colocalized with CLIPR-59, a tubulin-binding protein, shortly before it colocalized with tubulin (Sorice et al., 2010). Based on the assumption that GD3 is a marker for lipid rafts, it had been proposed that GD3 trafficking involved interactions between lipid rafts and the cytoskeleton

(Giammarioli et al., 2001; Sorice et al., 2009, 2010). However, these results also support an alternative hypothesis that GD3 trafficking is mediated by the selective binding of individual GD3 molecules directly to proteins associated with the cytoskeleton in absence of lipid rafts.

Overall, the results described in this section clearly demonstrate that the crosslinking of glycosphingolipids induces their redistribution into patches on the surfaces of immune cells. However, they fail to conclusively establish whether either favorable interactions between cholesterol and sphingolipids or specific glycosphingolipid-protein interactions are the driving force for this glycosphingolipid reorganization.

CERAMIDE-RICH MEMBRANE DOMAINS INDUCED BY EXTERNAL STIMULI

Ceramide's role as a second messenger that directly participates in signaling cascades began to gain recognition in the early 1990's (Kim et al., 1991; Dobrowsky and Hannun, 1992; Bielawska et al., 1993; Dobrowsky et al., 1993; Obeid et al., 1993; Cifone et al., 1994; Hannun, 1994). By the late 1990's, various stimuli were known to activate sphingomyelinases that hydrolyze sphingomyelin to ceramide, producing a transient increase in ceramide levels that is required for biological response (Wiegmann et al., 1994; Tepper et al., 1995; Grassmé et al., 1997; Brenner et al., 1998; Junge et al., 1999; Grullich et al., 2000). This section describes studies that probed the subcellular localization of sphingomyelinase and the ceramide it produces in response to external stimuli.

Among the stimuli that induce ceramide generation is the crosslinking of CD95 (Cifone et al., 1994; Tepper et al., 1995; Brenner et al., 1998; Grullich et al., 2000), which also induces GD3 production and its trafficking within the cell (*vide supra*). Immunoimaging studies established that CD95 activation induces acid sphingomyelinase translocation to the cell surface and subsequent CD95 clustering (Grassmé et al., 2001a; Lacour et al., 2004). Ceramide generation in the plasma membrane was initially postulated to occur in caveolae, which are flask-shaped plasma membrane invaginations that consist of the caveolin-1 structural protein (Liu and Anderson, 1995; Bilderback et al., 1997). This hypothesis was based on the finding that sphingomyelin levels decreased and ceramide levels increased in a caveolin-rich detergent-insoluble membrane fraction that could be isolated from cells (Liu and Anderson, 1995; Bilderback et al., 1997). The caveolin-containing detergent insoluble membrane fraction was also enriched with cholesterol, sphingolipids, and GPI-anchored proteins, so after the raft hypothesis was proposed, ceramide generation was postulated to occur in lipid rafts (Grassmé et al., 2001a). Efforts to investigate this hypothesis often combined immunolabels for sphingomyelinase detection with the aforementioned strategies used to assess the involvement of lipid rafts in receptor clustering, such as imaging immunolabeled GM1 as a proxy for rafts and probing the effects of cholesterol depletion. These studies demonstrated that after translocation to the cell surface, the signals from the acid sphingomyelinase overlapped with those from the clustered CD95 and cholera toxin-labeled GM1 on the surfaces of CD95-activated cells (Grassmé et al., 2001a; Bock et al., 2003). Depletion of cellular cholesterol reduced acid sphingomyelinase translocation to the cell surface, subsequent CD95 clustering, and CD95-induced apoptosis (Cremesti et al., 2001; Grassmé et al., 2001a; Lacour et al., 2004). The authors concluded that acid sphingomyelinase is transported to lipid rafts where it generates the ceramide that is required for receptor clustering and subsequent apoptosis. Noteworthy, this conclusion hinges on the assumptions that GM1 primarily resides in lipid rafts, and that cholesterol depletion eliminates lipid rafts without perturbing specific protein-protein or cholesterol-protein interactions.

The use of new ceramide-specific affinity labels to study the role of ceramide generation in receptor clustering yielded compelling evidence for the existence of ceramide-rich domains in the plasma membrane (Grassmé et al., 2001b, 2002; Bock et al., 2003; Lacour et al., 2004). Immunofluorescence imaging of a fluorescently labeled protein construct with an affinity for ceramide revealed large fluorescent patches at the perimeters of CD95-stimulated Jurkat cells (Grassmé et al., 2001b). CD95 clustering was inhibited by treating the cells with proteins that bind to the ceramide on the cell surface prior to CD95 activation, and by inhibition of acid sphingomyelinase, which confirms ceramide generation is required for biological response (Grassmé et al., 2001b). A subsequent report that employed anti-ceramide antibodies to detect ceramide also indicated the presence of large ceramide-rich patches on CD95-activated colon cancer cells that had been treated with the anticancer drug cisplatin (Lacour et al., 2004). Overlap between the large patches of ceramide-specific fluorescence and the clustered CD95 at the cell periphery was detected with immunofluorescence imaging; neither patches of ceramide-specific fluorescence nor CD95 clusters were found on untreated cells (Lacour et al., 2004). The possibility that the elevated patches of fluorescence from the ceramide-specific affinity labels detected in these studies may signify an excess of membrane caused by membrane folds and protrusions has not been directly assessed. However, electron microscopy images of intact and sectioned cells demonstrated that acid sphingomyelinase was localized within distinct regions on the surfaces of CD95-activated cells, and was not evenly distributed on their surfaces (Grassmé et al., 2001a,b). Because the production of ceramide on the cell surface is catalyzed by acid sphingomyelinase, this compartmentalized acid sphingomyelinase distribution indicates ceramide is produced at discrete regions on the cell surface. Consequently, the elevated patches of ceramide-specific fluorescence observed in the studies described above likely represent ceramide-enriched membrane domains, and not an excess of membrane.

Subsequent studies involving the imaging of immunolabeled ceramide show that many stimuli, including the activation of other immune cell receptors, induce the acid sphingomyelinase-mediated formation of ceramide-rich domains (Grassmé et al., 2002; Abdel Shakor et al., 2004; Korzeniowski et al., 2007). The activation of cluster of differentiation 40 (CD40), a member

of the TNF-receptor superfamily found on antigen presenting cells, induced the formation of ceramide patches that largely colocalized with clustered CD40 and acid sphingomyelinase (Grassmé et al., 2002). Similar to CD95, CD40 clustering, and subsequent signaling was inhibited by a loss of acid sphingomyelinase activity, neutralization of cell surface ceramide, and cholesterol depletion (Grassmé et al., 2002). Likewise, immunofluorescence imaging of ceramide showed the activation of Fc gamma receptor II (FcγRII), an immune cell receptor for IgG, induced acid sphingomyelinase activity at the cell surface and the formation of ceramide-rich membrane patches (Abdel Shakor et al., 2004; Korzeniowski et al., 2007). This ceramide production was required for the clustering of the crosslinked FcγRII, subsequent receptor phosphorylation, and signaling.

Some stimuli that ultimately trigger membrane internalization also induce acid sphingomyelinase translocation to the cell surface and the subsequent formation of ceramide-rich plasma membrane domains. This includes the internalization of pathogenic bacteria, viruses, cell-penetrating peptides, and nanoparticles functionalized with anti-intercellular adhesion molecule-1 (ICAM) antibodies (Grassmé et al., 1997, 2003a; Grassmé, 2005; Verdurmen et al., 2010; Serrano et al., 2012). Additionally, the binding of iron-loaded transferrin to the transferrin receptor results in the formation of ceramide-rich patches that are required for the recruitment transferrin/transferrin receptor complexes to clathrin-coated pits and their successive internalization (Abdel Shakor et al., 2012).

In the majority of these studies, the biological effects of ceramide production were hypothesized to involve changes in lipid-lipid interactions resulting from the hydrolysis of sphingomyelin in lipid rafts to ceramide. Cleavage of the phosphatidylcholine head group from sphingomyelin reduces the affinity between cholesterol and the newly formed ceramide (Megha and London, 2004). This hypothetically promotes a local loss of cholesterol and the formation of a ceramide-rich domain with a negative curvature that induces vesicle formation (Kolesnick et al., 2000; Cremesti et al., 2002; Megha and London, 2004; Bollinger et al., 2005). An alternative mechanism for ceramide-mediated receptor clustering and internalization invokes ceramide's role as a second messenger that mediates cytoskeletal remodeling and membrane internalization through selective ceramide-protein interactions. The ceramide produced in the plasma membrane by acid sphingomyelinase is known to selectively bind to and activate two protein phosphatases, PP2A and PP1 (Chalfant et al., 1999; Canals et al., 2010, 2012). These ceramide-activated serine/threonine phosphatases dephosphorylate ezrin, which abrogates the simultaneous binding of ezrin to actin and the plasma membrane, causing a loss of plasma membrane-cytoskeleton linkage, and cortical actin remodeling (Zeidan et al., 2008; Canals et al., 2010, 2012). Therefore, selective ceramide-protein interactions may mediate the cytoskeletal remodeling that is necessary for receptor clustering, internalization, and transport through the cortical actin network beneath the plasma membrane.

IMMUNOIMAGING MULTIPLE SPHINGOLIPID SPECIES IN PARALLEL WITHIN THE PLASMA MEMBRANE

The development of antibodies and non-toxic recombinant versions of toxin molecules that selectively bind to distinct sphingolipid subspecies has enabled simultaneously visualizing the distributions of multiple sphingolipid subspecies within the plasma membrane. Studies that imaged these new sphingolipid-specific affinity labels suggest that different sphingolipid subspecies are segregated within different regions of the plasma membrane (Fujita et al., 2007, 2009; Janich and Corbeil, 2007; Chen et al., 2008). One study probed the distributions of GM1, GM3, and prominin-1, a cholesterol-binding protein that resides in plasma membrane protrusions (Roper et al., 2000), on the apical surfaces of MDCK cells (Janich and Corbeil, 2007). This work showed that fluorescent cholera toxin B-labeled GM1 colocalized with antibody-labeled prominim-1 on microvilli on the apical surfaces of MDCK cells, whereas fluorescent antibody-labeled GM3 was excluded from these sites (Janich and Corbeil, 2007). In contrast, both fluorescent cholera toxin B-labeled GM1 and imunolabeled GM3 colocalized with the labeled prominin-1 on primary cilium, which are another type of protrusion on the apical surfaces of MDCK cells. A study that used near-field scanning optical microscopy (NSOM) and quantum dot-functionalized affinity labels to detect GM1 and GM3 on separate MDCK cells also indicated GM1 and GM3 were segregated on the apical cell surface (Chen et al., 2008). In this study, the GM3 and GM1 were primarily found on the peaks and valleys, respectively, of the microvillus-like protrusion on the apical surface of the MDCK cells (Chen et al., 2008).

A lack of co-localization between GM1 and GM3 on mouse fibroblast cells was also reported by Fujimoto and coworkers. They performed immunoelectron microscopy on flash-frozen and freeze-fractured mouse fibroblast cells that had been immunolabeled for GM1 and GM3 using orthogonal antibody pairs functionalized with different diameter colloidal gold particles (Fujita et al., 2007, 2009). Both GM3 and GM1 were clustered within separate plasma membrane domains that rarely overlapped. Cholesterol depletion reduced the abundances of the GM1 and GM3 clusters, which is consistent with the hypothesis that these gangliosides reside in rafts that are dependent on cohesive cholesterol-sphingolipid interactions (Fujita et al., 2007). However, chilling the cells on ice prior to flash-freezing, which was expected to promote the growth of the ordered lipid raft domains, actually reduced the clustering of GM1 and GM3 within the plasma membrane (Fujita et al., 2007). Interestingly, depolymerization of cellular actin by treatment with latrunculin A reduced the number of non-overlapping GM1 and GM3 domains in the plasma membrane, and increased GM1 and GM3 co-clustering (Fujita et al., 2009). Inhibition of Src-family kinases decreased the clustering of GM3 more significantly than GM1 (Fujita et al., 2009). The authors proposed that GM1 and GM3 might bind to different transmembrane proteins that associate with the cytoskeleton, and these different ganglioside-protein-cytoskeleton interactions are

differentially influenced by cholesterol depletion and Src-family kinase inhibition.

Altogether, the simultaneous imaging of multiple immunolabeled ganglioside species points to the existence of multiple types of sphingolipid domains in the plasma membrane. These studies indicate that the mechanism for plasma membrane organization is far more complex than one governed by the components' differential affinities for ordered domains that are induced by cohesive cholesterol-sphingolipid interactions.

IMAGING FLUOROPHORE-LABELED SPHINGOLIPIDS WITHIN THE PLASMA MEMBRANE

The presence of multiple different types of sphingolipid domains within the plasma membrane was also suggested by studies that probed the distributions of various fluorescent sphingolipid analogs on the surfaces of mammalian cells. In these experiments, fluorophore-labeled sphingolipid analogs are incorporated into the plasma membranes of living cells and imaged with fluorescence microscopy. A complication of this approach is that the fluorescent lipid analogs can be internalized and incorporated into intracellular membranes. Labeled intracellular membranes, such as endosomes or vesicles, adjacent to the plasma membrane produce regions of elevated fluorescence that are difficult to discriminate from fluorescent membrane patches that signify a local enrichment in the fluorescent lipid. To avoid this complication, Tyteca and coworkers probed the distribution of fluorescent sphingolipid analogs in erythrocytes (Tyteca et al., 2010; D'Auria et al., 2013), which lack nuclei, endosomes, endoplasmic reticulum, and other membrane-bound organelles, and are also incapable of lipid metabolism and membrane trafficking. They used BODIPY-labeled analogs of sphingomyelin, glucosylceramide (BODIPY-GlcCer), and lactosylceramide (BODIPY-LacCer) in which the BODIPY fluorophore was attached to the N-acyl fatty acid. All three of these BODIPY-labeled sphingolipid analogs formed micron-sized domains in the plasma membranes of erythrocytes. Similar domains were observed when other fluorophores were used in place of BODIPY, which indicates this sphingolipid clustering was not induced by the fluorophore (Tyteca et al., 2010). A series of control experiments argued that the regions of elevated BODIPY-sphingolipid fluorescence on the erythrocytes signify plasma membrane domains enriched with BODIPY-sphingolipids, and not membrane folds or protrusions. Interestingly, the abundances of these BODIPY-sphingolipid domains did not progressively increase as temperature decreased (Tyteca et al., 2010), which argues against a phase separation-like process.

Membrane domains enriched with BODIPY-sphingomyelin, BODIPY-GlcCer, and BODIPY-LacCer were also detected on nucleated cells. Compared to erythrocytes, the sphingolipid-enriched domains appeared to be more abundant and elongated on Chinese hamster ovary (CHO) cells (Tyteca et al., 2010). Control experiments argued against the possibilities that these fluorescent patches were caused by the detection of excess membrane or the nonspecific absorption of aggregated BODIPY-sphingolipid analogs. Double labeling experiments revealed the BODIPY-sphingomyelin and BODIPY-LacCer formed separate domains in the plasma membranes of CHO cells, whereas BODIPY-GlcCer and BODIPY-LacCer colocalized within the same domains (Tyteca et al., 2010). Additionally, a GPI-anchored green fluorescent protein (GFP) construct colocalized with the BODIPY-LacCer domains, but not the BODIPY-sphingomyelin domains. The BODIPY-sphingomyelin domains were not affected by latrunculin A-induced actin depolymerization, but they coalesced into larger structures following depletion of ATP or 70% of the cholesterol in CHO cells (Tyteca et al., 2010).

In a subsequent report, Tyteca and coworkers reported BODIPY-labeled analogs of GM1 (BODIPY-GM1) and phosphatidylcholine (BODIPY-PC) also formed micron-scale domains in the plasma membranes of erythrocytes (D'Auria et al., 2013). The mechanism for BODIPY-PC domain formation was not clear. The abundances of the membrane domains enriched with BODIPY-GM1, BODIPY-PC, BODIPY-sphingomyelin, and BODIPY-GlcCer decreased when membrane tension increased due to cell spreading (D'Auria et al., 2013). Cholesterol depletion had little effect on the BODIPY-GlcCer domains on erythrocytes. However, cholesterol depletion eliminated the BODIPY-sphingomyelin and BODIPY-PC domains (D'Auria et al., 2013) on erythrocytes, which seems to contrast with the prior finding that cholesterol depletion induced the formation of large BODIPY-sphingomyelin domains on CHO cells (Tyteca et al., 2010). The abundances of BODIPY-GlcCer and BODIPY-sphingomyelin domains on the erythrocytes increased when the membrane-spectrin linkage was uncoupled, and proteins involved in membrane-spectrin anchorage colocalized with the BODIPY-sphingomyelin domains (D'Auria et al., 2013). Overall, the lack of colocalization between the different sphingolipid domains, their dependency on membrane-cytoskeleton anchorage, and the differential effects of cholesterol depletion on these domains are inconsistent with hypothetical mechanisms of sphingolipid domain formation driven solely by cohesive cholesterol-sphingolipid interactions. The authors proposed that the differential sensitivity of the various sphingolipid domains to cholesterol abundance may indicate regulation of membrane-cytoskeleton anchorage by cholesterol (D'Auria et al., 2013). Consistent with their hypothesis, the band 3 anion transport protein, which links the plasma membrane to the underlying cytoskeleton, reportedly has an affinity for cholesterol (Klappauf and Schubert, 1977; Schubert and Boss, 1982).

SUPER-RESOLUTION FLUORESCENCE IMAGING OF FLUORESCENT SPHINGOLIPID ANALOGS IN THE PLASMA MEMBRANE

The expectation that lipid rafts are too small and dynamic to be detected with diffraction-limited fluorescence microscopy motivated attempts to detect lipid rafts with super-resolution

fluorescence microscopy techniques (Owen et al., 2012). Instead of imaging the sphingolipids and cholesterol in parallel at high spatial resolution, many studies focused on tracking the diffusion of fluorescent sphingolipid analogs or other putative raft components in the plasma membrane. The cohesive cholesterol- and sphingolipid interactions that hypothetically induce lipid raft formation would hinder the diffusion of these components in the plasma membrane, producing a detectable anomalous diffusion that would be sensitive to cholesterol depletion.

Perhaps the most influential super-resolution imaging studies of membrane organization revealed complex lipid dynamics that were ultimately inconsistent with partitioning into liquid-ordered membrane domains produced by favorable cholesterol-and sphingolipid interactions (Hiramoto-Yamaki et al., 2014; Honigmann et al., 2014; Andrade et al., 2015; Sevcsik et al., 2015). Stimulated emission depletion (STED) fluorescence microscopy imaging demonstrated fluorophore-labeled sphingomyelin, GM1, and a GPI-anchored protein were temporarily trapped within 20-nm-diameter areas in the plasma membrane of living cells, and this trapping was cholesterol-dependent (Eggeling et al., 2009). In comparison, identically labeled phosphatidylethanolamine appeared to diffuse freely in the membrane (Eggeling et al., 2009), which implied that lipid-cytoskeleton interactions were not responsible for the anomalous cholesterol-dependent sphingolipid diffusion. Noteworthy, this finding of unhindered phosphatidylethanolamine diffusion conflicts with a previous single molecule tracking study (Fujiwara et al., 2002), and subsequent STED-FCS and single molecule tracking studies reported by these authors and others (Andrade et al., 2015; Fujiwara et al., 2016; Komura et al., 2016). Although, the authors of the STED study never concluded that the cholesterol-dependent trapping of sphingomyelin, GM1 and GPI-anchored proteins was indicative of tiny lipid rafts, their results were often cited by others as support for the lipid raft hypothesis (Lingwood and Simons, 2010; Levental and Veatch, 2016). Subsequent studies showed that the transient trapping of the fluorescent sphingolipids and GPI-anchored proteins in the plasma membrane were both cholesterol- and cytoskeleton-dependent, and likely reflected binding to immobile membrane proteins, and not entrapment in lipid rafts (Mueller et al., 2011; Honigmann et al., 2014; Sevcsik et al., 2015). Super-resolution fluorescence microscopy imaging also revealed fluorescent cholesterol analogs diffuse freely in the plasma membranes of living cells (Hiramoto-Yamaki et al., 2014; Honigmann et al., 2014), which argues against the existence of lipid rafts.

DIRECT IMAGING OF SPHINGOLIPID DISTRIBUTION IN THE PLASMA MEMBRANE WITH HIGH-RESOLUTION SIMS

High-resolution SIMS performed on a NanoSIMS 50 instrument was used to decisively answer the question: How are cholesterol and sphingolipids distributed in the plasma membranes of intact mouse fibroblast cells? Transfected NIH 3T3 mouse fibroblast cells that stably expressed influenza hemagglutinin (Clone 15 cell line) were employed in these experiments because the micrometer-scale hemagglutinin clusters in their plasma membranes were hypothesized to colocalize with lipid rafts (Scheiffele et al., 1997; Hess et al., 2005; Polozov et al., 2008). This hypothesis suggested that these cells had sphingolipid- and cholesterol-rich membrane domains that could easily be detected with high-resolution SIMS. Untransfected NIH 3T3 mouse fibroblast cells were also analyzed for comparison. Distinct stable isotopes, ^{15}N and ^{18}O, were metabolically incorporated into the sphingolipids and cholesterol, respectively, in living Clone 15 and NIH 3T3 cells (Klitzing et al., 2013). High levels of rare isotope incorporation into the cellular sphingolipids and cholesterol were achieved to ensure that the majority of the sphingolipid and cholesterol molecules in the plasma membrane could be detected and imaged with high-resolution SIMS.

The low-voltage SEM image (**Figure 1A**) shows the morphology of a representative chemically fixed NIH 3T3 mouse fibroblast cell (Frisz et al., 2013a). High-resolution SIMS imaging of the lipid-specific isotope enrichments on the cell showed the plasma membrane contained ^{15}N-sphingolipid domains, evidenced by statistically significant local elevations in ^{15}N-enrichment, that were as large as 2 μm across (**Figure 1B**; Frisz et al., 2013a,b). In contrast, ^{18}O-cholesterol was uniformly distributed within the plasma membrane (**Figure 1C**) (Frisz et al., 2013a,b), and was not enriched at the sphingolipid domains (Frisz et al., 2013a). Similar sphingolipid and cholesterol distributions were observed on multiple other NIH 3T3 mouse fibroblast cells and Clone 15 cells (Frisz et al., 2013a,b).

The finding of sphingolipid domains with dimensions sufficient for detection with fluorescence microscopy is consistent with the abovementioned reports of micron-scale domains of fluorescent sphingolipid analogs in the membranes of living cells (Tyteca et al., 2010; D'Auria et al., 2013). Though unexpected, the relatively uniform cholesterol distribution observed is consistent with previous reports that intrinsically fluorescent sterols are evenly distributed in the membranes of mammalian cells (Wustner, 2007; Wüstner and Faergeman, 2008). This uniform cholesterol distribution is also supported by subsequently published super-resolution fluorescence imaging studies that showed fluorescent cholesterol analogs are not trapped in nanoscale domains within the plasma membranes of living cells (Hiramoto-Yamaki et al., 2014; Honigmann et al., 2014). Additionally, a comprehensive series of control experiments rigorously excluded the possibility that the lipid organizations imaged with high-resolution SIMS were artifacts of analysis. First, the imaging of fluorescent sphingolipids on fibroblast cells that had been metabolically labeled with fluorescent sphingosine showed that large sphingolipid domains were visible on the living cells, and the shapes, sizes, and positions of these fluorescent sphingolipid domains were not altered by glutaraldehyde fixation (**Figures 2A–C**; Frisz et al., 2013b). Thus, fixation did not induce sphingolipid clustering, and the lateral diffusion of lipids within the membrane during fixation did not disperse the sphingolipid domains that were present in the plasma membrane while the cells were alive. Next, experiments in which the rare stable isotope, ^{13}C, was incorporated into all lipid species and imaged in parallel with

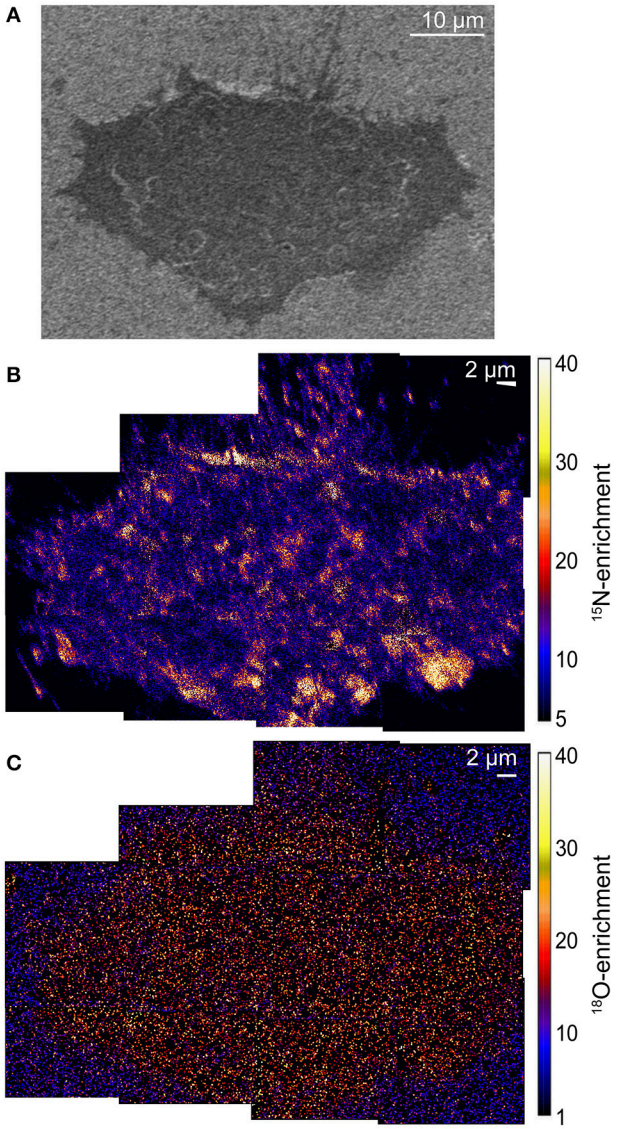

FIGURE 1 | SEM and SIMS images show the morphology of a NIH 3T3 mouse fibroblast cell and the sphingolipid and cholesterol distribution in its plasma membrane. (A) SEM image of a NIH 3T3 fibroblast. **(B)** Montage of ^{15}N-enrichment high-resolution SIMS images shows ^{15}N-sphingolipid domains (orange and yellow regions) in the plasma membrane. **(C)** The ^{18}O-enrichment images that were acquired in parallel show a relatively even ^{18}O-cholesterol distribution in the plasma membrane. Color scales show the number of times that the ^{15}N- or ^{18}O-enrichment is greater than standard abundance. Montages consist of several high-resolution SIMS images that were acquired with 87-nm-lateral resolution. Adapted with permission from research originally published in Frisz et al. (2013a). © The American Society for Biochemistry and Molecular Biology.

^{15}N-sphingolipids confirmed that the cells' plasma membranes were intact. Importantly, lack of ^{13}C-enrichment, which would signify an excess of all lipid species, at the ^{15}N-enriched domains conclusively demonstrated that the local elevations in ^{15}N-enrichment were not due to the detection of intracellular vesicles, organelles, or membrane folds, which would produce a co-committant increase (**Figures 2D–F**; Frisz et al., 2013b). Finally, control experiments ruled out the possibilities that the ^{15}N-enriched domains on the cells were caused by isotope-labeled lipid precursors nonspecifically adsorbed to the cells, cell topography, temperature-induced domain formation, or sample preparation (Frisz et al., 2013b). Published reports have also established that high-resolution SIMS imaging does not alter the lipid distribution in phase-separated supported lipid bilayers (Kraft et al., 2006; Anderton et al., 2011), and this technique has the sensitivity to detect nanoscale domains enriched with GM1 and cholesterol in model lipid membranes (Lozano et al., 2013).

The lack of cholesterol enrichment in the sphingolipid domains detected on the fibroblast cells suggests that the self-organizing potential of cholesterol and sphingolipids is not responsible for plasma membrane organization. This possibility was further assessed by imaging the distributions of ^{15}N-sphingolipids and ^{18}O-cholesterol following cholesterol depletion. SEM images of mouse fibroblast cells that had been treated with methyl-β-cyclodextrin, which reduced the cellular cholesterol by 30%, showed cholesterol depletion altered cell morphology and reduced cell spreading. High-resolution SIMS imaging revealed the abundance of ^{15}N-sphingolipid domains in the plasma membrane also decreased, but the remaining ^{18}O-cholesterol in the plasma membrane still appeared to be relatively uniformly distributed (Frisz et al., 2013a). No significant difference in the ^{18}O-cholesterol abundance in the sphingolipid domains and comparably sized non-domain regions was detected. Other mβCD-treated Clone 15 cells had similar cholesterol and sphingolipid distributions (Frisz et al., 2013a). Based on the lack of cholesterol enrichment in the sphingolipid-enriched domains either before or after cholesterol depletion, favorable cholesterol-sphingolipid interactions cannot be the driving force for plasma membrane organization.

The resemblance in the sphingolipid and cholesterol distributions in the plasma membranes of the Clone 15 and NIH 3T3 mouse fibroblast cells suggests the sphingolipid domains were not produced by favorable hemagglutinin-sphingolipid interactions. However, hemagglutinin might have an affinity for sphingolipids in the plasma membrane, which would cause the hemagglutinin to accumulate within the sphingolipid-enriched domains. This possibility was assessed by studying the stably expressed influenza hemagglutinin clusters in the membranes of uninfected Clone 15 cells instead of those in the membranes of influenza-infected cells to ensure that other viral proteins did not affect hemagglutinin localization within the plasma membrane. To permit visualization, the hemagglutinin on the metabolically labeled Clone 15 cells was labeled with a mouse anti-hemagglutinin antibody followed by an anti-mouse secondary antibody conjugated to a fluorinated colloidal gold particle (Wilson et al., 2012). High-resolution SIMS imaging of the $^{19}F^-$ ions distinctive to the immunolabeled hemagglutinin in parallel with the ^{15}N-sphingolipids and ^{18}O-cholesterol revealed the fluorine-rich patches that located the hemagglutinin clusters were neither enriched with cholesterol nor well colocalized with ^{15}N-sphingolipid domains (**Figures 3A–C**; Wilson et al., 2015). The low co-localization between the hemagglutinin and sphingolipid domains was confirmed by complementary

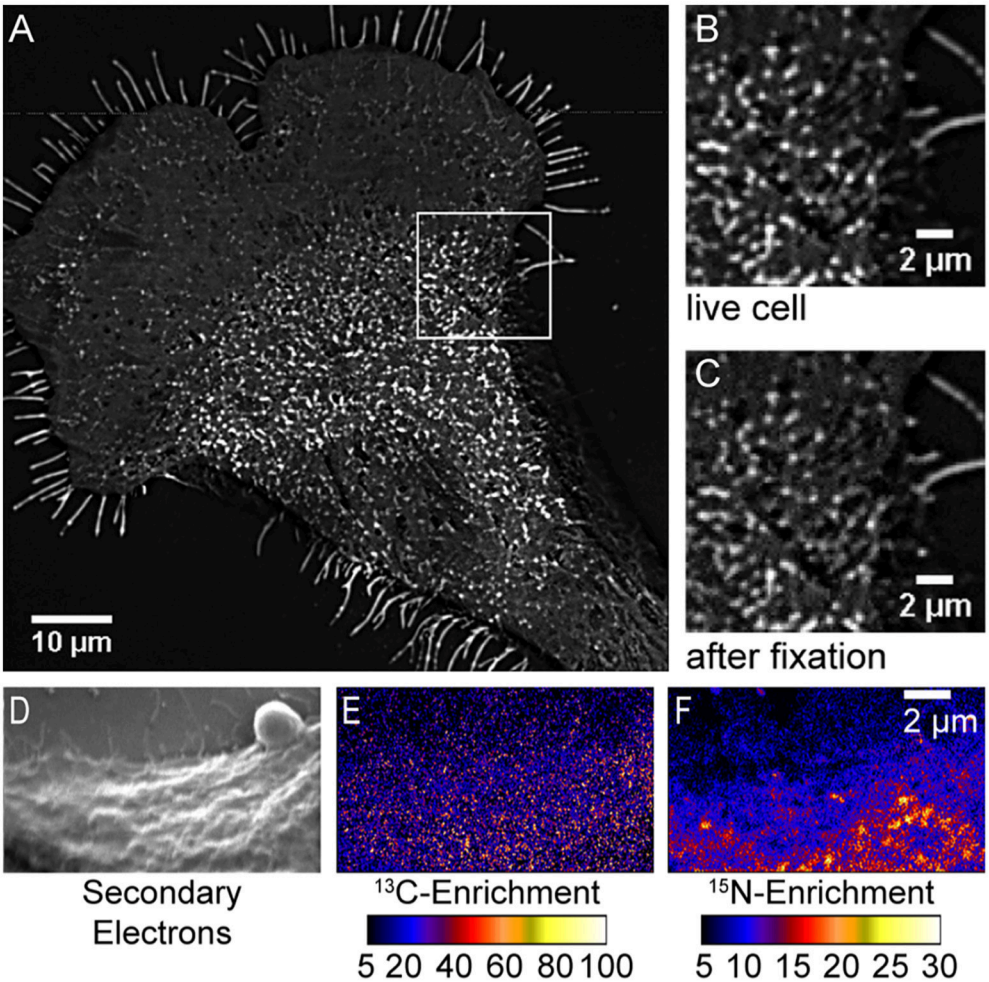

FIGURE 2 | Control experiments exclude possible artifacts caused by cell fixation or the detection of excess membrane caused by intracellular membranes adjacent to the plasma membrane. Total internal reflectance microscopy images (background subtracted and averaged through the stack) of BODIPY-sphingolipids in the plasma membrane of a fibroblast **(A,B)** before and **(C)** after fixation. Enlargement of outlined region in **(A)** shows no change in the domains that were present **(B)** in the living cell **(C)** after glutaraldehyde fixation. Fluorescent micro-extensions are artifacts of background correction. Reproduced with permission from Frisz et al. (2013b). Copyright 2013 National Academy of Sciences, U.S.A. The **(D)** secondary electron, **(E)** ^{13}C-enrichment, and **(F)** ^{15}N-enrichment images acquired with high-resolution SIMS shows that the ^{15}N-sphingolipid domains do not coincide with cell projections, folds, or other excesses of cellular lipids, which are labeled with carbon-13 and thus, would produce a co-elevation in ^{13}C-enrichment. The color scale represents the indicated isotope enrichment measured at each pixel compared to unlabeled cells. Adapted with permission from Frisz et al. (2013b). Copyright 2013 National Academy of Sciences, U.S.A.

experiments in which immunolabeled hemagglutinin and fluorescent sphingolipids in living Clone 15 cells were imaged with fluorescence microscopy (**Figures 3D–F**; Frisz et al., 2013b). The consistency between the findings of these two complementary techniques discounts the prospect that cell fixation or antibody labeling altered the membrane organizations observed with either technique. These findings disprove the hypothesis that hemagglutinin clustering is caused by an attraction to ordered plasma membrane domains that are enriched with cholesterol and sphingolipids. This conclusion is consistent with biophysical studies that indicated hemagglutinin is not located within cholesterol-rich liquid-ordered membrane domains (Hess et al., 2005, 2007; Polozov et al., 2008; Nikolaus et al., 2010).

The finding that cholesterol depletion reduced both cell spreading and sphingolipid domain abundance in the plasma membrane is consistent with the alternative hypothesis that the cytoskeleton and its associated proteins divide the plasma membrane into distinct lipid domains (Gheber and Edidin, 1999; Douglass and Vale, 2005; Kusumi et al., 2005; Hiramoto-Yamaki et al., 2014). This alternative hypothesis was also tested by using high-resolution SIMS to image the ^{15}N-sphingolipid distributions in the plasma membranes of NIH 3T3 cells that were treated with latrunculin A to depolymerize their cytoskeletons. Actin depolymerization altered cell morphology (**Figure 4A**) and eliminated the vast majority of large ^{15}N-sphingolipid domains in the plasma membrane (**Figure 4B**; Frisz et al., 2013b). This finding confirms the hypothesis

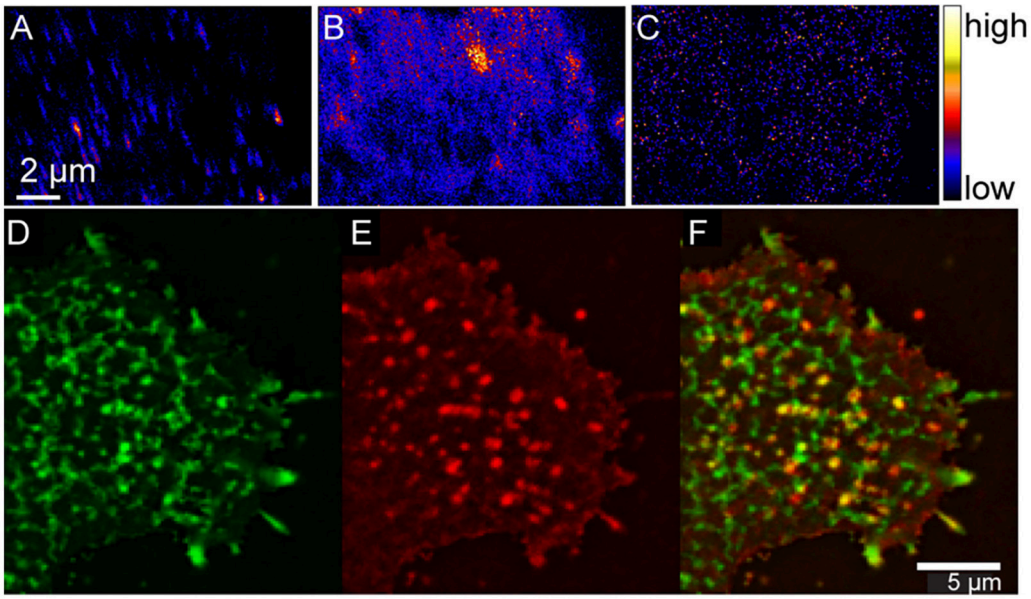

FIGURE 3 | High-resolution SIMS and complementary immunofluorescence imaging shows hemagglutinin does not cluster in plasma membrane domains that are enriched with cholesterol and sphingolipids. High-resolution SIMS images of a region on a mouse fibroblast cell that stably expressed influenza hemagglutinin (Clone 15 cell line). **(A)** High-resolution SIMS image of the $^{19}F^-$ counts shows the distribution of immunolabeled hemagglutinin in the plasma membrane. Comparison to the **(B)** ^{15}N-enrichment and **(C)** ^{18}O-enrichment images that were simultaneously acquired indicates hemagglutinin is not located in cholesterol- and sphingolipid-enriched domains. Reprinted from Wilson et al. (2015). Copyright (2015) with permission from Elsevier. Total internal reflectance microscopy detection of **(D)** BODIPY-sphingolipids (green) and **(E)** hemagglutinin (red) in the plasma membrane of a living Clone 15 cell. **(F)** Overlay shows little colocalization between the sphingolipids and hemagglutinin (yellow). Scale bar is 5 µm. Reproduced with permission from Frisz et al. (2013b). Copyright 2013 National Academy of Sciences, U.S.A.

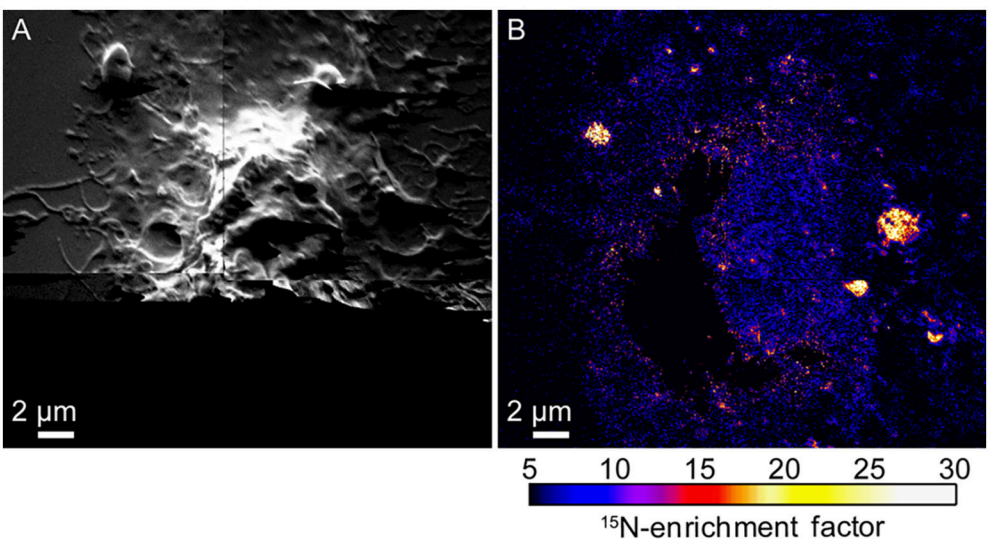

FIGURE 4 | Secondary electron and SIMS images of a NIH 3T3 fibroblast cell treated with latrunculin A to depolymerize the actin cytoskeleton. (A) Secondary electron images show cell morphology. Secondary electrons were not detected at the bottom of the image due to the low beam current used. **(B)** ^{15}N-enrichment images acquired with high-resolution SIMS show few ^{15}N-sphingolipid domains following actin depolymerization. Color scales show the number of times that the ^{15}N-enrichment is greater than standard abundance. Reproduced with permission from research originally published in Frisz et al. (2013a). © The American Society for Biochemistry and Molecular Biology.

that the cytoskeleton and its associated membrane proteins corral the sphingolipids within distinct domains in the plasma membrane.

IMPLICATIONS FOR PLASMA MEMBRANE ORGANIZATION HYPOTHESES

Independent experiments performed with complementary imaging techniques have yielded data that undeniably refutes the hypothesis that cohesive sphingolipid-cholesterol interactions are the driving force for plasma membrane organization. These findings include: (1) the lack of cholesterol- or hemagglutinin-enrichment in the sphingolipid domains that were detected in the plasma membranes of fibroblast cells with high-resolution SIMS (Frisz et al., 2013a; Wilson et al., 2015); (2) the unhindered diffusion of cholesterol analogs detected in the membranes of living cells with super-resolution fluorescence imaging (Hiramoto-Yamaki et al., 2014; Honigmann et al., 2014); and (3) the transient trapping of other putative raft components is inconsistent with interactions with rafts or lipid phase separation (Hiramoto-Yamaki et al., 2014; Honigmann et al., 2014; Sevcsik et al., 2015). Thus, although favorable cholesterol-sphingolipid interactions induce the formation of liquid-ordered domains that are enriched with cholesterol and sphingolipids in model membranes (Sankaram and Thompson, 1990) and membrane blebs (Baumgart et al., 2003, 2007), these interactions do not control lipid organization in the plasma membranes of actual cells. Given that cholesterol-sphingolipid interactions are a cornerstone of the lipid raft hypothesis and both high-resolution SIMS and super-resolution fluorescence techniques failed to detect lipid rafts, these results not only argue against the existence of rafts, they conclusively disprove their existence.

The discrepancies between experimental data and predictions of the raft hypothesis cannot be rectified by incorporating additional protein-protein or protein-lipid interactions into a revised model that is still based on cohesive sphingolipid-cholesterol interactions. Instead, alternative hypotheses that do not involve cohesive sphingolipid-cholesterol interactions must be developed, investigated, and discarded if they prove inconsistent with experimental results. These alternative hypotheses should account for the following observations:

i. The diffusion and distribution of proteins and lipids is influenced by the actin cytoskeleton (Fujiwara et al., 2002, 2016; Mueller et al., 2011; D'Auria et al., 2013; Frisz et al., 2013a,b; Honigmann et al., 2014; Andrade et al., 2015; Sevcsik et al., 2015; Komura et al., 2016).
ii. Actin accumulates under clusters of crosslinked membrane proteins (Ash et al., 1977; Bourguignon and Singer, 1977; Kellie et al., 1983; Pierini et al., 1996; Harder and Simons, 1999; Rodgers and Zavzavadjian, 2001; Delaguillaumie et al., 2004; Wilson et al., 2004; Goswami et al., 2008; Gowrishankar et al., 2012; Gudheti et al., 2013).
iii. Different sphingolipid subspecies form separate microdomains in the plasma membrane, and each domain of different sphingolipid subspecies may contain distinctly different membrane proteins (Fujita et al., 2007, 2009; Janich and Corbeil, 2007; Chen et al., 2008; Tyteca et al., 2010).
iv. Cellular processes are sensitive to sphingolipid catabolism and inhibitors of sphingolipid biosynthesis (Wiegmann et al., 1994; Tepper et al., 1995; Grassmé et al., 1997, 2001a,b, 2003a; Brenner et al., 1998; Junge et al., 1999; Grullich et al., 2000; Cremesti et al., 2001; Paris et al., 2001; Grassmé et al., 2003b; Abdel Shakor et al., 2004, 2012; Grassmé, 2005; Korzeniowski et al., 2007; Verdurmen et al., 2010; Serrano et al., 2012).
v. Cholesterol depletion affects protein clustering and cell signaling (Stauffer and Meyer, 1997; Harder et al., 1998; Harder and Simons, 1999; Huby et al., 1999; Janes et al., 1999; Cremesti et al., 2001; Grassmé et al., 2001a; Mitchell et al., 2002; Lacour et al., 2004; Hess et al., 2005).

The alternative hypothesis that the plasma membrane is segregated by cortical actin and its associated proteins is consistent with the numerous observations that the distribution and diffusion of lipids and proteins in the plasma membrane is influenced by drugs that affect cytoskeletal integrity (Kusumi and Sako, 1996; Ritchie et al., 2003; Kusumi et al., 2005). In this model, the cytoskeleton and its associated proteins establish diffusion barriers, and the energy-dependent constant delivery and removal of membrane proteins and lipids at the plasma membrane creates lateral variations in component abundance (Gheber and Edidin, 1999; Turner et al., 2005; Lavi et al., 2007; Fan et al., 2010). Indeed, localized trafficking hubs in the plasma membrane have been shown to produce stable domains of distinct protein compositions (Deutsch et al., 2012; Fox et al., 2013). Whether the sphingolipid domains in the plasma membrane are local hubs for sphingolipid trafficking might be assessed by performing high-resolution SIMS in a depth profiling mode to produce three-dimensional images of the intracellular sphingolipid distribution (Yeager et al., 2016).

Nonetheless, the true mechanism for plasma membrane organization is probably far more complex than the current cytoskeleton-based model. For example, cytoskeletal barriers combined with endocytosis and exocytosis events may not fully explain the reported redistribution of crosslinked gangliosides within the plasma membrane during capping. Therefore, the previous hypothesis that individual sphingolipid species selectively and reversibly interact with distinct proteins that are associated with the actin cortex may need to be reconsidered. These sphingolipid-protein interactions may be transient, regulated by external stimuli (i.e., ligand binding), and specific, where different sphingolipid subspecies bind to different protein partners. Such specific, inducible, and transient sphingolipid-protein interactions could direct the segregation of different glycosphingolipid species within different microdomains in the plasma membrane (Fujita et al., 2007, 2009; Janich and Corbeil, 2007; Chen et al., 2008), and mediate their clustering in response to crosslinking. This hypothetical mechanism may also account for colocalization between specific glycosphingolipid species and distinct proteins in the plasma membrane (D'Auria et al., 2013), and the accumulation of actin observed beneath clusters of membrane proteins (Ash et al., 1977; Bourguignon

and Singer, 1977; Kellie et al., 1983; Pierini et al., 1996; Harder and Simons, 1999; Rodgers and Zavzavadjian, 2001; Delaguillaumie et al., 2004; Wilson et al., 2004; Goswami et al., 2008; Gowrishankar et al., 2012; Gudheti et al., 2013). Given the existence of lipid binding proteins that selectively interact with phosphatidylinositols, phosphatidylcholines, and phosphatidylserines (Lemmon, 2008; Stahelin, 2009; Glatz, 2015), other lipid species may also selectively bind to distinctive proteins that are associated with the actin cortex.

The sensitivity of many cellular processes, including antigen capping and apoptosis, to enzymes that induce sphingolipid catabolism or drugs that inhibit sphingolipid biosynthesis can be attributed to the established role of sphingolipids and their metabolites as second messengers in diverse signaling processes (Hannun and Obeid, 2008; Zeidan et al., 2008; Kim et al., 2009; Milhas et al., 2010; Spiegel and Milstien, 2011; Canals et al., 2012). The cholesterol sensitivity of membrane protein clustering and other events that occur in the plasma membrane may be indicative of specific cholesterol-protein interactions (Lange and Steck, 2016). Cholesterol is known to selectively bind to specific sites on a few integral membrane proteins, thereby regulating their conformation and activity (Hanson et al., 2008; Fürst et al., 2014; Clay et al., 2015). The observation that cholesterol depletion reduces cell spreading may suggest that cholesterol binding regulates plasma membrane attachment to the cytoskeleton. Alternatively, cholesterol may indirectly affect membrane attachment to the cytoskeleton via its effects on the abundance of phosphoinositides in the plasma membrane, which help to recruit cytosolic proteins to the plasma membrane (Kwik et al., 2003). A combination of affinity labeling, mass spectrometry detection of protein complexes associated with distinct lipids or cholesterol, and super-resolution imaging of suspected binding partners in cells will be required to evaluate this hypothesis.

AUTHOR CONTRIBUTIONS

MK created figures and wrote the paper.

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Assembly and Turnover of Caveolae: What Do We Really Know?

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In addition to containing highly dynamic nanoscale domains, the plasma membranes of many cell types are decorated with caveolae, flask-shaped domains enriched in the structural protein caveolin-1 (Cav1). The importance of caveolae in numerous cellular functions and processes has become well-recognized, and recent years have seen dramatic advances in our understanding of how caveolae assemble and the mechanisms control the turnover of Cav1. At the same time, work from our lab and others have revealed that commonly utilized strategies such as overexpression and tagging of Cav1 have unexpectedly complex consequences on the trafficking and fate of Cav1. Here, we discuss the implications of these findings for current models of caveolae biogenesis and Cav1 turnover. In addition, we discuss how disease-associated mutants of Cav1 impact caveolae assembly and outline open questions in this still-emerging area.

Keywords: caveolae, caveolin-1, GFP, trafficking, degradation, breast cancer, pulmonary arterial hypertension, congenital generalized lipodystrophy

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INTRODUCTION

In addition to containing nanoclusters of proteins and lipids, the surface of many cell types also contain relatively stable flask-shaped invaginations that are 50–100 nm in diameter known as caveolae. Initially discovered nearly 60 years ago in the plasma membranes of endothelial cells of blood capillaries by electron microscopy, caveolae have been a target of scientific investigation for decades (Palade, 1953). The discovery of the first caveolae-associated protein caveolin-1 (Cav1) almost 40 years after the discovery of caveolae has greatly facilitated research into the structural and functional aspects of caveolae (Kurzchalia et al., 1992; Rothberg et al., 1992). To date, caveolae have been identified in a variety of tissues and cell types including endothelial cells, smooth muscle cells, fibroblasts, myoblasts, and adipocytes, among others (Hansen et al., 2013; Parton and del Pozo, 2013), and the importance of a series of accessory proteins in sculpting caveolae and regulating their dynamics is also now recognized (Hill et al., 2008; Hansen and Nichols, 2010; Hansen et al., 2011; Moren et al., 2012; Stoeber et al., 2012; Ariotti and Parton, 2013; Ludwig et al., 2013; Kovtun et al., 2014, 2015). It is also now clear that once formed, caveolae can flatten in response to membrane stretch and thus serve as membrane reservoirs (Gervasio et al., 2011; Sinha et al., 2011).

Unlike the more controversial case of lipid rafts (Owen et al., 2012; Kraft, 2013; LaRocca et al., 2013; Sevcsik and Schutz, 2016), caveolae are relatively stable structures and also thus readily detectable by conventional fluorescence and electron microscopy approaches. In addition, their presence in cells absolutely depends on the expression of Cav1, making them amenable to a range of biochemical and biophysical analyses as well as studies in animal models (Drab et al., 2001; Razani et al., 2001; Le Lay and Kurzchalia, 2005). Through these varied approaches, the importance of

caveolae in numerous cellular functions and processes has become well-recognized, and are thought to include roles in signal transduction, endocytosis, pathogen invasion, lipid homeostasis, and mechanotransduction (Parton and Simons, 2007; Hansen and Nichols, 2010; Ariotti and Parton, 2013; Parton and del Pozo, 2013; Cheng and Nichols, 2016). Furthermore, Cav1 and other caveolins have been implicated several pulmonary and vascular diseases, myopathies, lipodystrophies, and cancers (Hayashi et al., 2001; Razani and Lisanti, 2001; Cao et al., 2008; Kim et al., 2008; Mercier et al., 2009; Austin et al., 2012; Ariotti and Parton, 2013; Garg et al., 2015; Martinez-Outschoorn et al., 2015).

Given the importance of caveolae in both health and disease, it is critical to gain a clear understanding of how caveolae form and the mechanisms responsible for the turnover of their components. In this mini-review, we summarize current knowledge in these areas, including the unexpectedly complex consequences that overexpression and tagging of Cav1 can have on the trafficking and fate of Cav1 and caveolae biogenesis. In addition, we discuss how disease-associated mutants of Cav1 impact caveolae assembly and turnover and outline open questions in this emerging area.

WHAT CONDITIONS ARE NECESSARY FOR CAVEOLAE TO FORM CORRECTLY?

It is widely accepted that the assembly of caveolae requires the expression of Cav1 (Drab et al., 2001; Razani et al., 2001). A 178 amino acid-long protein, Cav1 is anchored to the membrane by an intra-membrane region that assumes a hairpin-like topology. The Cav1 protein contains four domains: the N-terminal domain (residues 1-81), scaffolding domain (CSD, residues 82-101), transmembrane domain (TMD, residues 102-134), and C-terminal domain (residues 135-178) (Root et al., 2015). The transmembrane domain is composed of two α-helices separated by three residue linker region containing a proline (P110) that induces a $\sim 50^{\circ}$ angle between the two α-helices (Root et al., 2015). This allows Cav1 to adopt a hairpin topology in the lipid bilayer such that both N- and C- termini are exposed to the cytoplasmic interior of the cell (Root et al., 2015). To date, however, the three dimensional structure of Cav1 remains unknown.

Cav1 is synthesized in the endoplasmic reticulum and undergoes a complicated series of oligomerization and trafficking events well before reaching the plasma membrane (**Figure 1**). Newly synthesized Cav1 is quickly organized into Cav1/Cav2 (caveolin-2) hetero-oligomers that contains 14-16 monomers (Monier et al., 1995; Sargiacomo et al., 1995) and partition as an 8S complex on sucrose gradients (Hayer et al., 2010a). This 8S-oligomerization step appears to be pivotal for the proper assembly of caveolae, because forms of Cav1 that fail to oligomerize are unable to independently assemble into caveolae (Mora et al., 1999; Lee et al., 2002; Ren et al., 2004; Shatz et al., 2010). Thereafter, 8S complexes are transported to the Golgi complex in a COPII-dependent mechanism where they serve as the subunits necessary for the assembly of filament-like 70S complexes that become enriched in cholesterol and lose their diffusional mobility. The cholesterol-rich membranes containing 70S Cav1 complexes are then transported to the cell surface (Hayer et al., 2010a).

At the plasma membrane, several accessory proteins are subsequently recruited to caveolin complexes to facilitate caveolae formation and assist in sculpting caveolar membranes as well as regulate caveolae dynamics. They include members of the cavin gene family, pacsin-2, and EHD-2 (Aboulaich et al., 2004; Hill et al., 2008; Hansen and Nichols, 2010; Hansen et al., 2011; Moren et al., 2012; Stoeber et al., 2012; Ariotti and Parton, 2013; Ludwig et al., 2013; Kovtun et al., 2014, 2015). Cavin-1 plays an important role in forming caveolae, as cavin-1 knock-down significantly reduces caveolae number in both mammalian cells and zebrafish (Hill et al., 2008) and cavin-1 knockout mice lack caveolae altogether (Liu et al., 2008). Additional cavin family members have also been identified, and recent studies have elucidated the organization and structure of multiple cavin-containing complexes (Hayer et al., 2010a; Ludwig et al., 2013; Gambin et al., 2014; Kovtun et al., 2014, 2015). These findings have been reviewed in detail elsewhere (Kovtun et al., 2015) and will not be discussed further here. EHD-2 is thought to help confine caveolae and reduce mobility at the plasma membrane through interactions with actin (Moren et al., 2012; Stoeber et al., 2012). Pacsin-2, which contains a membrane curvature-associated F-BAR domain, has also been reported to be recruited to and assist in sculpting caveolae (Hansen et al., 2011; Senju et al., 2011). Furthermore, post-translational modifications of Cav1 such as palmitoylation and phosphorylation also regulate steps in caveolae assembly and caveolae structure (Monier et al., 1996; Nomura and Fujimoto, 1999; Zimnicka et al., 2016). However, expression of Cav1 in a bacterial expression system can drive the formation of heterologous caveolae. Thus, Cav1 itself is capable of inducing membrane curvature in some membrane environments, even without the help of accessory proteins (Walser et al., 2012; Ariotti et al., 2015).

The use of fluorescent protein-tagged forms of Cav1 has made it possible to assess caveolae biogenesis and dynamics. Such experiments have often been carried out by expressing low levels of Cav1 in $Cav1^{-/-}$ mouse embryonic fibroblasts (Kirkham et al., 2008; Ariotti et al., 2015) or more recently at endogenous expression levels in genome-edited cell lines (Shvets et al., 2015). However, a large literature also exists where Cav1 has been studied in the context of overexpression systems. One potential caveat of such studies is that both overexpression and tagging strategies can interfere with caveolae biogenesis (Parton and del Pozo, 2013). For example, it has been reported that in some cell types, after a few hours of expression overexpressed Cav1 fails to co-localize with endogenous Cav1, implying that exogenous Cav1 is not always incorporated into caveolae (Hayer et al., 2010b). Indeed, caveolin-enriched organelles termed "caveosomes" were later shown to arise as a consequence of the accumulation of overexpressed caveolin in late endosomal structures (Pelkmans et al., 2001; Hayer et al., 2010b).

Studies from our own group further have revealed that the behavior of overexpressed Cav1 also depends on the type of the tag (Hanson et al., 2013; Han et al., 2015). In

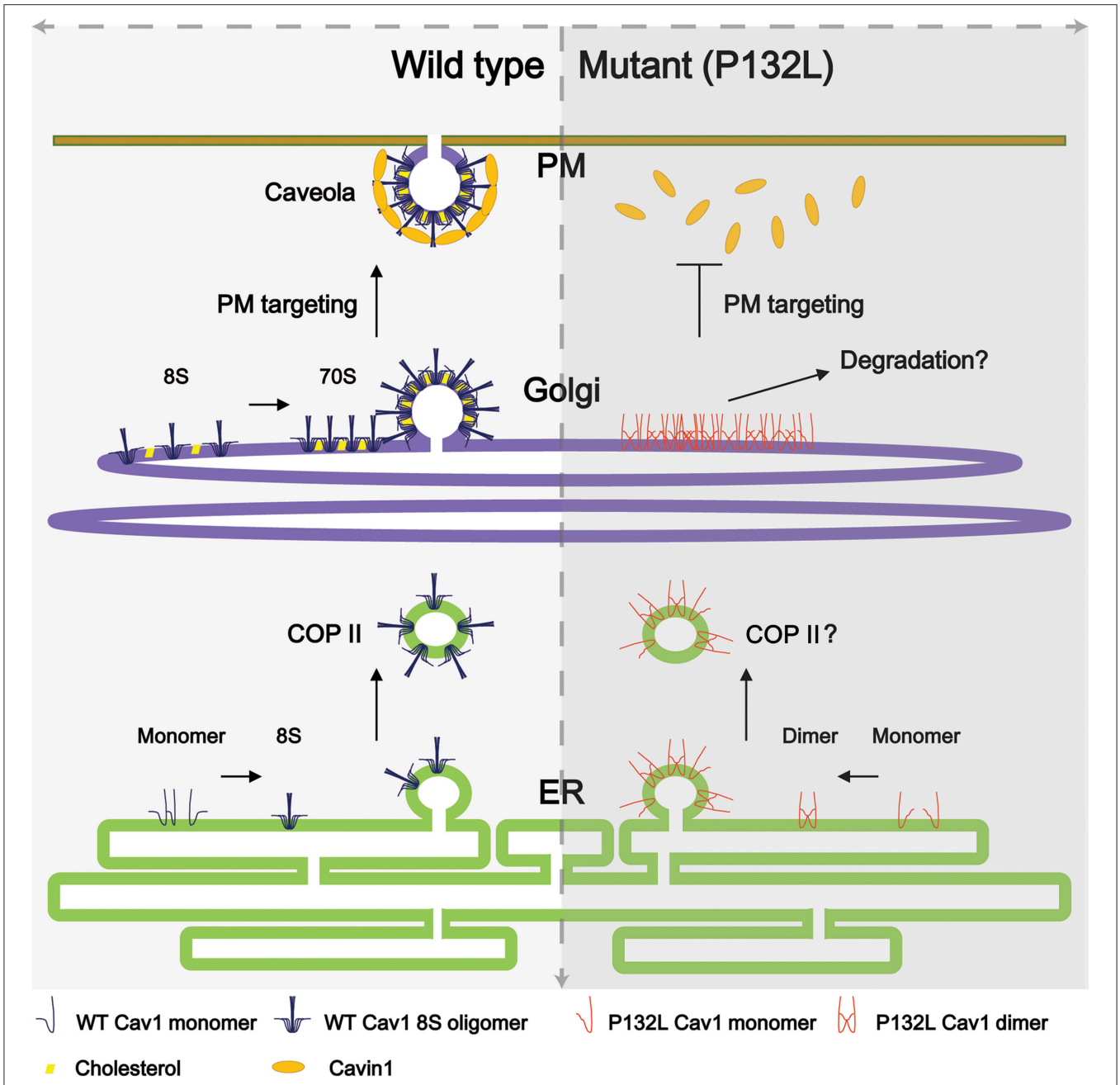

FIGURE 1 | Current model of caveolae biogenesis. (**Left**) Newly synthesized wild type Cav1 undergoes a series of oligomerization events as it passes through the secretory pathway. At the plasma membrane, accessory proteins interact with Cav1 complexes to form mature caveolae. (**Right**) In contrast, a breast-cancer associated mutant of Cav1, Cav1-P132L, is unable to oligomerize correctly and accumulates in the Golgi complex, where it is likely targeted for degradation. For simplicity, not all caveolae accessory proteins are illustrated here.

COS-7 cells, for example, Cav1-GFP strongly accumulates in a perinuclear compartment (Hanson et al., 2013) in the form of irregular aggregates that contain little if any endogenous Cav1 (Han et al., 2015). The behavior of Cav1-mCherry differs dramatically from that of Cav1-GFP in the same cell line, both in terms of its subcellular localization (Hanson et al., 2013) and biochemical properties (Han et al., 2015). Furthermore, the degree to which Cav1-GFP accumulates intracellularly depends on the cell type in which it is expressed (Hanson et al., 2013). Thus, the ability of Cav1 to form oligomers and traffic correctly to the plasma membrane is heavily dependent on how the protein is tagged as well as the cellular environment, pointing to the exquisitely sensitive nature of caveolae assembly.

WHAT MECHANISMS ARE RESPONSIBLE FOR THE TURNOVER OF CAV1 AND CAVEOLAE?

Cav1 is known to be a relatively long-lived protein; estimates of the half-life of endogenous Cav1 from metabolic labeling studies range from 5 to 36 h (Conrad et al., 1995; Forbes et al., 2007; Hayer et al., 2010b). Turnover of Cav1 is accelerated under conditions that compromise caveolar assembly and/or destabilize 70S caveolar scaffolds (Hayer et al., 2010b). Under these conditions, Cav1 is ubiquitinated and targeted to endosomal sorting complex required for transport (ESCRT) machinery via intraluminal vesicles of multi-vesicular bodies and subsequently is degraded within lysosomes (Hayer et al., 2010b). Thus, under these conditions Cav1 behaves as endocytic cargo that is targeted to early endosomes and follows a classical endocytic pathway leading to degradation.

More recent evidence has revealed additional cellular machinery involved in Cav1 turnover by this pathway. One major contributor is Valosin Containing Protein (VCP/p97), an AAA-ATPase that functions in processing of ubiquitinated cellular proteins. Along with its cofactor UBXD1, VCP binds to monoubiquitinated Cav1 on endosomes and in turn influences trafficking, endosomal sorting, and degradation of Cav1 (Ritz et al., 2011). The ubiquitination events required for targeting Cav1 into this pathway occur at the N-terminal region of the protein (Kirchner et al., 2013). Turnover of ubiquitinated Cav1 is aided by the Ankrd13 proteins, which contain a ubiquitin interacting motif that bind to polyubiquinated Cav1 oligomers on endosomes (Burana et al., 2016). While these studies have defined a distinct pathway that controls the turnover of Cav1, there are hints in the literature that additional machinery and mechanisms involved in Cav1 turnover remain to be discovered (Austin et al., 2012; Bakhshi et al., 2013; Cha et al., 2015; Mougeolle et al., 2015; Schrauwen et al., 2015).

HOW DO DISEASE-ASSOCIATED MUTATIONS AFFECT CAVEOLAE ASSEMBLY AND TURNOVER?

Cav1 has been implicated as a key player in a number of human diseases, and several disease-associated mutations in Cav1 have been identified (Hayashi et al., 2001; Razani and Lisanti, 2001; Cohen et al., 2004; Cao et al., 2008; Kim et al., 2008; Mercier et al., 2009; Austin et al., 2012; Ariotti and Parton, 2013; Garg et al., 2015; Martinez-Outschoorn et al., 2015). Perhaps the best known example is Cav1-P132L, originally identified as a somatic mutation associated with breast cancer (Hayashi et al., 2001). Although, the frequency with which this mutation occurs in humans has been highly debated (Hayashi et al., 2001; Lee et al., 2002; Koike et al., 2010; Lacroix-Triki et al., 2010; Ferraldeschi et al., 2012; Patani et al., 2012), Cav1-P132L has become a useful model for studying the behavior of mistrafficked forms of Cav1. This is because unlike wild type Cav1, Cav1-P132L typically localizes to the perinuclear region in a compartment proposed to correspond to the Golgi complex and does not form caveolae (Lee et al., 2002). Furthermore, Cav1-P132L primarily exists as monomer or dimer instead of the typical oligomers of wild type Cav1 observed in the cell (Lee et al., 2002; Ren et al., 2004; Hayer et al., 2010a; Rieth et al., 2012; Han et al., 2015). These features of Cav1-P132L differ substantially from the behavior of wild type Cav1 (**Figure 1**).

Interestingly, Cav1-P132L can also impact the behavior of wild type Cav1. In one of the earliest studies of Cav1-P132L, co-expression of Cav1-P132L with wild type Cav1 was shown to lead to a loss of wild type Cav1's affinity for detergent resistant membranes as well as to trap wild type Cav1 together with Cav1-P132L in a perinuclear compartment. Based on these findings, it was concluded that Cav1-P132L behaves in a dominant-negative manner, thereby interfering with caveolae formation (Lee et al., 2002). However, another study found that when co-expressed with wild type Cav1, Cav1-P132L had no effect on the localization of wild type Cav1 in FRT cells even though the mutant protein was localized in a perinuclear compartment (Ren et al., 2004). A different group showed that the number of caveolae increased upon stable expression of Cav1-P132L in H1299 cells, a cell line derived from human non-small cell carcinoma that endogenously expresses wild type Cav1 (Shatz et al., 2010). Thus, conflicting evidence exists as to how Cav1-P132L impacts caveolae assembly and function.

Why these behaviors of Cav1-P132L differ so much across studies is not yet clear. One potential clue comes from our recent observation that simply overexpressing Cav1-GFP causes a large fraction of the protein to be targeted to a perinuclear structure in COS-7 cells (Hanson et al., 2013). Furthermore, forms of Cav1 that were targeted to the plasma membrane when expressed separately became trapped intracellularly when they were co-expressed with Cav1-GFP (Hanson et al., 2013). Thus, in this system Cav1-GFP mimics the dominant negative trafficking defect originally reported for the Cav1-P123L mutant (Lee et al., 2002). Further, we observed that the majority of Cav1-GFP was degraded within 5 h, suggesting it may be improperly folded and thus targeted for degradation (Hanson et al., 2013). These findings raise the possibility that the dominant negative behavior reported for Cav1-P132L might at least in part be the result of misfolding induced by a combination of tagging and overexpression. They also raise questions about the identity of the perinuclear compartment that Cav1-GFP and Cav1-P132L accumulate in. In the case of Cav1-P132L, this compartment was originally proposed to correspond to the Golgi complex (Lee et al., 2002). However, given that perinuclear Cav1-GFP forms irregular aggregates, another possibility is that Cav1-GFP associates with aggresomes, structures that form in response to the accumulation of protein aggregates too large to be degraded by the proteasome (Wojcik et al., 1996; Johnston et al., 1998; Kopito, 2000; Garcia-Mata et al., 2002; Hyttinen et al., 2014). If this is the case, it would have important consequences for our current understanding of trafficking defects ascribed to mutant forms of both Cav1 and other caveolin family members, including a dominant negative P104L mutation in caveolin-3 associated with muscular dystrophy that corresponds to the P132L mutation in Cav1 (Carozzi et al., 2002; Sotgia et al., 2003; Pol et al., 2005; Cai et al., 2009).

In addition to Cav1-P132L, in recent years several additional disease-associated mutants of Cav1 have been identified, including one homozygous null mutation and three heterozygous frameshift mutations in the *Cav1* gene identified in patients with pulmonary arterial hypertension (PAH), lipodystrophy, or both (Kim et al., 2008; Austin et al., 2012; Garg et al., 2015; Schrauwen et al., 2015). The first mutation, c.G112T (p.E38X), is linked to lipodystrophy and leads to a complete loss of Cav1 protein expression (Kim et al., 2008). Two of the frameshift mutations, c.474delA (p.P158P fsX22), and c.473delC (p.P158H fsX22), generate a novel 21 amino acid-long C-terminus beyond amino acid position 158 associated with PAH (Austin et al., 2012). The third non-sense mutation, c.479_480delTT (p.F160X), introduces a premature stop codon that results in a truncated mutant protein lacking the last 19 amino acids of wild type Cav1 C-terminus. Interestingly, this mutation is associated with both PAH and congenital generalized lipodystrophy (Garg et al., 2015; Schrauwen et al., 2015).

How these mutant forms of Cav1 contribute to the development of PAH and/or congenital generalized lipodystrophy is not yet clear. However, one notable similarity shared by P158P/H and F160X is that they occur in the distal C-terminus of Cav1. This domain of Cav1 is thought to be important for Cav1 homo-oligomerization, Golgi-plasma membrane trafficking, and DRM association (Song et al., 1997; Machleidt et al., 2000). Initial studies in patient skin fibroblasts show that the presence of either P158P fsX22 or the truncation mutant F160X lead to decreased Cav1 protein levels (Austin et al., 2012; Schrauwen et al., 2015). It is thus possible that at least some of the newly identified PAH-associated Cav1 mutants are targeted for degradation, and may also function as dominant negatives against wild type Cav1. Caveolae assembly appears to be at least partially preserved for the case of the F160X mutation (Garg et al., 2015), although pathway analysis indicates its expression impacts signaling pathways that are important adipose tissue homeostasis (Schrauwen et al., 2015). It will be interesting to determine whether caveolae form correctly for the P158P mutants and whether Cav1 follows a conventional trafficking and degradative pathway in these patients.

CONCLUSION

In summary, our understanding of how Cav1 assembles to form caveolae and is turned over outside of caveolae has increased tremendously over the past few years, yet is far from complete. A clear model of caveolae biogenesis has emerged, but additional work is needed to understand how disease-associated Cav1 mutants impact this process. Indeed, how wild type Cav1 itself is packed into caveolae is not yet entirely clear. How cells dispose of Cav1 in response to stress, and whether similar or different mechanisms are utilized to target various disease-associated mutants of Cav1 for degradation also remain to be more fully investigated. Some of these processes may be mimicked by overexpression of tagged forms of Cav1. Thus, further investigation of what may at first glance appear to be an artifact of tissue culture may ultimately reveal mechanisms that are of physiological and/or pathophysiological importance. Finally, it is important to recognize that a consensus model for how caveolae function does not yet exist (Cheng and Nichols, 2016). An important challenge for the future will be to better understand how abundance and structure of caveolae control the many functions currently ascribed to this intriguing class of membrane domains.

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Drafted the manuscript or revising it critically for important intellectual content: BH, AT, CC, AK. Approved the final version of the manuscript to be published: BH, AT, CC, AK. Agree to be accountable for all aspects of the work: BH, AT, CC, AK.

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REVIEW
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New Insights into How Trafficking Regulates T Cell Receptor Signaling

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There is emerging evidence that exocytosis plays an important role in regulating T cell receptor (TCR) signaling. The trafficking molecules involved in lytic granule (LG) secretion in cytotoxic T lymphocytes (CTL) have been well-studied due to the immune disorder known as familial hemophagocytic lymphohistiocytosis (FHLH). However, the knowledge of trafficking machineries regulating the exocytosis of receptors and signaling molecules remains quite limited. In this review, we summarize the reported trafficking molecules involved in the transport of the TCR and downstream signaling molecules to the cell surface. By combining this information with the known knowledge of LG exocytosis and general exocytic trafficking machinery, we attempt to draw a more complete picture of how the TCR signaling network and exocytic trafficking matrix are interconnected to facilitate T cell activation. This also highlights how membrane compartmentalization facilitates the spatiotemporal organization of cellular responses that are essential for immune functions.

Keywords: TCR signaling, exocytic trafficking, LAT, Rabs, SNAREs

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INTRODUCTION

The key signaling molecules involved in the T Cell Receptor (TCR) signaling network have been well-characterized. T cell signaling is initiated upon TCR engagement by major histocompatibility complex (MHC) molecules bound to peptide antigens (pMHC). Upon TCR engagement, the TCR-associated CD3 dimers are phosphorylated by the kinase Lck on intracellular immunoreceptor tyrosine-based activation motif (ITAM) consensus sites, leading to the recruitment and activation of downstream signaling molecules, such as the adaptor protein Linker for Activated T cells (LAT). Subsequent activation responses including the secretion of lytic granules (LG) target infected or cancer cells for lysis. The formation of a structured interface between a T cell and an antigen-presenting cell (APC), termed the immunological synapse, is critical for the efficient delivery of effector molecules to the APC and intracellular signals in the T cell. The architecture of the immunological synapse was first described in 1998 as a "bull's eye pattern," consisting of the central supramolecular activation cluster (cSMAC), where signaling molecules such as the TCR accumulated, surrounded by a ring of adhesion molecules known as the peripheral SMAC (pSMAC), with other molecules such as CD45 being excluded and localized in the distal SMAC (dSMAC, **Figure 1**; Monks et al., 1998; Grakoui et al., 1999; Freiberg et al., 2002).

Fluorescence microscopy has aided our understanding of the mechanisms underlying the coalescence and segregation of receptors and signaling molecules in the plasma membrane during synapse formation. Many studies focused on how the two-dimensional organization of proteins

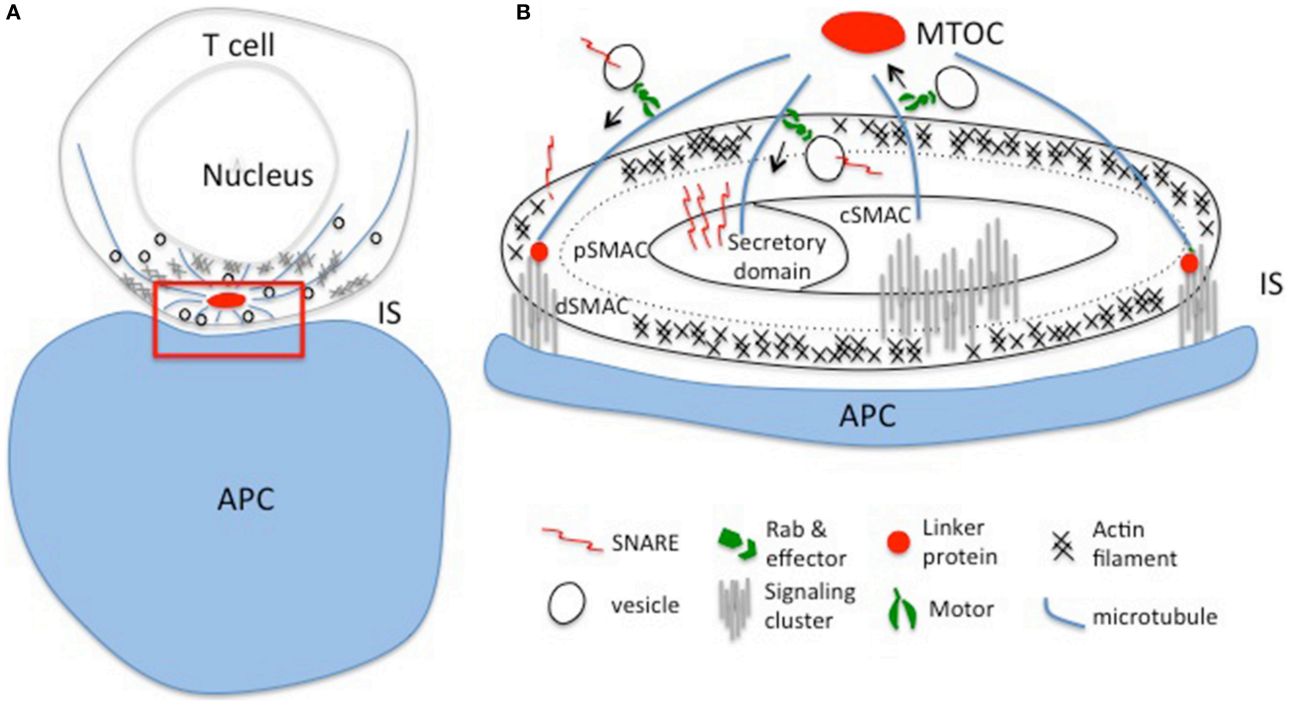

FIGURE 1 | Polarized exocytosis initiated by cytoskeleton reorganization upon TCR activation. (A) T cell receptors encounter their cognate antigen on the APC leading to the formation of an immunological synapse and polarized intracellular trafficking established by the reorganization of actin and microtubule networks. Polarized intracellular trafficking is essential for directed release of effectors and local accumulation of signaling molecules at the immunological synapse. **(B)** Enlargement of the red region in panel **(A)**. Vesicles travel along actin and mainly microtubule networks beneath the synapse. Rabs and SNAREs, and linker proteins connect the cytoskeletal network with the plasma membrane to ensure precise spatio-temporal control of the delivery of effectors and signaling molecules to the different domains of the synapse: the secretory domain, the central (cSMAC), peripheral (pSMAC), and distal (dSMAC) central supramolecular activation clusters. IS, immunological synapse; APC, antigen presenting cell; MTOC, microtubule organizing center.

within the synapse contributes to T cell activation. However, as three-dimensional objects, cells can utilize an additional layer of regulatory mechanisms to control TCR signaling outcomes. Emerging evidence suggests that intracellular vesicular trafficking plays an important role in orchestrating TCR signaling. Engaged TCRs are internalized and targeted for degradation, but can also remain phosphorylated and signaling-competent (Luton et al., 1997; Coombs et al., 2002; Yudushkin and Vale, 2010; Benzing et al., 2013). Continuous delivery of the TCR into the immunological synapse is essential for sustained signaling and T cell activation (Grakoui et al., 1999; Lee et al., 2003; Soares et al., 2013b; Choudhuri et al., 2014; Martin-Cofreces et al., 2014). For example, although Lck activity is enhanced by TCR ligation (Stirnweiss et al., 2013), it is thought that the spatial organization and subcellular redistribution of Lck, in conjunction with antigen-binding induced conformational changes of the TCR-CD3 complex (Martinez-Martin et al., 2009; Swamy et al., 2016), control the extent of TCR-CD3 phosphorylation (Ehrlich et al., 2002; Thoulouze et al., 2006; Anton et al., 2008; Nika et al., 2010; Rossy et al., 2013). In the case of the adaptor protein LAT, the docking of sub-synaptic vesicles was observed in response to the initial wave of TCR signaling to sustain TCR signaling (Bonello et al., 2004; Billadeau, 2010; Purbhoo et al., 2010; Williamson et al., 2011; Larghi et al., 2013). In addition to the classical process of vesicles forming inside the cell, docking, and fusing at the immunological synapse, TCR-enriched microvesicles can also form and be released from the center of the IS to transmit signals to the APC (Choudhuri et al., 2014).

In T cells, there is a continuous flow of proteins and membranes along the endocytic and exocytic pathways. When a T cell encounters an APC, ligation of the TCR with cognate pMHC molecules leads to rapid cytoskeletal reorganization/polarization, which ensures the initiation of receptor and signaling protein endocytosis, and polarized secretion of LG and other vesicles (Dustin and Cooper, 2000; Angus and Griffiths, 2013). The internalization of the TCR-CD3 complex and downstream signaling proteins including LAT and SLP76 (SH2 domain containing leukocyte protein of 76 kDa) is relatively well-studied. Upon TCR activation, both engaged and bystander TCR can be internalized by clathrin independent, and/or dependent endocytosis (Monjas et al., 2004). Internalized engaged TCR-CD3 complexes are mainly degraded, while bystander TCR-CD3 complexes are predominately recycled back to the immunological synapse (Liu et al., 2000; Monjas et al., 2004; von Essen et al., 2004). SLP76 and LAT are first recruited to TCR and ZAP70 clusters in the plasma membrane and then segregate from these clusters during the transport of engaged TCR toward the central region of the immunological synapse

within the membrane. This initiates LAT and SLP76 endocytosis (Barr et al., 2006). Internalized LAT were found to co-localize with transferrin positive vesicles, indicating a clathrin-mediated endocytic trafficking route, as well as cholera toxin B positive vesicles that do not co-localize with transferrin-positive vesicles in T cells and distinct SLP76-positive vesicles (Balagopalan et al., 2009). The E3 ubiquitin ligase Cbl and Cbl-b are known to play a key role in both TCR and LAT internalization (Naramura et al., 2002; Balagopalan et al., 2007). Noteworthy, no detectable LAT degradation seems to occur in response to TCR activation, indicating LAT is mainly recycled instead of being degraded after internalization, although LAT was ubiquitinated in response to TCR signaling (Balagopalan et al., 2007).

Vesicles containing cargo such as signaling molecules or effector molecules are transported along the microtubules or actin filament networks by molecular motors and delivered to the immunological synapse. The precise sorting and delivery of cargo are dependent on a subfamily of Ras GTPases, called Rab proteins (Fukuda, 2008; Wandinger-Ness and Zerial, 2014), and on membrane docking and fusion machinery proteins, known as soluble N-ethylmaleimide-sensitive-factor accessory-protein receptors (SNAREs; Das et al., 2004; Jahn and Scheller, 2006; **Figure 1**). Endosomal sorting complexes required for transport (ESCRT) components mediate the release into the extracellular space of vesicles that form at the immunological synapse (Choudhuri et al., 2014). In this review, we will summarize the known Rab and SNARE proteins involved in both the delivery of signaling molecules and the secretion of effectors at the immunological synapse in response to TCR activation. We will also summarize the known protein–protein interactions that may facilitate efficient and precise delivery of signaling molecule-containing vesicles in the activated TCR signaling network, using LAT-containing vesicles as an example.

EXOCYTOSIS MACHINERY OF EFFECTOR AND SIGNALING MOLECULES

The TCR and downstream signaling proteins undergo constant internalization and exocytosis in resting T cells (Geisler, 2004). Upon TCR activation, polarized exocytosis is initiated by cytoskeletal rearrangements, which may be sufficient for receptors and signaling molecules to locally accumulate in the immunological synapse. So far, little is known about the molecular machinery involved in exocytosis of TCR and TCR signaling proteins, neither in resting nor in activated T cells. The monomeric G protein Rabs are the coordinators of intracellular membrane trafficking of TCR and downstream signaling proteins. Soares and colleagues have evaluated he role of 17 Rabs known to be involved in exocytic processes and examined TCR, Lck, and LAT exocytosis during TCR activation. They demonstrated that TCRζ can be delivered to the immunological synapse from fast recycling Rab4b compartments. A newly synthetized pool of TCR is also brought to the immunological synapse *via* Rab3d and Rab8b-positive compartments (Soares et al., 2013a). In addition, the TCR has been reported to co-localize with Rab35 and transferrin-positive compartments. It has been further shown that a Rab35-dominant negative mutation impairs TCR enrichment at the immunological synapse (Das et al., 2004; Patino-Lopez et al., 2008). Lck-containing vesicular compartments co-localized with the recycling endosome marker Rab11b (Soares et al., 2013a). Further, LAT vesicles co-localized with late endosome marker Rab7, newly synthesized protein pool Rab8 positive compartments, as well as Rab27a and Rab37, two Rab molecules known to regulate LG and cytokine secretion, respectively (Hong, 2005; Fischer et al., 2007; Purbhoo et al., 2010; Fukuda, 2013; Soares et al., 2013a; **Figure 2**). Hence, according to these studies, there is very little overlap between the identities of vesicles containing the TCR, Lck, and LAT, which suggest that distinct trafficking mechanisms exist for different signaling molecules. Hence, the intracellular reservoir of TCR, Lck, and LAT destined for transport to the plasma membrane and/or exocytosis are clearly distinct from each other, and range from slow and fast recycling compartments, to Golgi, and late endosome/lysosome (**Figure 2**). The contribution of this complex organization to T cell signaling and activation remains to be fully understood.

In general, GTP-bound active Rab proteins regulate many steps of intracellular membrane trafficking by recruiting different effectors to restricted membrane domains (Grosshans et al., 2006). Rab effectors are very diverse and can be divided into three groups according to the vesicle trafficking steps they are involved in, such as vesicle formation, trafficking along cytoskeletal networks, and vesicle fusion. Within the LG secretion pathway, the role of Rab27a and its effectors has been identified in relation to familial hemophagocytic lymphohistiocytosis (FHLH) and Griscelli syndrome type2 (GS; Fukuda, 2008, 2013; Krzewski and Cullinane, 2013). Granule exocytosis by cytotoxic T lymphocytes (CTLs) is defective in these patients, and this was found to be due to dysfunctional Rab27a (Menasche et al., 2000; Haddad et al., 2001). Rab27a effectors are synaptotagmin-like proteins (Slp) and typically contain a Slp homology domain (SHD, Rab27a-binding domain) and two C2 domains (for binding to phospholipids and potentially calcium). Slp3-a forms a complex with Rab27a and the motor protein kinesin-1 and was demonstrated to mediate the terminal transport of LG to the immune synapse (Kurowska et al., 2012). There is evidence that Slp2-a is also involved in docking of LG in CTLs by binding to Rab27a and the plasma membrane (Menasche et al., 2008). Mutations of another Rab27a effector (Holt et al., 2008), Munc13-4, caused immunodeficiency in patients with type 3 FHLH. Munc13-4 plays a role in LG maturation and also drives the SNARE assembly process and is critical for the priming/fusion step of LG exocytosis (Feldmann et al., 2003). In addition, Munc13-4 has been also demonstrated to mediate fusion of Rab11-positive recycling vesicles with Rab27-positive late vesicles, constituting a pool of vesicles destined for regulated exocytosis (Menager et al., 2007). Munc18-2 promotes SNARE complex assembly (Hackmann et al., 2013; Spessott et al., 2015) and is not a direct effector but binds to another Rab27a effector, Slp-4a (Fischer et al., 2007; Jenkins and Griffiths, 2010; Hackmann et al., 2013; Krzewski and Cullinane, 2013).

Until now, limited information has been obtained regarding the role of Rab effectors in the regulation of exocytosis of

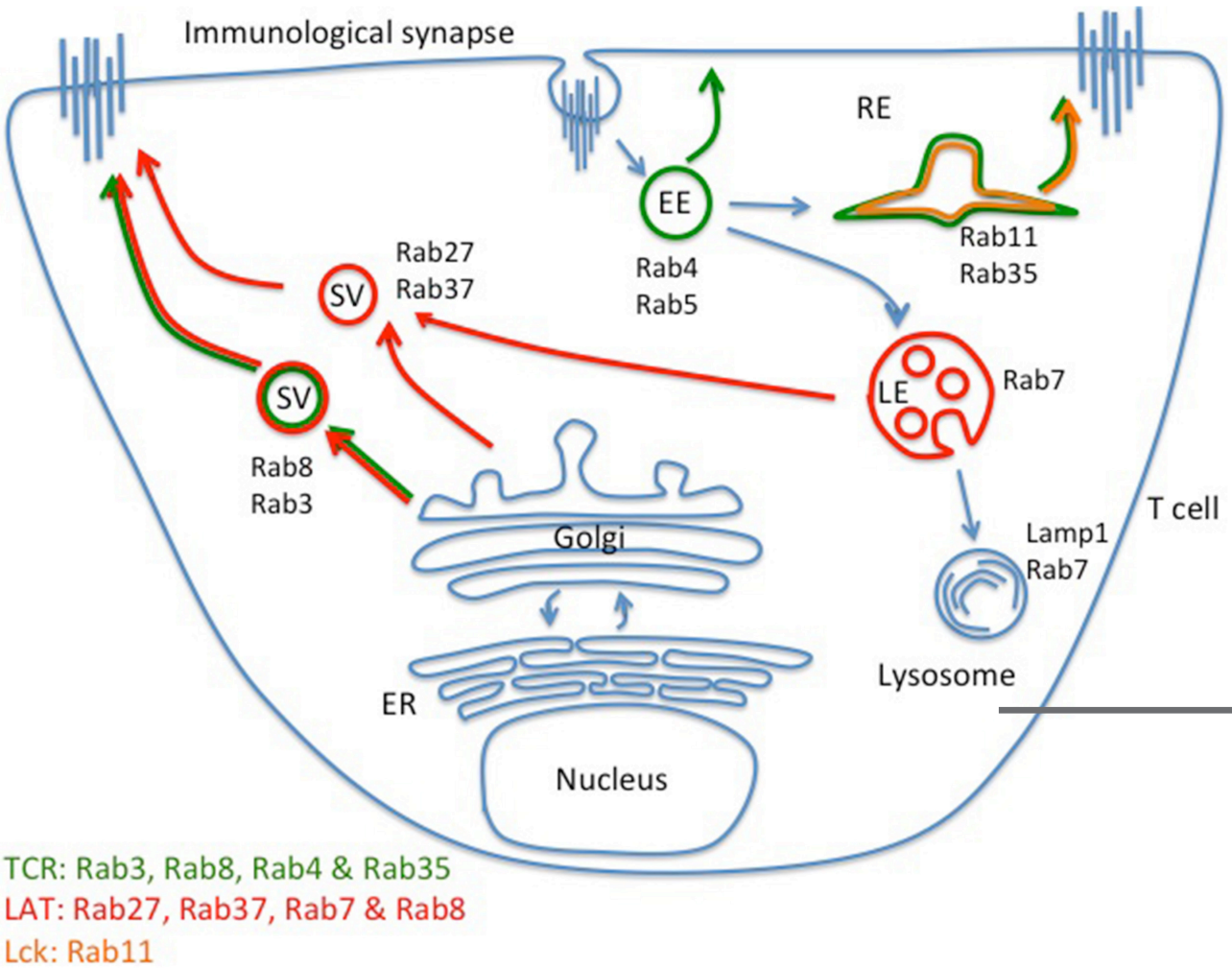

FIGURE 2 | Distinct intracellular compartments control TCR, LAT, and Lck exocytosis. Regulated exocytosis of receptors and signaling molecules is initiated upon TCR activation. According to current understanding, exocytosis of the TCR-CD3 complex is mainly facilitated by newly formed Rab8- and Rab3-positive vesicles. Fast (Rab4-positive vesicles) and slow (Rab11-positive vesicles) recycling compartments can also contribute to the delivery of the TCR-CD3 complex to the immunological synapse. Lck co-localizes with Rab11-positive vesicles. LAT vesicles mainly contain the late endosome/lysosome markers of Rab27 and Rab37 but LAT also co-localizes with newly synthesized Rab8-positive vesicles. There appears to be little overlap between the TCR, LAT, and Lck exocytic trafficking pathways, indicating that distinct trafficking routes of signaling molecules may facilitate segregation before stimulation, and efficient delivery to and high number of encounters within the immunological synapse after stimulation. EE, early endosome; LE, late endosome; RE, recycling endosome; SV, secreting vesicle.

TCR and signaling molecules. **Table 1** summarizes the known effectors that are expressed in T cells for Rabs involved in exocytosis of TCR and signaling molecules (Grosshans et al., 2006; Fukuda, 2013; Wandinger-Ness and Zerial, 2014). Through Rab effectors, the Rab network is connected to the SNARE system, linking the transportation of exocytic vesicles in the cytosol to their docking and fusion with the plasma membrane. Prior to membrane fusion, SNAREs on opposing membranes are able to form four-helix bundles that lead to a tight connection of vesicular and target membranes. SNARE complexes can be divided into two groups, vesicle SNAREs (vSNAREs), and target membrane SNAREs (tSNAREs). SNAREs can also be divided into either Q- (Qa, Qb, Qc, or Qbc, mainly tSNAREs) or R-SNAREs (mainly vSNAREs) based on their structure. In general, the four helix-bundle of a functional SNARE complex must consist of one Qa-SNARE, one Qb-SNARE, one Qc-SNARE, and one R-SNARE. In some cases, the two helices of the Qb- and the Qc-SNARE can be provided by one protein (Jahn and Scheller, 2006). In addition to the core SNARE complex, the calcium sensors synaptotagmins, and synaptotagmin-like proteins facilitate the assembly of the SNARE complex and trigger the final membrane fusion step.

Pattu and colleagues have examined the expression of 28 SNAREs and their co-localization with LGs and TCR-CD3 complex in synapses in primary human CD8+ T cells. They found that Stx16 Vtib and Stx8 had the highest co-localization with LGs while TCR-CD3 co-localized with Stx3, Stx4, Stx7, Stx13, Vtib, Stx6, Stx8, VAMP3, and VAMP4 (Pattu et al., 2012).

TABLE 1 | Effectors for Rabs involved in the exocytosis of TCR and TCR signaling molecules.

Signaling molecule	Rabs	Effectors	Effector expression in T cell	Proposed functions
TCR	Rab3d	Regulating synaptic membrane exocytosis 1/2(RIMS1/2)	Low/medium	Regulates synaptic vesicle exocytosis, regulates voltage-gated calcium channels, scaffolding protein (Millar et al., 2002; Gandini et al., 2011)
		Rabphilin 3A like (without C2 domain; RPH3AL)	Low	A direct regulatory role in calcium ion-dependent exocytosis (Li et al., 1994; Millar et al., 2002; Grosshans et al., 2006)
	Rab4b	Rabaptin, Rab GTPase-binding effector protein 1 (RabEP1)	High	Acts as a linker between adaptin and Rab4 and Rab5, involved in endocytic membrane fusion (Vitale et al., 1998)
		RUN and FYVE domain containing 1 (RUFY1)	Medium	Binds to phospholipid vesicles and participates in early endosomal trafficking (Cormont et al., 2001; Fouraux et al., 2004)
		RAB11 family-interacting protein 1 (RAB11FIP1)	Medium	Regulates Rab GTPases (Lindsay et al., 2002)
		CD2-associated protein (CD2AP)	Medium	Regulates actin cytoskeleton (Kirsch et al., 1999; Cormont et al., 2003)
		GRIP1 associated protein 1 (GRIPSP1)	Medium	Interacting with endosomal SNARE syntaxin 13 (Hoogenraad et al., 2010)
	Rab8b	Otoferlin (OTOF)	Low	Calcium sensor, regulates vesicle membrane fusion in calcium-dependent manner (Roux et al., 2006)
		Synaptotagmin-like 1 (SYTL1)/SLP1/exophilin-7	Medium	Binds to PI3,4,5P3 (Hattula et al., 2006)
		Optineurin/RAB11 family-interacting protein 2 (OPTN/RAB11FIP2)	Medium	Interacts with myosin VI (Ying and Yue, 2012)
		Mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2)	Medium	Serine/threonine protein kinase (Katz et al., 1994; Ren et al., 1996)
		MICAL (microtubule associated monooxygenase, calponin, and LIM domain containing)-like 1 (MICALL1)	Medium	Linking EHD1 and Rab8 on recycling endosomal membrane tubules (Sharma et al., 2009)
		MICAL (microtubule associated monooxygenase, calponin, and LIM domain containing)-like 2 (MICALL2)/junctional Rab13-binding protein (JRAB)	Low	Regulates the endocytic recycling of occludins, claudins and E-cadherin to the plasma membrane, may regulate actin cytoskeleton (Yamamura et al., 2008)
		Oculocerebrorenal syndrome of Lowe (OCRL)	Mixed reports of low and high expression	Phosphatase enzyme involved in actin polymerization and may function in lysosomal membrane trafficking (Hagemann et al., 2012; Luo et al., 2012)
		Myosin 5B (MYO5B)	Low	Motor protein, travels toward the plus end of actin filaments (Khandelwal et al., 2013)
	Rab35	Oculocerebrorenal syndrome of Lowe OCRL	Mixed reports of low and high expression	Phosphatase enzyme involved in actin polymerization and may function in lysosomal membrane trafficking (Dambournet et al., 2011)
		Fascin actin-bundling protein 1 (FSCN1)	Medium	Actin crosslinking protein (Zhang et al., 2009)
		Run and SH3 domain containing 1 (RUSC1/NESCA)	Low	RUN and SH3 domain containing protein (Fukuda et al., 2011; Chaineau et al., 2013)
		Microtubule associated monooxygenase, Calponin and LIM domain containing 1 (MICAL1)	Medium/High	Disassemble actin filament (Chaineau et al., 2013)
		MICAL like protein 1 (MICAL-L1)	Medium/High	Interacting with EHD1 (Rahajeng et al., 2012)
		ArfGAP with coiled-coil, Ankyrin repeat and PH domains 2 (ACAP2)	Medium	Arf GAP (Kobayashi and Fukuda, 2012)

(Continued)

TABLE 1 | Continued

Signaling molecule	Rabs	Effectors	Effector expression in T cell	Proposed functions
Lck	Rab11b	Optineurin/RAB11 family-interacting protein 2 (OPTN/RAB11FIP2)	Medium	Interacts with myosin VI (Wandinger-Ness and Zerial, 2014)
		Phosphatidylinositol 4-kinase beta (PI4KB)	High	Regulates the trafficking from Golgi to plasma membrane (de Graaf et al., 2004)
		TBC1 domain family membrane 14 (TBC1D14)	Low	Regulates autophagosome formation (Longatti et al., 2012)
		WD repeat domain 44/Rabphilin-11/rab11-binding protein (WDR44)	High	Plays a role in endosome recycling (Wandinger-Ness and Zerial, 2014; Vetter et al., 2015)
		Zinc finger FYVE domain-containing 27 (ZFYVE27)	Medium	Functions as an upstream inhibitor of Rab11 (Shirane and Nakayama, 2006)
		Exocyst complex component 6 (EXOC6)/Sec15	Medium	Essential for vesicular traffic from Golgi apparatus to the cell surface (Zhang et al., 2004; Wu et al., 2005)
		Myosin 5B (MYO5B)	Low	Motor protein, travels toward the plus end of actin filaments (Roland et al., 2011)
LAT	Rab27a	Synaptotagmin-like 2 (SYTL2)/SLP2-a/exophilin-4	High	Calcium sensor, binds to PS, PI(4,5)P2 (Galvez-Santisteban et al., 2012)
		Synaptotagmin-like 1 (SYTL1)/SLP1/exophilin-7	Medium	Calcium sensor, binds to PI(3,4,5)P3 (Brzezinska et al., 2008)
		Synaptotagmin-like 3 (SYTL3)/SLP3-a/exophilin-6	Low	Forms Rab27a/kinesin-1/SYTL3 complex (Gibbs et al., 2004; Fukuda, 2013)
		Unc-13 homolog D (Unc13d)/Munc13-4	High	Controls the priming/fusion step of LG exocytosis (Feldmann et al., 2003; Brzezinska et al., 2008)
		Myosin 5B (MYO5B)	Low	Travels toward the plus end of actin filaments (Gibbs et al., 2004; Fukuda, 2013)
		Rabphilin 3A like (without C2 domain; RPH3AL)	Low	A direct regulatory role in calcium ion-dependent exocytosis (Izumi, 2007; Fukuda, 2013)
		Melanophilin (MLPH)	Medium	Forms a ternary complex with Rab27a and myosin Va (Strom et al., 2002)
		Coronin, actin-binding protein, 1C (CORO1C)	Low	Binds to GDP-bound form of Rab27a, regulates endocytosis of secretory membrane (Kimura et al., 2008)
	Rab37	Regulating synaptic membrane exocytosis 1 (RIMS1)	Low	Regulates synaptic vesicle exocytosis, regulates voltage-gated calcium channels, scaffolding protein (Wandinger-Ness and Zerial, 2014)

In other reports, vSNARE VAMP2, VAMP8, tSNARE Stx7, Stx 11 Vti1b were found to be involved in LG secretion (Dressel et al., 2010; Pattu et al., 2011; Qu et al., 2011; Halimani et al., 2014; Marshall et al., 2015). For TCR exocytosis, vSNARE, VAMP2, VAMP3, VAMP7 as well as tSNARE Vti1B have been shown to play a role (Das et al., 2004; Pattu et al., 2011; Qu et al., 2011; Matti et al., 2013; Soares et al., 2013a; Finetti et al., 2015b). In Jurkat cells, tSNARE SNAP23 and Stx4 accumulated at the immunological synapse indicating that those SNAREs may also play a role in regulated exocytosis upon TCR activation (Das et al., 2004). Exocytosis of Lat vesicles in response to TCR activation relies on VAMP7 and synaptotagmin 7 (Syt7). Although vesicle fusion is the classical role of Syt7, no evidence has emerged to date that VAMP7- and Syt7-positive LAT vesicles fuse with the plasma membrane but it remains an open question whether LAT vesicles in activated T cells dock at or fuse with the plasma membrane (Williamson et al., 2011; Larghi et al., 2013; Soares et al., 2013a). Further work is required to draw a more complete picture of how SNARE complexes regulate the secretion of LG and the exocytosis of receptors and signaling molecules. The ultimate goal is to understand how such a great diversity and redundancy in the tethering-fusion apparatus during T cell activation regulate the fusion of TCR, Lat, or Lck containing vesicles to specific areas of the immunological synapse.

ADDITIONAL TRAFFICKING MECHANISMS FOR TCR SIGNALING MOLECULES

In addition to the classical machinery mediating trafficking—facilitated by Rab proteins—and fusion to the plasma membrane—facilitated by SNARES—other proteins have also been demonstrated to regulate the targeting of TCR, Lck,

and LAT to the immune synapse. Sorting nexin 17 (SNX17) and SNX27 are implicated in recycling TCR toward the immunological synapse (Rincon et al., 2011; Osborne et al., 2015). Intraflagellar transport (IFT) particles generally mediate the assembly of cilia, but in T cells, IFT20 was found to co-localize with the microtubule organizing center (MTOC), and Golgi and post-Golgi compartments. Knocking down IFT20 resulted in the TCR-CD3 complex accumulating in Rab5 endosomes and failure to be properly recycled toward the immunological synapse (Finetti et al., 2009, 2015a; Finetti and Baldari, 2013). Recently, Vivar and colleagues demonstrated that IFT20 was also required for the delivery of the intracellular pool of LAT to the immunological synapse in primary murine CD4+ T cell (Vivar et al., 2016). The polarized recycling of TCR signaling proteins appears to be highly regulated. Indeed, Lck delivery to the immunological synapse relies on Unc119, which controls the transportation of Lck-positive endosomal compartments by regulating Rab11 activation, and orchestrating the recruitment of the actin-based motor protein, myosin 5B (Gorska et al., 2009). In addition, the membrane protein MAL, which is redistributed to the cSMAC upon T cell activation, contributes to target the delivery of Lck and LAT to the center of the immunological synapse (Anton et al., 2008, 2011). Microtubule plus end binding protein EB1 may contribute in directing LAT vesicles to the TCR-ZAP70 signaling complex. Indeed, in activated cells, knocking down EB1 impairs TCR clustering at the plasma membrane and the sustained activation of LAT and PLCγ1. But more importantly, the absence of EB1 prevents the encounters between LAT vesicles and CD3 vesicles at the immunological synapse (Martin-Cofreces et al., 2012).

Together, these studies show that several regulators are involved in fine-tuning how and when vesicles from the recycling pathway are redirected to bring components of the TCR signaling pathway to the immunological synapse. This suggests that targeted recycling has a critical role in T cell activation, which probably goes beyond the mere shuttling of membrane proteins to the plasma membrane, and might contribute to the spatiotemporal regulation of TCR signaling.

TRAFFICKING VS. SIGNALING—THE EXAMPLE OF LAT-POSITIVE VESICLES

LAT is an essential adaptor protein that is recruited to phosphorylated TCR-CD3 complexes at the cell surface. The kinase ZAP70 is responsible for phosphorylating tyrosine residues in cytoplasmic tail of LAT. Due to the nine tyrosine residues, LAT acts as a scaffolding protein for downstream signaling molecules including SLP-76, PLC-γ1, Grb2, Gads, Sos1, and so on. Recently the interactome of LAT was mapped out, which revealed 112 unique interactions in the ZAP70-LAT-SLP-76 signaling axis (Malissen et al., 2014). Previous imaging and biochemistry studies identified two pools of LAT, a cytoplasmic vesicular pool, and a plasma membrane pool that forms nano-clusters. To date, the functional differences between these two pools of LAT is not clear but the notion was put forward that LAT at the plasma membrane is involved in initial TCR signaling while vesicular LAT is required for signal amplification (Bonello et al., 2004; Purbhoo et al., 2010; Williamson et al., 2011; Balagopalan et al., 2013; Larghi et al., 2013; Soares et al., 2013a).

With respect to LAT vesicles, calcium fluxes in the context of Lck-mediated TCR signaling have emerged as one of the key factors in regulating the trafficking of these vesicles. Calcium influx initiated by the first wave of TCR signaling triggers the sub-synaptic LAT vesicle exocytosis which further propagates TCR signaling (Soares et al., 2013a). TCR activation initiates Lck spatial reorganization that conditions subsequent LAT vesicle delivery. Interfering with Lck translocation by knocking down MAL protein impairs LAT vesicle exocytosis. However it can be rescued by artificially creating calcium influx (Soares et al., 2013a). Reducing LAT exocytosis, by interfering with Lck exocytosis, or silencing calcium sensor Syt7 or vesicle SNARE VAMP7 decreased LAT phosphorylation and IL2 production in general (Williamson et al., 2011; Larghi et al., 2013; Soares et al., 2013a). Single molecule localization microscopy (SMLM) revealed that LAT and pLAT clusters number as well as cluster size decreased by blocking LAT vesicle exocytosis during TCR activation (Larghi et al., 2013). Moreover, the presence of interacting signaling nano-territories between LAT and SLP76 was also impaired by blocking LAT exocytosis (Soares et al., 2013a). Interestingly, the long, tubular-shaped LAT clusters almost disappeared when LAT exocytosis was blocked (Soares et al., 2013a). These long tubular LAT vesicles travel toward the immunological synapse during TCR activation (Bonello et al., 2004; Billadeau, 2010), supporting the idea that LAT vesicles do not fuse with the immunological synapse. Larghi et al. expressed LAT molecule that presented a HA-TEV-tag on the extracellular site. By cleaving the HA tag with TEV protease in plasma membrane LAT population before T cell activation, despite the enrichment of HA-TEV-LAT vesicles at the sub-synaptic membrane after TCR stimulation, LAT was not recognized with antibodies to HA in non-permeabilized cells, indicating that HA-TEV-LAT vesicles had not fused with the plasma membrane (Larghi et al., 2013). Since LAT vesicles appear to carry phosphorylated LAT in activated T cells (Williamson et al., 2011), it is possible that vesicular LAT is phosphorylated in *trans* by the TCR-ZAP70 complex in the plasma membrane. Given that LAT vesicles appear to dock at the plasma membrane for only ∼1 min (Purbhoo et al., 2010; Williamson et al., 2011), many LAT molecules could be rapidly phosphorylated in that manner, leading to signal amplification and spreading throughout the cell.

CONCLUSION

Extensive research has focused on understanding the TCR signaling network and signal-regulating mechanisms during T cell activation due to the essential roles of T cells in the adaptive immune system. Surprisingly, current knowledge of the trafficking machineries involved in the intracellular movement of TCR and signaling molecules remains very limited. In this review, we attempted to summarize the known trafficking machinery involved in the exocytosis of TCR, downstream signaling molecules and effectors during TCR activation. By putting this information into context, we tried to draw a more complete

picture of trafficking networks involved in the regulation of TCR signaling.

It emerges that key signaling molecules involved in early TCR signaling reside in distinct vesicle subpopulations that contain non-overlapping SNARE molecules (Das et al., 2004; Pattu et al., 2012; Larghi et al., 2013; Matti et al., 2013; Finetti et al., 2015b). Despite their differences, it seems that fusion with the plasma membrane might not be the final destination of some populations of vesicles, raising the question of how trafficking *per se* can contribute to regulate T cell signaling. Rather than distributing signaling molecules across the cell, and regulating signaling by changing the local concentration of available signaling molecules, vesicles themselves may be signaling entities. This is illustrated in the emerging concept of LAT vesicles that do not appear to fuse with the plasma membrane but are nevertheless phosphorylated upon TCR stimulation (Williamson et al., 2011; Larghi et al., 2013). Such vesicles resemble signaling endosomes (Benzing et al., 2013) but contain the trafficking machinery for exocytosis (Larghi et al., 2013; Soares et al., 2013a). Thus, LAT vesicles blur the lines between exocytosis, endocytosis, and signaling. Quantitative imaging may reveal whether LAT vesicles indeed amplify the initial TCR signals in the coming years. This would constitute a new perspective, as the trafficking machinery is not solely used to deliver molecules to and from the plasma membrane but to directly facilitate the signaling process in a highly controlled manner.

It is tempting to speculate that the different trafficking machinery is required to segregate Lck and LAT into different vesicles from those that contain TCR and the CD3 complex. Such segregation may be necessary to achieve the distinct spatial organization of the immunological synapse. Emerging imaging techniques such as the lattice light-sheet microscope (Chen et al., 2014) may soon reveal how vesicle trafficking is functionally linked to synapse organization. Segregation into different membrane compartments could both prevent signaling in resting T cells and facilitate sustained signaling in activated T cells. Already the actin cytoskeleton at the immunological synapse has been identified as a gatekeeper for the secretion of LG (Ritter et al., 2015) and new rapid, super-resolution imaging will undoubtedly bring further insights into the interconnectedness between the structural organization of the synapse, signaling activities, compartmentalization of signaling components, and vesicle movement.

AUTHOR CONTRIBUTIONS

JL and QD wrote the manuscript, KG, JR, and SP provided critical reviewing and editing.

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Effect of Spatial Inhomogeneities on the Membrane Surface on Receptor Dimerization and Signal Initiation

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Important signal transduction pathways originate on the plasma membrane, where microdomains may transiently entrap diffusing receptors. This results in a non-random distribution of receptors even in the resting state, which can be visualized as "clusters" by high resolution imaging methods. Here, we explore how spatial in-homogeneities in the plasma membrane might influence the dimerization and phosphorylation status of ErbB2 and ErbB3, two receptor tyrosine kinases that preferentially heterodimerize and are often co-expressed in cancer. This theoretical study is based upon spatial stochastic simulations of the two-dimensional membrane landscape, where variables include differential distributions and overlap of transient confinement zones ("domains") for the two receptor species. The *in silico* model is parameterized and validated using data from single particle tracking experiments. We report key differences in signaling output based on the degree of overlap between domains and the relative retention of receptors in such domains, expressed as escape probability. Results predict that a high overlap of domains, which favors transient co-confinement of both receptor species, will enhance the rate of hetero-interactions. Where domains do not overlap, simulations confirm expectations that homo-interactions are favored. Since ErbB3 is uniquely dependent on ErbB2 interactions for activation of its catalytic activity, variations in domain overlap or escape probability markedly alter the predicted patterns and time course of ErbB3 and ErbB2 phosphorylation. Taken together, these results implicate membrane domain organization as an important modulator of signal initiation, motivating the design of novel experimental approaches to measure these important parameters across a wider range of receptor systems.

Keywords: spatial stochastic modeling, membrane domains, ErbB receptors, ErbB2, ErbB3

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INTRODUCTION

The plasma membrane is the initiation site for signaling pathways that govern cell differentiation, proliferation and survival (Groves and Kuriyan, 2010; Radhakrishnan et al., 2012). The membrane provides a platform for the reversible binding of ligands to receptors, initiating critical processes such as dimerization, activation of catalytic activity and recruitment of binding partners

(Groves and Kuriyan, 2010). Given its importance in cell signaling, the structure and composition of membranes have been probed by many different groups. Singer and Nicholson, in their landmark paper of the fluid mosaic model, proposed membranes to be largely homogenous with randomly distributed mixtures of integral membrane proteins and lipids (Singer and Nicolson, 1972). However, the authors also showed electron microscopy images of major histocompatibility antigen "patches," providing early evidence for membrane organization. Since then, considerable evidence has accumulated showing that membrane proteins and lipids can be transiently confined in specific domains (Kaizuka et al., 2007; Chung et al., 2010; Treanor et al., 2010; Radhakrishnan et al., 2012; Goñi, 2014). The anomalous diffusion of membrane constituents, observed through single molecule tracking methods (Fujiwara et al., 2002), is likely due, at least in part, to their transient entrapments within heterogeneous domains (Marguet et al., 2006). Multiple theories exist to explain the richness of the plasma membrane topography, including lipid rafts which are enriched in unsaturated fatty acids and cholesterol (Pike, 2003), corrals formed by the actin cortical cytoskeleton network (Jaqaman et al., 2011; Kalay, 2012; Cambi and Lidke, 2015) and protein islands (Lillemeier et al., 2006). Even very short periods of confinement within domains give rise to lateral heterogeneity and an uneven distribution of proteins on the membrane surface that can be captured in "snap-shot" images by electron microscopy of membrane rip-flips (Wilson et al., 2000; Prior et al., 2001; Andrews et al., 2009). More recently, super-resolution microscopy methods have also been employed to document the clustering of membrane proteins (van den Dries et al., 2013; Itano et al., 2014). The exchange of proteins between domains is highly variable, ranging from very low exchange rates observed in yeast membranes (Spira et al., 2012) to very rapid exchanges described for the EGFR in mammalian cell membranes (Low-Nam et al., 2011).

Many important receptors exhibit varying degrees of clustering prior to ligand engagement, including members of the EGFR/ErbB family (Nagy et al., 2002; Yang et al., 2007) and the ITAM-bearing immunoreceptors (FcεRI, BCR, TCR) (Pike, 2003; Lillemeier et al., 2006; Andrews et al., 2009; Tolar et al., 2009; Treanor et al., 2010; Dinic et al., 2015). Experimental evidence has suggested that membrane domains can both enhance and inhibit signaling in different settings (Marmor and Julius, 2001; Miura et al., 2001; Douglass and Vale, 2005; Allen et al., 2007; Bénéteau et al., 2008; Ganguly et al., 2008). Computational studies have also supported the concept that membrane organization has cell and receptor-specific outcomes (Lim and Yin, 2005; Hsieh et al., 2008; Costa et al., 2011; Abel et al., 2012; Kalay et al., 2012). For example, the formation of different signaling clusters has been proposed to support distinct TCR signaling patterns (Singleton et al., 2009). Vale and colleagues recently demonstrated in model membranes that phase separation of signaling partners can create distinct signaling compartments (Su et al., 2016). Members of the ErbB family of receptor tyrosine kinases have been shown to have distinct distribution patterns on cancer cell membranes (Yang et al., 2007; Steinkamp et al., 2014), leading to computational studies from our group that predict the impact of critical variables such as receptor co-expression, density and dimer off-rates (Hsieh et al., 2008; Pryor et al., 2013, 2015).

Deterministic models based upon Ordinary Differential Equations (ODEs) are not well suited to explore spatial aspects of signaling, since they assume molecules in a system are well mixed. Stochastic modeling approaches offer greater flexibility to consider effects of membrane topography, receptor clustering and diffusion dynamics on signaling events (Mayawala et al., 2006; Nicolau et al., 2006; Hsieh et al., 2008; Costa et al., 2009; Chaudhuri et al., 2011). These versatile mathematical models provide a platform for rapid exploration of key factors that are difficult to vary (and measure) experimentally. In this study, we take advantage of this powerful approach to consider the effect of two parameters, membrane domain overlap and domain retention, on ErbB3 and ErbB2 homo- and heterodimerization. Our group previously evaluated the domain occupancy and distribution of ErbB2 and ErbB3 stably expressed as recombinant proteins in Chinese Hamster Ovary (CHO) cells (Steinkamp et al., 2014; Pryor et al., 2015). Analysis of dual-color single particle tracking data, which permitted independent observations of each species, indicated that domains confining the two ErbB receptors were only partially overlapping in the CHO cell membrane (Pryor et al., 2015). We then built a spatial stochastic model based upon this distribution, as well as experimentally measured values for dimer off-rates, kinase/phosphatase activity and receptor diffusion (Pryor et al., 2015). However, we speculate that the degree to which there is differential segregation of these two closely related receptors will vary widely as a property of cell type, because of dissimilar receptor ratios, density, cytoskeletal features, membrane composition and on-going signal transduction from other cell surface receptors triggered by circulating or local ligands. In this paper, we focus on two specific parameters that affect the degree to which ErbB2 and ErbB3 experience periods of co-confinement: domain overlap and retention, where the latter is expressed as a function of escape probability.

MATERIALS AND METHODS

Spatial Stochastic Model for ErbB2 and ErbB3 Homo- and Hetero-Dimerization

Reactions

The spatial stochastic model of ErbB2 and ErbB3 interactions was described previously (Pryor et al., 2015). Briefly, the model includes two members of the EGFR family, ErbB2 and ErbB3, which diffuse within the simulation space and interact with each other.

The following reactions are accounted for in the model:

(i) Dimerization: Homo- and heterodimerization of ErbB2 and ErbB3 receptors.
(ii) Phosphorylation: Receptors are phosphorylated through intrinsic phosphorylation rates.
(iii) Dephosphorylation: Receptors are dephosphorylated through experimentally determined dephosphorylation rates.
(iv) Dissociation: Dimer dissociation occurs through experimentally determined dimer off rates.

We assume that the dimerization of receptors occurs through the interaction of the dimerization arms on the extracellular domain of receptors. In the absence of ligand, the ErbB3 extracellular domain fluxes from a closed (tethered) to an open (dimer-competent) conformation. The open conformation of ErbB3 is stabilized by ligand binding (Pryor et al., 2015). Unliganded ErbB3 is assumed to be predominately closed (99.99% closed). At any given time step, there is a 10^{-4} probability for unoccupied ErbB3 receptors to assume the upright dimer-competent state while all ligand-bound ErbB3 monomers are dimer-competent (Hsieh et al., 2008). ErbB3 ligand concentrations vary in the simulations as described in the legends. ErbB2 receptors are assumed to be in open conformation and dimerization competent (Cho et al., 2003; Garrett et al., 2003). In the model, ErbB2 has a single representative tyrosine phosphorylation site based on uniform dephosphorylation kinetics over two tested phosphorylation sites (Pryor et al., 2015). ErbB3 has two representative phosphorylation sites based upon (Y1289; Y1197). **Table 1** lists the reaction parameters used in our model including receptor dimerization, phosphorylation/dephosphorylation, and receptor dissociation as previously described (Pryor et al., 2015). For receptor phosphorylation events, the model takes into consideration the asymmetric orientation of kinase domains which occurs during ErbB receptor activation (Ward and Leahy, 2015). Reactions are governed by binding radii estimated using SMOLDYN, a software application that takes into consideration receptor on-rates, diffusion coefficients and simulation time steps to construct a binding radius (Andrews and Bray, 2004). An unbinding radius of 5 times the binding radius was used to decrease rebinding events.

Simulation Landscape

The simulation landscape contains receptor specific domains (**Figure 1A**) and receptors can diffuse across domains and domain-free areas. An exit penalty limits receptor escape from the domains. **Figure 1A** depicts domains that were identified in previous work (Pryor et al., 2015). Represented by a rectangular box measuring 0.1995 μm^2 in area (**Figure 1A**), the space contains 5 ErbB2 and 9 ErbB3 receptor domains. These domains were derived from domain analysis of two-color single particle tracking data where ErbB3 was labeled with HRG-conjugated quantum dot (QD) and HA-tagged ErbB2 was labeled with anti-HA Fab conjugated QD (Pryor et al., 2015). The total ErbB2 domain area is 0.0502 μm^2; the total ErbB3 domain area is 0.0274 μm^2.The free area outside the domains is 0.1219 μm^2. We then created three distinct domain overlap conditions for comparison:

(i) 100% overlap: 100% of the ErbB3 domain area is overlapping with the ErbB2 domain area. This resulted in complete mixing of ErbB3 and ErbB2 domains (**Figure 1D**).
(ii) 50% overlap: 50% of the ErbB3 domain area is overlapping with ErbB2 domain area. This resulted in partial overlapping of ErbB3 and ErbB2 domains (**Figure 1C**).
(iii) 0% overlap: 0% of the ErbB3 domain area is overlapping with the ErbB2 domain area. This resulted in complete separation of ErbB3 and ErbB2 domains (**Figure 1B**).

Number and Density of Receptors

The model was populated with 50,000 ErbB2 and 50,000 ErbB3 receptors/cell. Since the total area of a cell is 314.16 μm^2 (with a diameter of 10 μm), this translates into a receptor *density* of $\sim$159 receptors/μm^2 for each receptor. Adjusted for a simulation area of 0.1995 μm^2, the total *number* of receptors is 31 of each receptor species.

Receptor Diffusion

Receptor diffusion occurs in the two dimensional membrane simulation space (x and y direction) through Brownian motion. Receptor jumps in these two directions are calculated using diffusion coefficients generated from SPT data and normally distributed random numbers.

Boundary Conditions

As in Pryor et al. (2015) and Pryor et al. (2013), the periodic boundary condition is applied to the edges of the simulation space. If a receptor jump takes the receptor across the edge of the simulation space, the jump distance is divided between the distances covered before and after the boundary is crossed. The receptor then traverses the distance to the boundary and the remaining distance is calculated from the opposite edge of the simulation space. Hence, the receptor "re-enters" the simulation space from the opposite boundary. Reflective boundary conditions are applied when a receptor reaches the edge of a membrane domain. Like the periodic boundary conditions, the jump distance is divided between the distances covered before and after reaching the boundary. A probability for crossing/escaping from the membrane boundary is calculated and if the probability of escaping is not met, then the receptor hits the boundary and is deflected back into the domain. If the probability of escape is met, then the receptor continues across the boundary. Escape rates in Pryor et al. (2015) were estimated by parameter fitting to the ratio of domain-confined receptors experimentally measured in CHO cell membranes; this rate is a key variable of the present study (**Table 2**).

Simulation Code

Input files containing the initial simulation space, receptor locations and ligand concentrations are generated in Matlab. These files are then accessed by a program written in Fortran, which simulates brownian diffusion and molecular interactions between the two receptors. At the end of the simulations, all output files are processed in Matlab for analysis of results. Code is available upon request.

RESULTS

Domain Overlap Affects the Frequency of Hetero-Interactions and Receptor Phosphorylation Events

It is unknown to what extent different receptors share the same membrane domains, how fluid these domains are over time, and whether activation of receptors alter domain overlap. Therefore, we explored these possibilities through simulations, reporting results as changes in homo- and hetero-dimerization

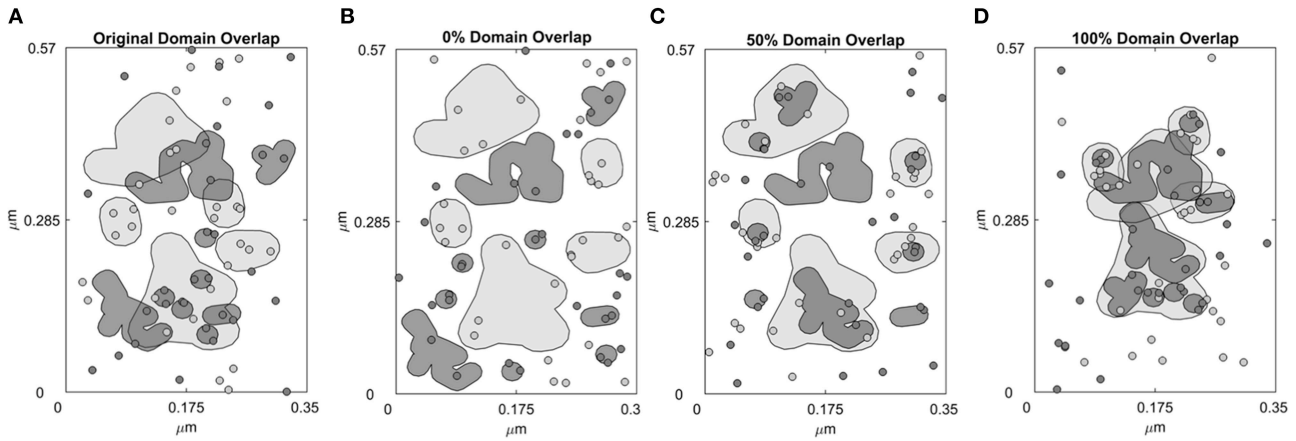

FIGURE 1 | Four domain configurations of the simulation space. Simulation space was partitioned into receptor-specific domains with defined domain overlaps. **(A)** A simulation space that mimics the domain properties of CHO cells overexpressing ErbB2 and ErbB3 based on domain analysis of SPT data. ErbB2 (light gray, shaded) and ErbB3 (dark gray, shaded) membrane domains overlap by 42.4%. ErbB2 receptors (light gray, circled) and ErbB3 receptors (dark gray, circled) are randomly distributed within their own domains as well as outside the domains (white region). **(B–D)** Domains were rearranged to create a simulation space where the ErbB2 and ErbB3 domains are completely non-overlapping (0% overlap, **B**), partially overlapping (50% overlap, **C**) or completely overlapping (100% overlap, **D**). In the initial configuration, ErbB2 and ErbB3 receptors were positioned to randomly occupy their respective domains.

TABLE 1 | Model parameters of receptor monomers and dimers.

	ErbB2	ErbB3	ErbB2 ErbB3	ErbB3 ErbB3	ErbB2 ErbB2
Diffusion coefficient ($\mu m^2/s$)[a,b]	0.0272	0.013	0.015	0.0185	0.015
Diffusion coefficient (phosphorylated) ($\mu m^2/s$)[a,b]			0.0046	0.0028	0.015
Dimer on rate ($\mu m^3/s$)[c]			0.00009	0.00009	0.00009
Dimer off rate (0 ligand) (1/s)[a,b]			0.436	0.436	4.36
Dimer off rate (1 ligand) (1/s)[b]			0.408	0.234	
Dimer off rate (2 ligand) (1/s)[b]				0.13	
Basal Phosphorylation rate (1/s)[d,e]	0.073	0.00007			
Phosphorylation rate (1/s)[d,e]	0.146	0.078			
Dephosphorylation rate (1/s)[a]	0.2	0.013 (PY1197) 0.06 (PY1289)			

[a] *Pryor et al. (2015).*
[b] *Steinkamp et al. (2014).*
[c] *Pryor et al. (2013).*
[d] *Kleiman et al. (2011).*
[e] *Shi et al. (2010).*

TABLE 2 | Escape rates of receptor monomers and dimers.

	ErbB2	ErbB3	ErbB2 ErbB3	ErbB3 ErbB3	ErbB2 ErbB2
Nominal escape rate[a]	0.5128	0.2401	0.3764	0.2401	0.5128
Escape rate reduced by 1/2[b]	0.2564	0.1200	0.1882	0.1200	0.2564
Escape rate reduced by 1/4[b]	0.1282	0.0600	0.0941	0.0600	0.1282

[a] *Pryor et al. (2015).*
[b] *Simulation data in this paper.*

and phosphorylation status. Unlike prior work fit to cells overexpressing ErbB family members (Pryor et al., 2013, 2015), we used receptor densities within the range of expression values expected for normal cells (50,000 receptors/cell). The simulation landscape included either no domains or ErbB2 and ErbB3-specific domains with partial, full or no overlap (**Figure 1**).

The rapid cycling of ErbB3 receptors through different states is illustrated in **Figure 2**, where simulations were initially performed in a landscape lacking domains. Here, ligand-bound ErbB3 freely diffuse, encountering other ErbB3 or ErbB2 monomers with no barriers imposed. They constantly cycle through homodimer (red), heterodimer (orange) and monomer (white) states by binding and unbinding to other receptors as they diffuse through the simulation space (**Figure 2A**). Off-rates for hetero- and homodimers are assigned probabilities based upon experimental measures for unoccupied and ligand bound dimers (Steinkamp et al., 2014). The catalytic activity of each monomer in a dimer is tracked throughout the simulation. Activity is dependent on the stochastically-governed orientation of the monomer in the asymmetric model, where one of the monomers is the "activator" and the other monomer is the "receiver." Further, ErbB3 monomers are assumed to require phosphorylation by a "receiver" ErbB2 in a prior hetero-dimerization event. A phosphorylated ErbB3 monomer remains a competent "receiver" during subsequent encounters only until it is dephosphorylated. Simulation time steps are 1×10^{-6} s and observations are recorded every 0.05 s. Plots in **Figure 2B** show

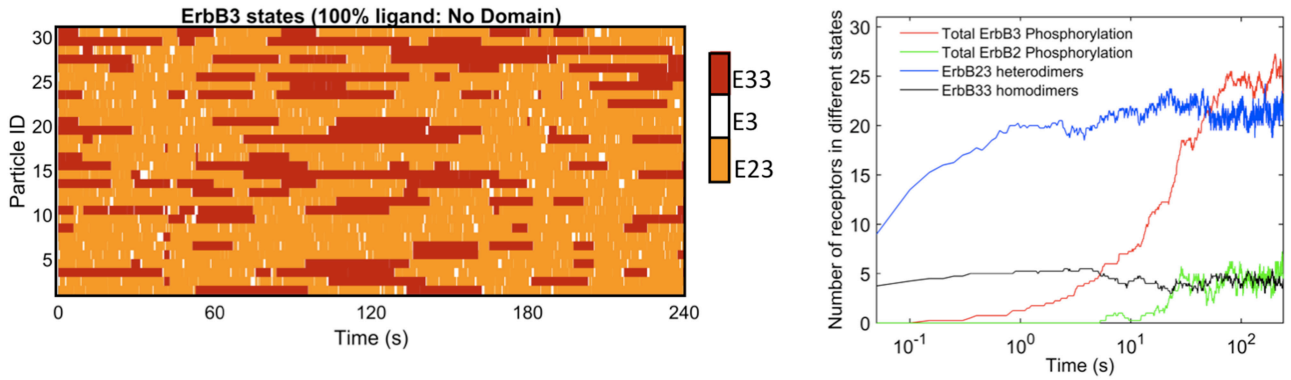

FIGURE 2 | Kinetics of ErbB3 dimerization and phosphorylation. (A) Representative plot of individual ErbB3 receptors showing changes in receptor state over time. ErbB3 receptors cycle between homodimer, heterodimer and monomer states. **(B)** Plot showing the kinetics of dimer formation and phosphorylation of ErbB2 and ErbB3. ErbB2/3 heterodimer and ErbB3/3 homodimer formation are plotted with total ErbB2 and ErbB3phosphorylation over time for 100% ligand in the absence of domains. Data in B are the averages of 4 runs.

that dimerization is already occurring by the earliest observation interval and continues to rise over the first 10 s of the simulation. Phosphorylation kinetics are delayed, observable within 0.5 s of the simulation and rising to steady state values by 50 s.

In **Figure 3**, we report the effect of adding domains to these simulations. The extreme cases of completely overlapping vs. non-overlapping ErbB2 and ErbB3 domains are shown in **Figures 3A–H**. Color keys in these plots indicate shifting profiles of monomers and dimers, as well as report phosphorylation states. Clearly, confinement in shared domains favors heterodimer interactions with a corresponding decrease in ErbB3 homodimers and ErbB2 monomers (**Figures 3A,B**). Phosphorylation kinetics is affected by co-confinement with a delayed but steep rise in phosphorylation (**Figures 3C,D**). Therefore, the overall signaling response is likely increased with shared domains.

Results in **Figure 4** report dimers at steady state (240 s) using the three distinct domain configurations shown in **Figures 1B–D** as well as no domain configuration. Simulations with completely overlapping domains produced the greatest number of heterodimers regardless of ligand concentration, although the greatest difference can be seen with 100% ligand (**Figure 4A**). At lower ligand concentrations, the effect of overlapping domains on dimer formations was diminished. This phenomenon is best explained by segregation of the few ligand bound receptors. ErbB3 homodimers displayed the opposite trend to that of heterodimers, where the highest number of homodimers were seen when ErbB3 domains did not overlap with ErbB2 (**Figure 4B**). This was notable for conditions of 100% and 50% liganded ErbB3.

Steady state phosphorylation levels are also affected by the configuration of domains (**Figures 4C,D**). Phosphorylation levels of both ErbB2 and ErbB3 decreased as domain overlap decreased, highlighting the importance of heterointeractions for maximal signaling. ErbB2 phosphorylation was most affected by domain overlap, particularly in simulations with 100% liganded ErbB3 (**Figure 4D**). Note that ErbB3 phosphorylation, which is heavily dependent on interactions with ErbB2 is not favored under conditions where ErbB2 homodimers are predominant.

Stronger Domain Retention Affects Receptor Dimerization and Phosphorylation Events Only When the Domains Partially Overlap or Non-overlap

Although the clustering of receptors in domains is important for signaling, little is known about the movement of receptors into and out of membrane domains or the extent to which this movement is altered with receptor activation. Since it is difficult to measure experimentally receptor residency times within domains, Pryor et al estimated an escape rate based on the ratio of domain-confined to free receptors in CHO cells under low ligand conditions (Pryor et al., 2015). To examine the effect of this parameter on signaling outcome, we ran simulations where we varied the escape rate to model changes in domain retention. The affinity of receptors for their domains was increased by reducing the escape rate of both monomers and dimers. We compared simulations run with the original nominal escape rate, or with the escape rate reduced by ½ or ¼. The effect of these escape rates were examined with different ligand concentrations in the four domain overlap configurations (**Figure 5**). Reducing the escape rates had no effect on heterodimer formation for domains that were completely overlapping. However, when the domains were partially overlapping or non-overlapping, heterodimer formation was significantly reduced as the escape rate decreased. For instance, in the case of 100% liganded ErbB3, when the escape rate was reduced to ¼ and the domains were partially overlapping, the number of heterodimers at steady state was 35% lower than with the original escape rate. With non-overlapping domains, heterodimers were reduced by 70% (**Figure 5A**). Similar trends were seen in 50% and 20% ligand conditions

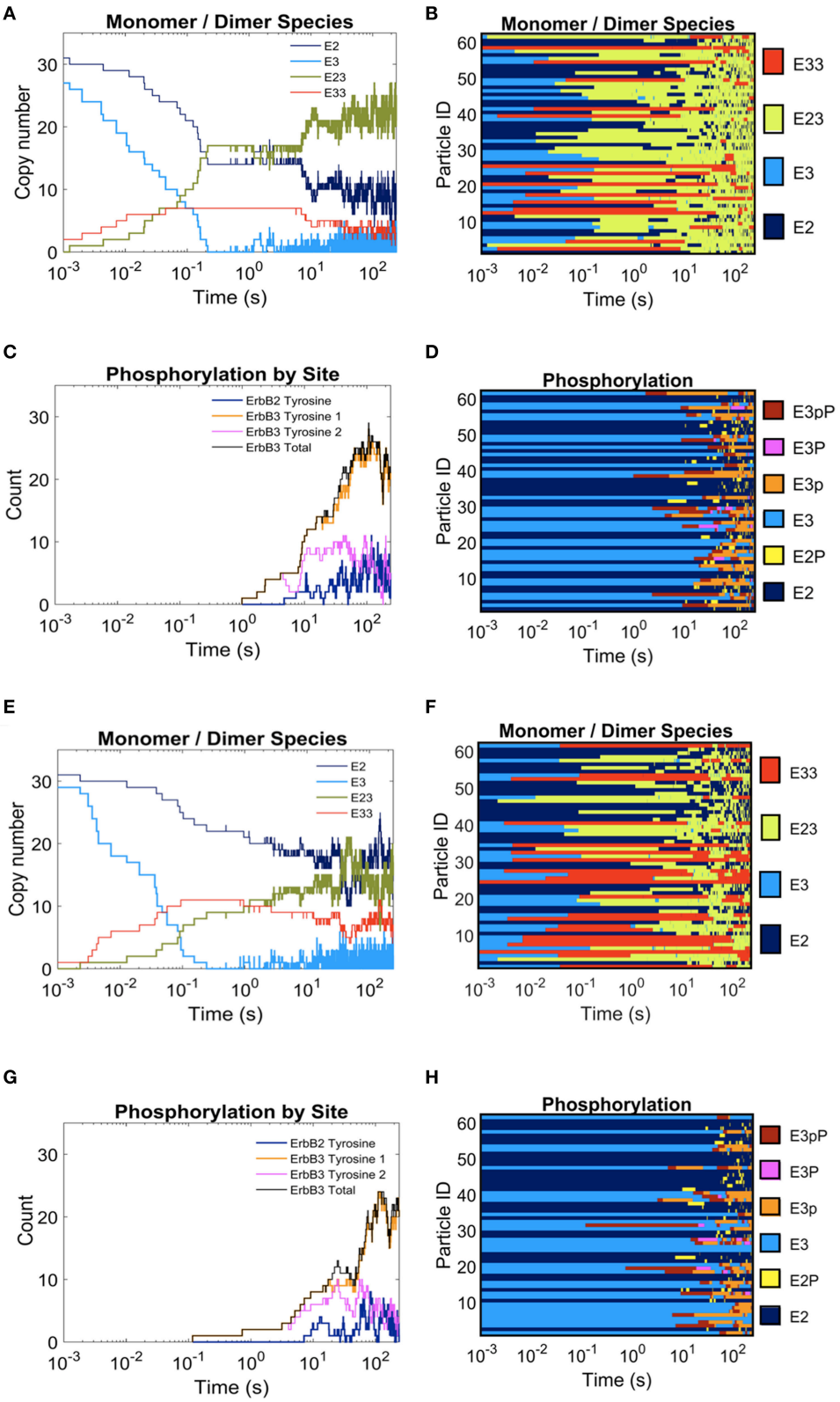

FIGURE 3 | The effect of overlapping domains on ErbB2/ErbB3 dimerization and phosphorylation kinetics with 100% ligand-bound ErbB3. Plots for the completely overlapping domain configuration **(A–D)**: The kinetics of dimer formation **(A)**, representative plots of dimerization state for individual receptors over the simulation time **(B)**, the kinetics of receptor phosphorylation **(C)**, and a representative plot of phosphorylation state for receptors over time **(D)**. **(E–H)**: Plots for the non-overlapping domain configuration. Plots are arrayed as in **(A–D)**.

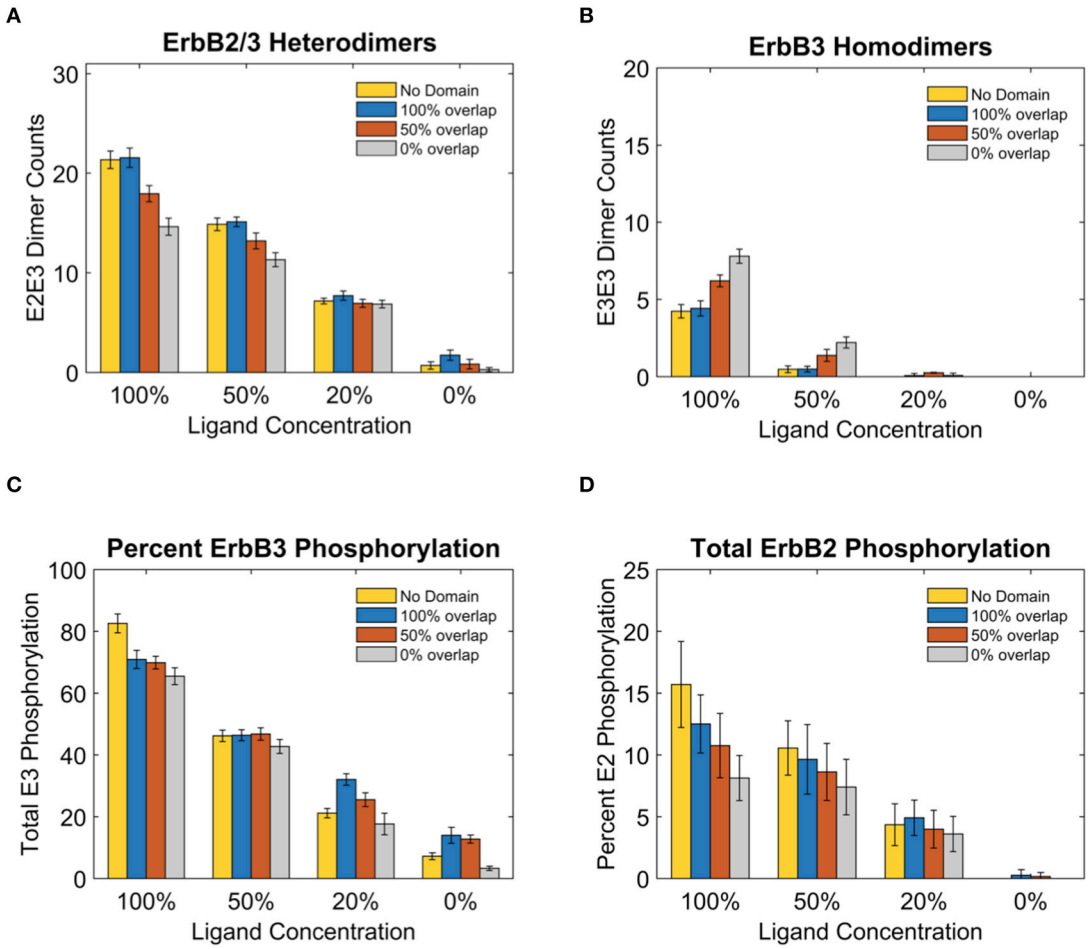

FIGURE 4 | Overlapping domains influence dimer formation and phosphorylation. (A,B): Dimer counts across different ligand concentrations with 4 different membrane configurations- 100% (blue bars), 50% (orange bars), and 0% overlap (gray bars) as well as no domain simulations (yellow bars) for ErbB2/ErbB3 heterodimers **(A)** and ErbB3 homodimers **(B)**. **(C,D):** Total receptor phosphorylation across different ligand concentrations and all four domain configurations for ErbB3 **(C)** And ErbB2 **(D)**. All bars are the averages of 4 runs ± standard deviation.

(**Figures 5B,C**). With unliganded ErbB3, heterodimerization was rare (**Figure 5D**). With completely overlapping domains, reducing the escape rates did not affect erbB3 homodimer formation either (**Figures 5A–D**). With overlapping domains, reducing the escape rate increased ErbB3 homodimers for partially and non-overlapping domains (**Figures 5E–G**). Escape rates ¼ of the original rate yielded maximum increase of 63%, which occurred with non-overlapping domains and 100% ligand (**Figure 5E**). Similar trends were seen with lower ligand concentrations (**Figures 5F,G**). Unliganded ErbB3 is not shown since there were no homodimers in this condition.

The significant changes in dimerization with increased domain retention had variable effects on downstream signaling as assessed by steady state phosphorylation levels of ErbB3 and ErbB2 (**Figure 6**). For ErbB3, phosphorylation levels are relatively stable with increased domain retention (**Figures 6A–D**). The greatest effect on phosphorylation levels occurred in the case of no domain overlap, where the ErbB3 monomers were more restricted from encounters with ErbB2. In the case of fully-liganded ErbB3, a four-fold reduction in escape rate led to a 28% reduction in phosphorylation (**Figure 6A**, gray bar for 0% overlap). For lower ligand concentrations, varying domain overlap had a greater effect on phosphorylation than domain retention (**Figures 6C,D**).

ErbB2 phosphorylation was markedly sensitive to increases in domain retention. Reduced ErbB2 phosphorylation corresponded to decreases in heterodimer formation (**Figures 6E–G**). Once again, little change was seen with completely overlapping domains. However, increasing domain retention lowered ErbB2 phosphorylation with either partially or non-overlapping domains. Results were striking for simulations run with a four-fold lower escape rate and 100% liganded ErbB3.

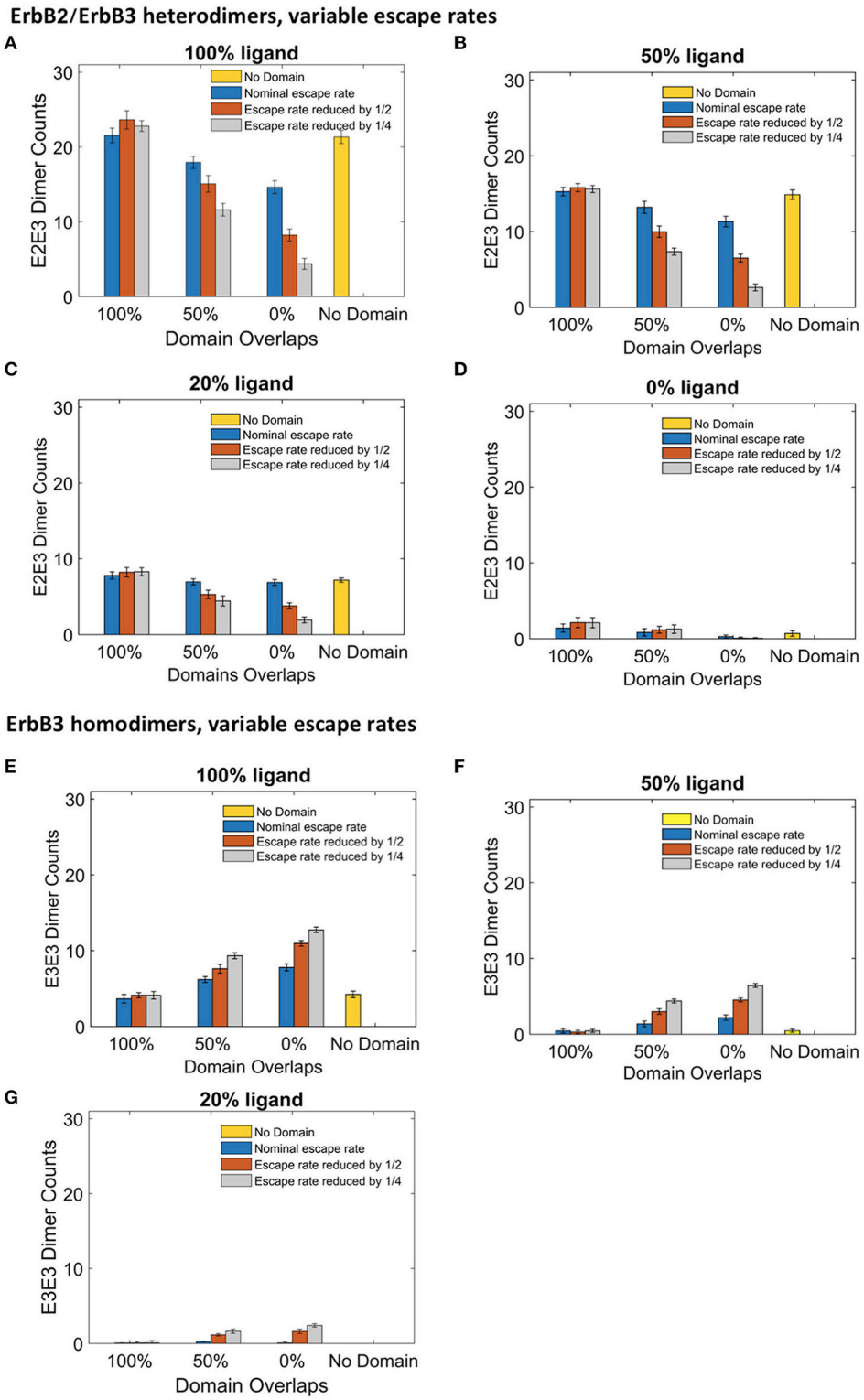

FIGURE 5 | The effect of changes in domain retention on ErbB2/3 heterodimer and ErbB3/3 homodimer counts across different ligand concentration and domains. Dimer counts across different membrane configurations, ligand concentration and three different escape rates- nominal escape rate (blue bars), escape rate reduced by ½ (orange bars), and escape rate reduced by ¼ (gray bars) as well as no domain simulations (yellow bars). **(A)** ErbB2/3 heterodimer for 100% liganded ErbB3. **(B)** ErbB2/3 heterodimer for 50% liganded ErbB3. **(C)** ErbB2/3 heterodimer for 20% liganded ErbB3. **(D)** ErbB2/3 heterodimer for 0% liganded ErbB3. **(E)** ErbB3/3 homodimer for 100% liganded ErbB3. **(F)** ErbB3/3 homodimer for 50% liganded ErbB3. **(G)** ErbB3/3 homodimer for 20% liganded ErbB3. The ErbB3/3 homodimer count was 0 for 0% liganded ErbB3. All bars are the averages of 4 runs ± standard deviation.

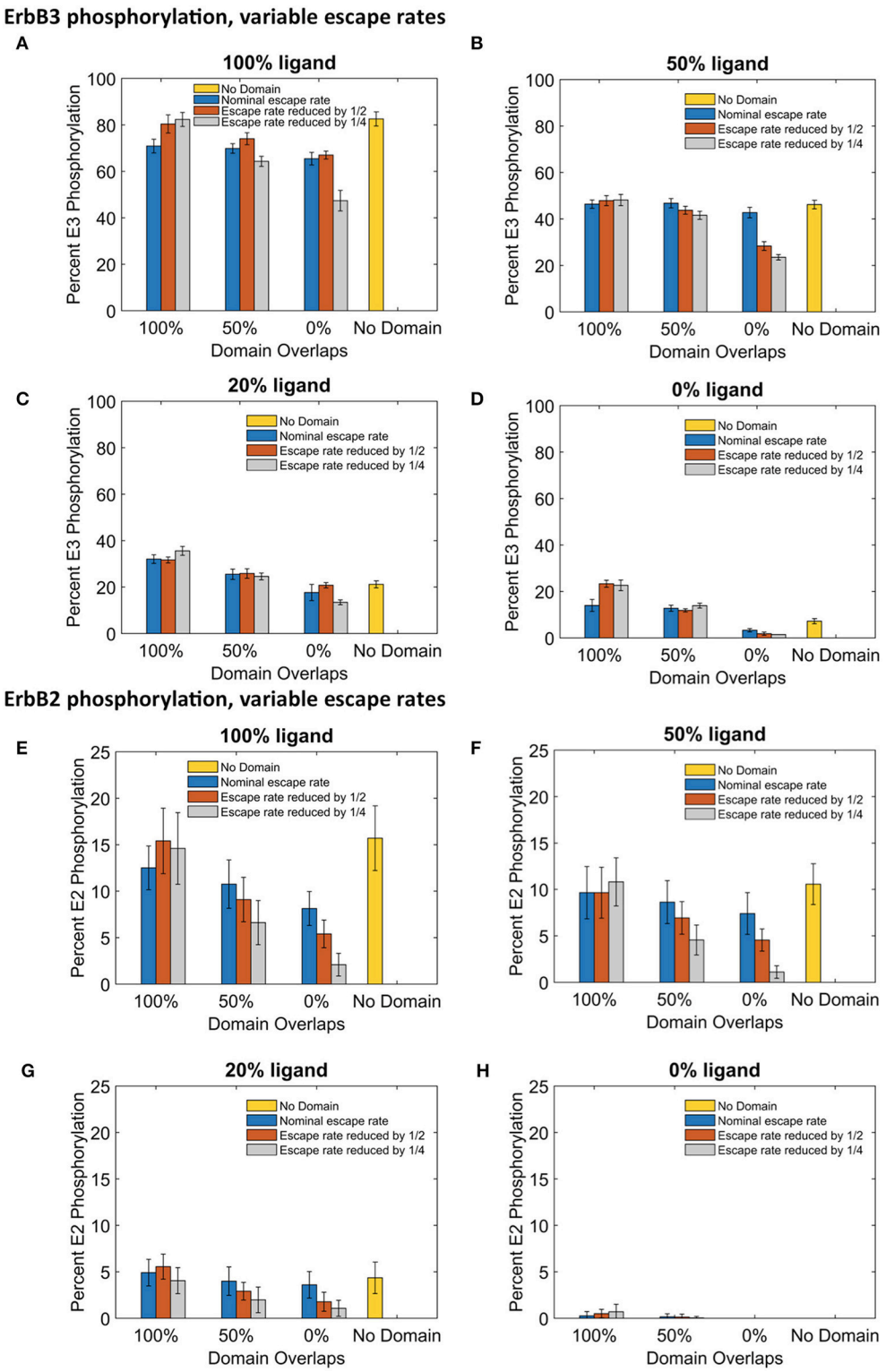

FIGURE 6 | The effect of changes in domain retention on ErbB3 and ErbB2 phosphorylation across different ligand concentration and domains. Total receptor phosphorylation across different membrane configurations, ligand concentration and three different escape rates- nominal escape rate (blue bars), escape rate reduced by ½ (orange bars), and escape rate reduced by ½ (gray bars) as well as no domain simulations (yellow bars). **(A)** Total ErbB3 phosphorylation for 100% liganded ErbB3. **(B)** Total ErbB3 phosphorylation for 50% liganded ErbB3. **(C)** Total ErbB3 phosphorylation for 20% liganded ErbB3. **(D)** Total ErbB3 phosphorylation for 0% liganded ErbB3. **(E)** Total ErbB2 phosphorylation for 100% liganded ErbB3. **(F)** Total ErbB2 phosphorylation for 50% liganded ErbB3. **(G)** Total ErbB2 phosphorylation for 20% liganded ErbB3. **(H)** Total ErbB2 phosphorylation for 0% liganded ErbB3. All bars are the averages of 4 runs ± standard deviation.

Here, ErbB2 phosphorylation was reduced by 39% (partially overlapping domains) or 74% (non-overlapping domains).

DISCUSSION

ErbB2 and ErbB3 are members of the ErbB family of receptor tyrosine kinases that are often co-expressed in cells. Under physiological conditions, neither receptor is active on its own. However, through heterointeractions these receptors activate two key pro-survival pathways. ErbB3 primarily activates the PI3K/Akt pathway and ErbB2 favors the MAP kinase pathway (Yarden and Sliwkowski, 2001). Activation of the ErbB2/ErbB3 signaling unit via overexpression of the receptors, gain-of-function oncogenic mutations, or autocrine release of the ErbB3 ligand, heregulin, have been identified in many types of cancer (Holbro et al., 2003; Wolf-Yadlin et al., 2006; Sheng et al., 2010; Jaiswal et al., 2013; Capparelli et al., 2015). Given the potency of this interaction, normal cells must maintain tight control over ErbB2/ErbB3 interactions. In the absence of ligand, dimerization is limited by the constant fluxing of the ErbB3 extracellular domain from a tethered, inactive conformation to an upright, active conformation with the active conformation stabilized by ligand binding (Dawson et al., 2007). Another way to control ErbB2/ErbB3 interactions may be through dynamic reorganization of membrane domains. Sequestration of ErbB2 and ErbB3 in separate domains could prevent spurious signaling in the absence of ligand, while reorganization into overlapping domains upon ligand binding could encourage the formation of signaling clusters (Vámosi et al., 2006). Evidence for reorganization can be seen in electron microscopy studies of SKBR3 breast cancer cell membranes. ErbB2 and ErbB3 are dispersed in the absence of ligand, but in the presence of ligand, ErbB3 forms large clusters with areas of co-localized ErbB2 and ErbB3 (Yang et al., 2007). It has also been shown that ErbB2 clusters within lipid rafts and that disruption of these rafts reduces both ErbB2 clustering and the association of ErbB2 and ErbB3 (Nagy et al., 2002). The remodeling of domains during active signaling has not yet been explored by simulation, in part due to difficulties in accurately measuring the dynamics of these changes. Here, we have examined how domain remodeling, represented in our model by varying domain overlap and domain retention, will effect heterodimer formation and signaling.

Our spatial stochastic model of ErbB2/ErbB3 interactions provides a useful system in which to explore how changes in domain configuration might affect receptor activation. We began with a model parameterized based on single particle tracking data acquired under low (nanomolar) ligand conditions. We then explored how changes in domain characteristics, as well as ligand occupancy, influences dimerization and phosphorylation in this system. The sensitivity of the model to these parameters illustrates that variations in domain characteristics amongst different cell and tissue types are likely unappreciated modulators of signaling by these (and other) receptors.

Previous spatial stochastic models have shed insight on the effect of domains on signaling (Hsieh et al., 2008; Costa et al., 2009, 2011; Chaudhuri et al., 2011; Kalay et al., 2012). Kalay et al. evaluated movement of tracer molecules within lattice-based domains and found that confinement increased reaction rates (Kalay et al., 2012). Addressing ErbB receptor family interactions with rectangular subdomains, Hsieh et al found that domains created local densities that favored EGFR interactions on the membrane surfaces (Hsieh et al., 2008). Our model increases the complexity by introducing two interacting receptor types with unique behaviors and overlapping, experimentally-defined domains. Thus, the model provides a mechanistic understanding of the interplay between domain overlaps and domain retention on the complex interactions of ErbB2 and ErbB3. The model relies on previously described characteristics of these receptors. For example, ErbB2 homodimers are not favored due to evidence for electrostatic repulsion (Garrett et al., 2003); this translates in the model to a low probability for ErbB2 homointeractions. In addition, ErbB3 has very low kinase activity unless activated by ErbB2 (Steinkamp et al., 2014). Thus, in cells where these are the two predominant ErbB species, they are predicted to be mutually dependent on each other for activation. It follows that differential preference of the two species for unique confinement zones or membrane domains should have a strong influence.

Accordingly, we found that phosphorylation of the two ErbB species was differentially affected by domain overlap. This was particularly evident in the case of 100% liganded ErbB3, where ErbB2 phosphorylation dropped by 50% between completely overlapping to non-overlapping domains (**Figure 4D**). At these physiological receptor levels, ErbB2 homo-encounters are largely unproductive due to the low on-rate. Simulations with more domain overlap had a larger number of heterodimer interactions than those with partial or no domain overlap. This was most notable when all ErbB3 were occupied with ligand (**Figure 4**). ErbB3 relies heavily on heterodimerization for activation. However, once ErbB3 receptors are activated by ErbB2, they can go on to homodimerize and activate other ErbB3 receptors. Therefore, steady state ErbB3 phosphorylation was less dependent on domain overlap.

It should be noted that the amount of hetero- and homodimers and phosphorylation levels were nearly the same between no domain spatial stochastic simulations and 100% domain overlapping conditions. This finding differs from our previous work with EGFR which showed that domains greatly improved phosphorylation of EGFR receptors, indicating that the introduction of multiple receptor types to these simulations further complicates outcome (Pryor et al., 2013). True domain overlaps are likely to fall somewhere between non-overlapping and completely overlapping configurations, indicating the need for spatial simulations that take this into account. Ligand binding to ErbB3 in SKBR3 breast cancer cell membranes leads to formation of large ErbB3 clusters with modest levels of co-localized ErbB2; this indicates that domain reorganization can occur during signaling (Yang et al., 2007). The remodeling of domains during active signaling has not yet been explored by simulation, in part due to difficulties in accurately measuring the dynamics of these changes.

SPT has revealed a range of non-brownian motion for proteins on the membrane plane. Anomalous diffusion is a term often used to explain the characteristic restricted movements of proteins that "hop" between membrane domains. There are also reports of specific membrane proteins that undergo directed (motor-driven) motion (Kusumi and Sako, 1996; Saxton and Jacobson, 1997; Schütz et al., 1997; Kusumi et al., 2005). These different modes of motion can have a profound impact on reaction kinetics on the membrane surface by perturbing reaction rates (Saxton and Jacobson, 1997; Melo and Martins, 2006). Thus, it is important to continue evaluating factors, such as diffusion coefficients, corral sizes and escape probability of proteins from their confined domains (Saxton and Jacobson, 1997), that are expected to impact signal initiation and propagation. In this work, we used a simulation approach to study the effect of escape probabilities on the reaction kinetics of the ErbB2/3 signaling pathway. We show that membrane segregation can influence signaling in non-intuitive ways that are linked to the individual characteristics of receptors. Given the technical challenges associated with measuring the dynamics of domain confinement, extent of mixing and escape rates in live cell membranes, simulation offers a powerful tool to explore these variables.

AUTHOR CONTRIBUTIONS

Conception and design: ÁH, JE. Development of computation framework: RK, ÁH. Acquisition and interpretation of data: RK, ÁH, MS, BW, JE. Writing, review, and/or revision of the manuscript: BW, RK, MS, ÁH, JE. Administrative support and study supervision: BW.

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REVIEW
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Tetraspanins Function as Regulators of Cellular Signaling

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Tetraspanins are molecular scaffolds that distribute proteins into highly organized microdomains consisting of adhesion, signaling, and adaptor proteins. Many reports have identified interactions between tetraspanins and signaling molecules, finding unique downstream cellular consequences. In this review, we will explore these interactions as well as the specific cellular responses to signal activation, focusing on tetraspanin regulation of adhesion-mediated (integrins/FAK), receptor-mediated (EGFR, TNF-α, c-Met, c-Kit), and intracellular signaling (PKC, PI4K, β-catenin). Additionally, we will summarize our current understanding for how tetraspanin post-translational modifications (palmitoylation, N-linked glycosylation, and ubiquitination) can regulate signal propagation. Many of the studies outlined in this review suggest that tetraspanins offer a potential therapeutic target to modulate aberrant signal transduction pathways that directly impact a host of cellular behaviors and disease states.

Keywords: tetraspanins, signal transduction, tetraspanin-enriched microdomains, adhesion-mediated signaling, receptor-mediated signal transduction

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INTRODUCTION

Tetraspanins are membrane-spanning proteins with a conserved structure that function primarily as membrane protein organizers. Phylogenetic analysis identified 33 tetraspanins in humans, 37 in *Drosophila melanogaster* (Charrin et al., 2014), and 20 in *Caenorhabditis elegans* (Huang et al., 2005), while only 17 were identified in *Arabidopsis thaliana* (Boavida et al., 2013). Tetraspanins have also been identified in the ameoba, *Dictyostelium discoideum*, which exists as both a unicellular and multicellular organism (Albers et al., 2016). While some tetraspanins are expressed ubiquitously in humans, others are cell or tissue specific (Maecker et al., 1997; de Winde et al., 2015), providing a means to regulate the signal transduction associated with a breadth of cellular processes.

Members of the tetraspanin family of proteins have four transmembrane domains, which contribute to the creation of a small (EC1) and large (EC2) extracellular loop (**Figure 1**). The large extracellular loop contains a conserved Cys-Cys-Gly amino acid motif (CCG-motif), as well as two other conserved cysteine residues. EC2 of CD81 was resolved using crystallography (Kitadokoro et al., 2001), where the authors demonstrated that the four conserved cysteine resides within EC2 promote the formation of disulfide bridges, as had been suggested by previous reports (Levy et al., 1991; Tomlinson et al., 1993; Maecker et al., 1997). Moreover, molecular modeling studies using the CD81 EC2 structure as a template predicted the topography of several other tetraspanins including CD37, CD53, CD82, and CD151 (Seigneuret et al., 2001; Seigneuret, 2006). These studies demonstrated that the EC2 domain of tetraspanins consist of one conserved and one variable domain, with the conserved domain consisting of a three-helix bundle while the variable domain is unique to particular tetraspanins. A recent report resolved a crystal structure of full-length CD81, finding that the four transmembrane domains create a cholesterol-binding pocket (Zimmerman et al., 2016).

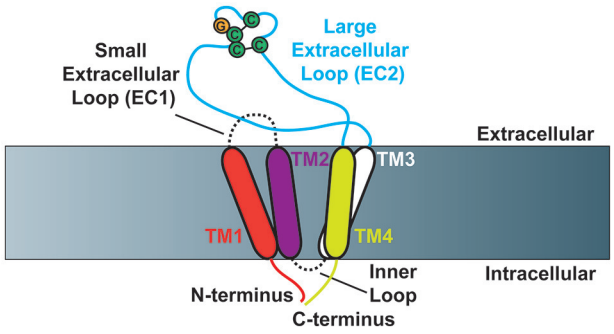

FIGURE 1 | Schematic of tetraspanin molecular structure (Based on Zimmerman et al., 2016). Cartoon depicting the structural characteristics of tetraspanins. Tetraspanins have four transmembrane domains (TM1-TM4), which create one small (EC1) and one large (EC2) extracellular loop as well as a short inner loop. The N- and C-termini of tetraspanins are localized to the intracellular side of the membrane. The Cys-Cys-Gly amino acid motif is depicted in addition to the two characteristic disulfide bonds that are formed in EC2.

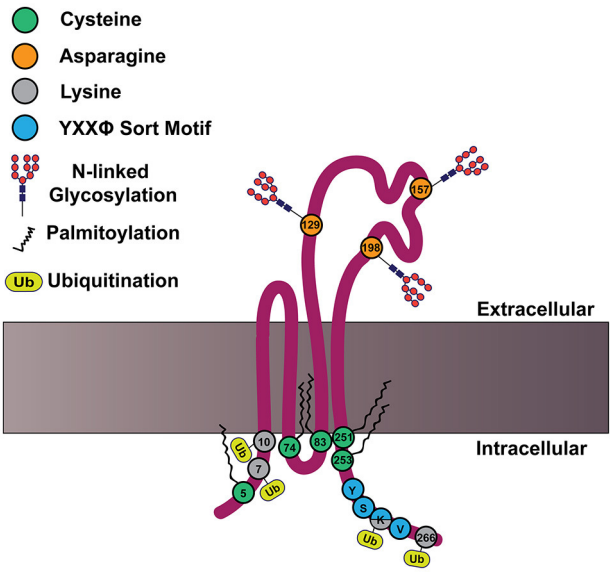

FIGURE 2 | CD82 structure and motifs. Cartoon depicting CD82 topology within the plasma membrane and important motifs. CD82 contains five membrane proximal cysteine residues (shown in green) at residues 5, 74, 83, 251, and 253, which can be palmitoylated. There are three asparagine residues in EC2 (shown in orange) that are predicted to be N-linked glycosylated at residues 129, 157, 198. There are four cytoplasmic lysine residues 7, 10, 263, and 266 (shown in gray), which are predicted to be ubiquitinated. The C-terminal tyrosine based sort motif (YXXø) is depicted in blue at amino acids 261–264; for CD82 this motif is Tyr-Ser-Lys-Val.

Furthermore, the authors performed molecular dynamics simulations that suggest CD81 can adopt an open or closed conformation depending on whether or not cholesterol is bound.

In addition to the defining features of tetraspanins, many members of the tetraspanin family also contain post-translational modifications. For example, tetraspanins may be palmitoylated at membrane proximal cysteine residues, which was demonstrated to regulate protein-protein interactions (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002, 2004). Meanwhile, tetraspanins can also be N-linked glycosylated at asparagine residues, which is less clearly understood (Ono et al., 1999; Stuck et al., 2012; Marjon et al., 2016). Tetraspanins may also be ubiquitinated at cytoplasmic sites, which contributes to their down-regulation (Lineberry et al., 2008; Wang Y. et al., 2012). An example structure of tetraspanin CD82 is depicted in **Figure 2**, with the post-translational modifications highlighted. How these tetraspanin post-translational modifications impact signal transduction will be addressed in more detail later in this review.

Through their function as molecular scaffolds, tetraspanins contribute to organismal development, reproduction, and immunity (Kaji et al., 2000, 2002; Le Naour et al., 2000; Miyado et al., 2000; García-Frigola et al., 2001; Levy and Shoham, 2005; Jarikji et al., 2009; van Spriel, 2011; Han et al., 2012). Consistent with their expression being primarily found in multicellular organisms, it is not surprising that many processes to which tetraspanins contribute center around cell-cell- interactions. Additionally, numerous tetraspanins are also associated with the development and progression of disease, in particular, with respect to cancer and cancer cell-niche interactions (Zoller, 2009; Hemler, 2013). Although tetraspanins do not have known adhesive ligands or catalytic activity, they contribute to cellular physiology by organizing molecules within the plasma membrane into microdomains.

The proposed function of tetraspanins is to organize the plasma membrane by facilitating the formation of what are termed tetraspanin enriched microdomains (TEMs). TEMs consist of homophilic and heterophilic interactions amongst tetraspanins, interactions between tetraspanins and other membrane proteins, as well as interactions between tetraspanins and proteins at the membrane/cytoplasm interface (Hemler, 2005; Charrin et al., 2009, 2014; Stipp, 2010). Moreover, these protein associations can occur through direct binding between tetraspanins and other proteins or through tetraspanin interactions with a common binding partner.

Interactions between tetraspanin and signaling molecules have been detected for various types of proteins, including adhesion and signaling receptors, and cytosolic signaling molecules, which are depicted in **Figure 3**. The downstream cellular consequences of these interactions vary, ranging from regulation of cellular adhesion, migration, contractility and morphology. As recent comprehensive reviews focused on tetraspanin regulation of immune signaling are available (Levy and Shoham, 2005; Halova and Draber, 2016), we will discuss other major classes of signaling molecules regulated by tetraspanins, as well as the cellular consequences of such regulations.

TETRASPANINS AND ADHESION-MEDIATED SIGNALING

One of the most prominent classes of adhesion receptors which tetraspanins are known to regulate is the integrin family of

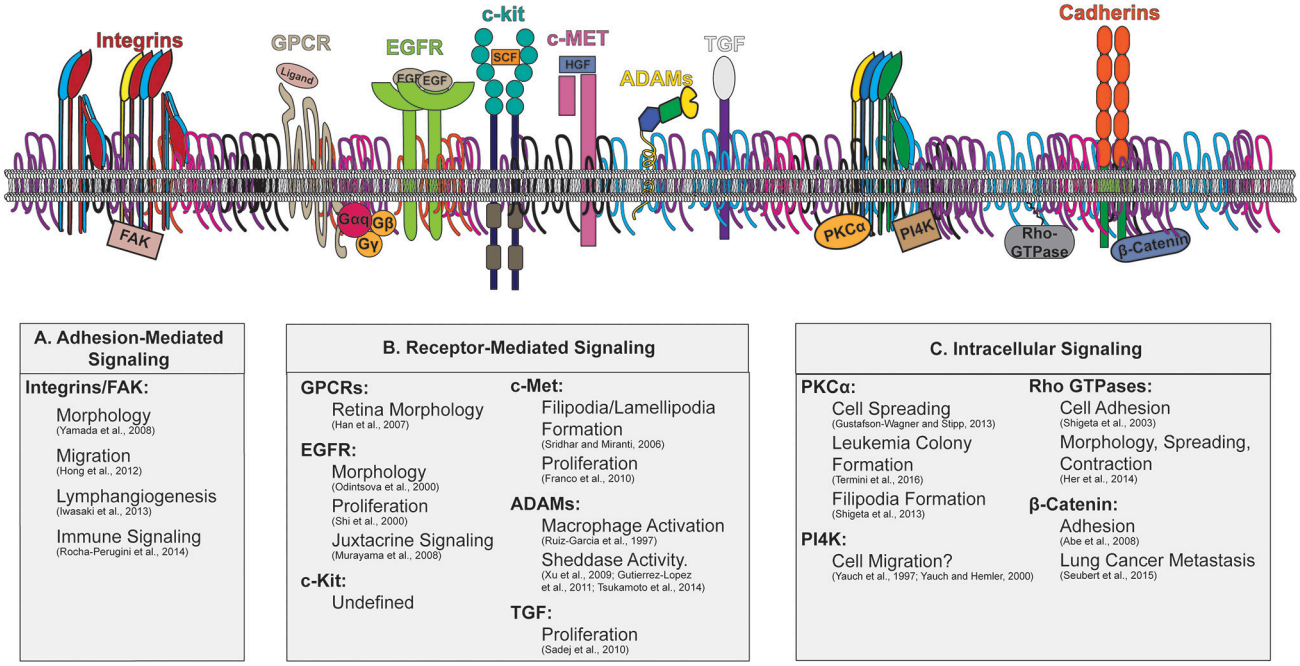

FIGURE 3 | Tetraspanin enriched microdomains with signaling molecules. Illustration of the plasma membrane depicting tetraspanin interactions with membrane and cytosolic signaling molecules. The downstream signaling consequences attributed to tetraspanin regulation are indicated beneath. Key signaling molecules modulated by tetraspanins include: **(A)** Adhesion-Mediated Signaling (Integrins/FAK), **(B)** Receptor-Mediated Signaling (GPCRs, EGFR, c-kit, c-Met, ADAMs, TGF), and **(C)** Intracellular signaling (PKC, PI4K, Rho-GTPases, and β-catenin).

proteins. Integrins are heterodimeric proteins consisting of one α and one β subunit, and this combination of subunits dictates their ligand specificity (Humphries et al., 2006). Numerous studies identified direct and indirect interactions between integrins and tetraspanins (Slupsky et al., 1989; Rubinstein et al., 1994; Berditchevski et al., 1996; Mannion et al., 1996; Yáñez-Mo et al., 2001, 1998; Stipp and Hemler, 2000; Berditchevski, 2001). Though integrins lack intrinsic catalytic activity, they propagate signals through a variety of cytoplasmic signaling molecules, many of which are components of focal adhesions (Schwartz, 2001). Through a combination of imaging and biochemical studies, researchers showed that tetraspanins colocalize with the focal adhesion proteins vinculin and talin as well as myrstoylated alanine-rich C-kinase substrate (MARCKS), which is involved in PKC-mediated signaling (Berditchevski and Odintsova, 1999). Moreover, signaling downstream of integrins is also mediated by the focal adhesion kinase, which is further regulated by tetraspanins as indicated below.

Focal Adhesion Kinase

Focal adhesion kinase (FAK) is a cytosolic protein which can interact directly with the integrin cytoplasmic tail, thereby allowing integrins to link to the actin cytoskeleton and promote downstream signaling (Schlaepfer et al., 1999). Immunoprecipitation studies demonstrated that tetraspanins CD9, CD63, CD81, CD82, and CD151 interact with the phosphorylated form of FAK (Berditchevski and Odintsova, 1999). Additionally, cells plated on anti-tetraspanin monoclonal antibodies demonstrated reduced FAK phosphorylation, further suggesting that tetraspanin scaffolding can contribute to FAK activation.

As suggested, a number of tetraspanins have been implicated in FAK regulation. It was shown that the siRNA knockdown of CD151 resulted in diminished phosphorylation of FAK, p130Cas, paxillin, and Src (Yamada et al., 2008). In fact, treatment with a CD151 monoclonal antibody, which reduced CD151 interactions with α3β1, also led to a reduction in FAK phosphorylation. In an attempt to rescue this phenotype, control or CD151 knockdown cells were treated with a β1 integrin activating antibody and these data demonstrated that FAK phosphorylation could not be rescued under enforced integrin activation. As such, this study provides evidence that tetraspanins may regulate integrin-mediated signaling through a mechanism independent of initial integrin activation. The authors quantified FAK autophosphorylation (Tyr397), which is a FAK modification stimulated by integrin clustering (Schlaepfer et al., 1999). As tetraspanins have been previously demonstrated to regulate integrin clustering (van Spriel et al., 2012; Termini et al., 2014), perhaps the loss of CD151 diminishes integrin clustering, thereby reducing FAK phosphorylation. Additionally, the presence of CD151 increased FAK and Src phosphorylation in response to plating on extracellular matrix components, which modulated GTPase activation and downstream cell migration (Hong et al., 2012). The authors demonstrated that there is a greater increase

in FAK and Src activation in response to plating on laminin than fibronectin, which is consistent with previous findings that CD151 is closely associated with laminin binding integrins (Berditchevski et al., 2002; Stipp, 2010).

Another tetraspanin identified to regulate FAK activity is CD9. In the case of lymphatic dermal endothelial cells, CD9 knockdown diminished FAK phosphorylation in response to VEGF-1 administration, demonstrating that tetraspanin regulation of FAK signaling can occur through multiple activating stimuli (Iwasaki et al., 2013). The authors further demonstrated that this CD9-mediated reduction in post-adhesion signaling impaired lymphangiogenesis. Consistent with previous studies of CD151 (Yamada et al., 2008), Rocha-Perugini et al. demonstrated that silencing of CD151 or CD9 reduced the expression of phospho-FAK and phospho-ERK in response to T-cell engagement (Rocha-Perugini et al., 2014). A decrease in the accumulation of activated β1 integrins and phospho-FAK was also detected at the immune synapse in CD9 and CD151 knockdown cells, suggesting that CD9 and CD151 promote the recruitment to and retention of integrins at the immune synapse, which results in diminished integrin downstream signaling. Therefore, the influence that tetraspanins have on integrin localization provides a critical means to regulate integrin-mediated signaling.

Though not technically considered a tetraspanin, the L6 tetraspan protein, TM4SF5, has sequence characteristics and structural properties similar to tetraspanins (Wright et al., 2000). It was shown that the intracellular loop of tetraspan TM4SF5 is critical for promoting an interaction between TM4SF5 and FAK (Jung et al., 2012). The authors performed *in vitro* pull-down assays using the N- or C-terminal cytoplasmic regions of TM4SF5 or the TM4SF5 intracellular loop to assess FAK binding. It was found that only the intracellular loop interacted with FAK, although the precise sites of association remain unknown. Future studies focused on identifying the particular amino acid residues within tetraspans that promote this association may offer potential targets to attenuate FAK signaling, which can be deregulated in numerous types of cancer (Sulzmaier et al., 2014).

TETRASPANINS AND RECEPTOR-MEDIATED SIGNALING

G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are seven membrane-spanning proteins that transmit signals with the help of intracellular G proteins (Kobilka, 2007). Upon ligand binding, GPCRs can be coupled to Gα, Gβ, and Gγ subunits to activate numerous cellular responses including calcium and potassium channel regulation, as well as phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) signaling (Tuteja, 2009). With the use of model systems such as *Drosophila*, it was determined that tetraspanins can regulate GPCR-mediated signaling. For example, the *Drosophila*-specific tetraspanin, Sunglasses or Sun, is required for the light-induced down-regulation of rhodopsin, a light-sensitive GPCR (Xu et al., 2004). Interestingly, Sun was concentrated in the retina and removal of Sun resulted in retinal degeneration. Moreover, the authors determined that in flies with reduced Sun expression, extended exposure to light resulted in the diminished ability to down regulate rhodopsin. In line with these findings, Sun is most closely related to human tetraspanin, CD63, which is enriched within the lysosome (Metzelaar et al., 1991). Therefore, it is likely that Sun assists with GPCR signal attenuation by directing its endosomal trafficking in a similar manner to CD63. Additionally, an interaction between Sun and the Gq subunit of rhodopsin was identified, which was further proposed to help Sun promote the endocytosis of rhodopsin (Han et al., 2007).

The regulation of GPCRs by human tetraspanins has also been explored. It was shown that the GPCR, GPR56, associates with tetraspanins CD9 and CD81 (Little et al., 2004; Xu and Hynes, 2007), two tetraspanins which have also been demonstrated to interact with one another (Stipp et al., 2001). Through the use of mass spectrometry, it was also determined that the G protein subunits, $G\alpha_{11}$ $G\alpha_q$ and Gβ associate with CD81 and further immunoprecipitation studies demonstrated that this association is not detected with tetraspanins CD63 or CD151 (Little et al., 2004). The authors postulate that perhaps the regulatory role of tetraspanins with respect to GPCRs may be to enhance ligand binding and downstream signaling, though this has yet to be directly tested. Important future studies will involve the analysis of downstream signaling through tetraspanin-mediated changes in GPCRs, including the potential regulation of GPCR-ligand affinity.

Epidermal Growth Factor Receptor

In addition to GPCRs (Metzelaar et al., 1991; Xu et al., 2004; Han et al., 2007) and integrins (He et al., 2005; Winterwood et al., 2006; Termini et al., 2014), tetraspanins have also been demonstrated to regulate the trafficking and signaling downstream of the epidermal growth factor receptor (EGFR). EGFR is a transmembrane receptor that can be activated by numerous ligands including epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). Ligand binding induces EGFR dimerization, which enhances EGFR catalytic activity (Jura et al., 2009; Valley et al., 2015). Moreover, EGFR endocytosis can serve as both a positive and negative regulatory signaling mechanism (Tomas et al., 2014). The contribution of tetraspanins in mediating EGFR trafficking has been extensively studied (Odintsova et al., 2000, 2003; Berditchevski and Odintsova, 2007).

Through a series of immunoprecipitation studies, it was shown that tetraspanin CD82 associates with EGFR and the overexpression of CD82 controls the phosphorylation kinetics of EGFR, Grb2, and Shc (Odintsova et al., 2000). It was determined that this regulation mediates the morphological response of HB2 cells to EGF stimulation. Interestingly, in cells expressing CD82, there was a more rapid down-regulation of EGFR upon EGF stimulation compared to cells that do not express CD82, indicating that CD82 contributes to EGFR down-regulation through modified internalization kinetics. This led the authors to suggest that the presence of CD82 modulates the signaling potency of the receptor even before it is activated. Furthermore, the authors speculate that the combination of reduced CD82 and

increased EGFR expression may lead to uncontrolled signaling. Therefore, CD82, and likely other tetraspanins, may provide a means to attenuate signaling through modulations in EGFR trafficking. A follow-up study found that CD82 negatively regulates ligand-induced dimerization of EGFR, but does not affect the dimerization of ErbB2 or ErbB3 (Odintsova et al., 2003). Although the authors did not examine the downstream effects of altered dimerization, they suggest that the differential compartmentalization of EGFR by CD82 might alter cellular signaling.

Further studies examined the role of the vesicular associated membrane protein (VAMP), TI-VAMP, and CD82 in regulating the surface dynamics of EGFR. In this study, knockdown of CD82 led to increased EGFR endocytosis upon EGF stimulation through increased AP-2 recruitment (Danglot et al., 2010). Furthermore, CD82 knockdown also altered the EGFR diffusion patterns on the plasma membrane and reduced ERK phosphorylation upon EGF stimulation, providing evidence that tetraspanins can regulate the spatial dynamics of proteins for controlling downstream signaling. This report provides a unique mechanism by which CD82, through cooperation with TI-VAMP and AP-2, can regulate EGFR signaling and surface dynamics. Moreover, the authors propose that these regulatory mechanisms may be in part controlled by CD82-mediated alterations in actin dynamics or the membrane lipid composition.

EGFR regulation by CD82 was also shown to mediate ganglioside production. More specifically, the overexpression of CD82 in combination with inhibition of ganglioside production resulted in increased EGFR phosphorylation in response to EGF stimulation (Li Y. et al., 2013). The authors speculate that significant interplay occurs between glycosphingolipid enriched microdomains and TEMs, which cooperatively regulate cellular signaling. The overexpression of CD82 might promote EGFR clustering, which may stimulate dimerization and thereby enhance downstream EGFR signaling. Alternatively, the reduction in ganglioside production might improve EGFR phosphorylation by reorganizing the receptors into clusters within TEMs, since gangliosides have been demonstrated to contribute to TEM organization (Odintsova et al., 2006).

Beyond the prominent role of CD82 in regulating EGFR, additional studies also identified CD9 as a mediator of EGFR signaling. With the use of an autocrine system of MDCK cells co-expressing CD9 and TGF-α, TGF-α stimulation promoted EGFR activation (Shi et al., 2000). The authors also utilized a paracrine system whereby CHO cells expressing TGF-α alone or TGF-α and CD9 together were plated with 32D cells expressing EGFR. This experiment demonstrated that co-expression of TGF-α and CD9 increases EGFR activation, although the precise mechanism by which CD9 modulates EGFR signaling remains unclear. Regardless, this study provides unique insight into how CD9 may regulate cellular signaling initiated through contact between adjacent cells. Interestingly, another report investigated the effect of CD9 expression on EGFR signaling, finding that increased expression of CD9 resulted in decreased phosphorylation of EGFR, Shc, and total Grb2 expression (Murayama et al., 2008). Though these studies demonstrate opposing effects of CD9 on EGFR, they also indicate that TNF-α plays a role in mediating EGFR activation through CD9. These studies open the possibility that other tetraspanins such as CD82 may also work in concert with TNF-α, similar to CD9 and TNF-α in mediating EGFR activation. Therefore, future analyses would benefit from examining the interplay of TNF-α with other tetraspanins in regulating EGFR signaling.

c-Kit

The stem cell factor receptor or c-Kit (CD117) is a receptor tyrosine kinase that binds to its ligand, stem cell factor (SCF), which is also known as steel factor (SLF) or kit ligand (Lennartsson and Rönnstrand, 2012). c-Kit signaling can activate several signaling cascades, including PI3K, Src family kinases, and MAPK to name a few. Moreover, c-Kit mediated signaling can control numerous cellular processes including migration, survival and the differentiation of hematopoietic progenitor cells. With the use of immunoprecipitation studies, it was determined that c-Kit associates with tetraspanins CD9, CD63, and CD81 and this interaction was enhanced upon treatment with SCF (Anzai et al., 2002). Although the authors found increased phosphorylation of c-Kit within the immunoprecipitated fraction, they determined that this does not enhance kinase activity in response to SCF treatment. Rather, the kinetics of SCF binding to c-Kit were altered when c-Kit associated with CD63. The authors suggest that this might be because free c-Kit is internalized upon SCF binding, implying that perhaps the CD63/c-Kit complex is more stable on the cellular surface. While this study alludes to a role for tetraspanins in regulating c-Kit phosphorylation, further analysis is necessary to determine the downstream consequences of tetraspanin mediated c-Kit activation. Additionally, the possibility that tetraspanins, such as CD63, might stabilize c-Kit and modulate signaling through alterations in protein trafficking could have significant impact on specific leukemias where c-Kit expression and activation are known to be dysregulated (Ikeda et al., 1991; Goemans et al., 2005; Boissel et al., 2006; Corbacioglu et al., 2006; Paschka et al., 2006).

c-Met

c-Met is a receptor tyrosine kinase that can activate numerous pathways to promote cellular survival, motility, and proliferation (Birchmeier et al., 2003). Hepatocyte growth factor (HGF) binding to c-Met causes c-Met dimerization, which helps to initiate various cellular signaling cascades including AKT, ERK/MAPK, and JNK (Organ and Tsao, 2011). Furthermore, the overexpression of CD82 diminished the phosphorylation of c-Met in response to integrin ligand engagement, resulting in reduced Src phosphorylation (Sridhar and Miranti, 2006). In the case of invasive tumor situations, the authors' data suggest that the loss of CD82 leads to enhanced activation of c-Met through integrin activation. Although the regulatory mechanism remains unknown, this study provides a clear indication that tetraspanins can modulate c-Met mediated signaling downstream of integrin engagement.

It was also shown through immunoprecipitation studies that CD82 and c-Met interact (Takahashi et al., 2007). Moreover, the authors demonstrated that upon the ectopic expression of

CD82, activation of c-Met with HGF led to increased formation of lamellipodia and filipodia through modulations in GTPase activities. Additionally, the ectopic expression of CD82 also prevented c-Met association with Grb2 and PI3K, implicating that CD82 has an inhibitory role with respect to these binding events. As such, perhaps the Grb2 and PI3K binding sites within c-Met become inaccessible in the presence of the c-Met/CD82 interaction.

The regulatory role of CD82 with respect to c-Met-mediated signaling has also been extended to controlling ERK1/2 and AKT signaling in hepatocellular carcinoma cells (Li Y. et al., 2013). An alternative report focused on CD151 with respect to Met signaling, showing that knockdown of CD151 led to diminished HGF-induced proliferation (Franco et al., 2010). The researchers determined that CD151 knockdown decreased tyrosine phosphorylation of the β4 integrin subunit, which decreased MAPK signaling through ERK in response to HGF. Therefore, this study suggests that the c-Met-CD151-β4 complex is critical for MAPK signaling. While the molecular link between tetraspanins and ERK or AKT downstream of c-Met remains an open question, this work implicates integrins as a possible connection.

Transforming Growth Factor Signaling

Transforming growth factor α (TGF-α) is synthesized as a transmembrane protein, which can become cleaved by metalloproteinases to release soluble TGF-α (Pandiella and Massagué, 1991). This cleavage is stimulated by endotoxins (Breshears et al., 2012; Liu Z. et al., 2013) and ROS (Boots et al., 2009) and is mediated primarily by ADAM17 (Peschon et al., 1998), but also by ADAM10 (Hinkle et al., 2003) and MeprinA (Bergin et al., 2008; Minder et al., 2012; Singh and Coffey, 2014). Moreover, TGF-α can interact with and activate EGFR on neighboring cells (Schlessinger and Ullrich, 1992; Thorne and Plowman, 1994; Moral et al., 2001). An association between CD9 and transmembrane TGF-α was identified and found to be dependent on the TGF-α ectodomain (Shi et al., 2000). The experimenters illustrated that the cleavage of TGF-α was inhibited by CD9, implicating a role for the association between CD9 and TGF-α as a means of protecting TGF-α from proteolytic cleavage. The authors suggested that the inhibition of TGF-α cleavage feeds into enhanced TGF-α induced EGFR activation, which can increase cellular proliferation. This study provides evidence that tetraspanins, such as CD9, can promote cellular signaling by stabilizing transmembrane proteins, thereby providing a potent activation stimulus to mediate juxtacrine signaling. Protein kinase C (PKC) and MAPK signaling can also regulate TGF-α cleavage (Baselga et al., 1996; Fan and Derynck, 1999). As tetraspanins can regulate PKC and MAPK signaling (Zhang et al., 2001; Termini et al., 2016), a closer examination into the interplay between these molecules in mediating TGF-α signaling may provide a more comprehensive view of the complex regulatory networks at play within TEMs.

A follow up study demonstrated that CD9 expression enhances TGF-α expression at the cell surface using MDCK cells (Imhof et al., 2008). Here, CD9 was shown to promote the trafficking of TGF-α from the Golgi to the cell surface by stabilizing the glycosylated and prodomain-removed forms of TGF-α. Furthermore, the authors demonstrated that the expression of TGF-α and CD9 alters actin organization and focal adhesion formation, supporting the notion that the combination of CD9 and TGF-α expression produces dramatically different signaling responses than the expression of TGF-α alone. Therefore, the tetraspanin expression profile should be considered when characterizing TGF-α signaling, particularly in many cancers where TGF-α expression is thought to support cancer progression (Kenny and Bissell, 2007).

Additionally, the contribution of tetraspanins to the regulation of the TGF isoform TGF-β1 has been assessed. Researchers used CD151 knockdown MDA-MB-231 cells and determined that in the presence of TGF-β1, CD151 knockdown cells had a significantly decreased proliferative rate compared to control cells (Sadej et al., 2010). More specifically, in the CD151 knockdown cells, TGF-β1 stimulation led to reduced p38 phosphorylation, resulting in decreased metastasis. Mechanistically, the authors propose that CD151 modulations of the plasma membrane may alter the distribution of TGF-β1 receptors and downstream signaling. Future studies may focus on determining how CD151 modulates the molecular organization of the TGF receptor, as this may provide a mechanism to regulate downstream signaling.

A Disintegrin and Metalloproteases

The A Disintegrin and Metalloprotease (ADAM) family of transmembrane and secreted proteins contribute to the regulation of cellular adhesion, migration, proliferation, and signaling (Seals and Courtneidge, 2003). As the name states, ADAMs contain a disintegrin and a metalloprotease domain. While the metalloprotease domain can cleave extracellular matrix (ECM) components and mediate ectodomain shedding of cytokines, growth factors, the disintegrin domain can interact with integrins. Recent comprehensive reviews provide insight on the role that tetraspanins play in regulating membrane proteases, with a particular emphasis on their role in regulating ADAM10 and ADAM17 (Yáñez-Mo et al., 2011; Matthews et al., 2016). Initial reports demonstrated that ADAM10 is associated with CD9, CD81, and CD82, indicating that ADAM10 likely exists within TEMs. Interestingly, treatment with anti-tetraspanin antibodies stimulated the release of TNF-α and EGF in an ADAM10-mediated manner. Furthermore, through mass spectrometry studies and extensive immunoprecipitation studies, Tspan12 was found to associate with ADAM10, which contributed to the ability of ADAM10 to process amyloid precursor protein for shedding (Xu et al., 2009). Using several mutated TSPAN12 constructs, this association was determined to be regulated by EC1, the C-terminal tail and TSPAN12 palmitoylation. More recent co-immunoprecipitation studies revealed that the subgroup of TspanC8 tetraspanins (Tspan5, 10, 14, 15, 17, and 33) interact with ADAM10 (Dornier et al., 2012). Additionally, ADAM17 was also found to associate with tetraspanin CD9 in leukocytes and endothelial cells, which diminishes ADAM17-mediated TNF-α and ICAM-1 shedding. Interestingly, CD9 can regulate the catalytic activity of ADAM17 with regards to shedding of LR11 in monocytes, promonocytes

and B-lymphoblastoid cell lines (Tsukamoto et al., 2014). As ADAMs are implicated in regulating various cancer cell types (Mochizuki and Okada, 2007), the role of tetraspanins in regulating ADAMs in malignant cells will provide significant insight and perhaps a means to attenuate aberrant ADAM activity.

ADAMs are produced as immature, inactive, preforms in the endoplasmic reticulum. During trafficking from the ER to the plasma membrane, the enzyme's prodomain is removed and ADAMs are then rendered catalytically active (Seals and Courtneidge, 2003). Interestingly, it was determined that TspanC8 contributes to ADAM10 maturation and ultimately the stabilization of ADAM10 at the cell surface (Prox et al., 2012). Furthermore, Tspan33 knockdown in erythrocytes resulted in diminished ADAM10 surface expression. Meanwhile, ADAM10 surface expression remained unchanged in platelets, demonstrating that tetraspanin regulation of ADAM10 is likely cell-type specific (Haining et al., 2012). Additionally, the role of Tspan33 in regulating ADAM10 for the control of macrophage activation was recently explored (Ruiz-García et al., 2016). Researchers utilized Tspan33 overexpressing Raw 264.7 cells and demonstrated that increased Tspan33 expression results in increased ADAM10 processing, consistent with the earlier aforementioned studies.

TETRASPANINS AND INTRACELLULAR SIGNALING

Although tetraspanins are known to primarily affect the properties of other membrane proteins, they have also been shown to regulate cytoplasmic signaling molecules. Signaling proteins are often recruited to the cytoplasmic interface of the plasma membrane where they initiate signaling and TEMs can serve as a potential membrane recruitment site. Therefore, in the following section, we will review how tetraspanins control the localization, kinetics, and signaling properties of cytosolic proteins.

Protein Kinase C

The protein kinase C (PKC) family of intracellular signaling proteins consists of isoforms, which are further classified into conventional, novel or atypical isoforms (Newton, 1995). PKCs can phosphorylate several targets, including the myosin light chain II (Liu X. et al., 2013), PKD2 (Waldron et al., 2001; Navarro and Cantrell, 2014), Ras GEFs (Jun et al., 2013), and the β1 integrin tail (Stawowy et al., 2005), which collectively contribute to the regulation of cell proliferation, apoptosis, and adhesion amongst other cellular behaviors (Kang, 2014). The interaction between tetraspanins and PKC was originally demonstrated in K562 cells using an elaborate series of immunoprecipitation experiments (Zhang et al., 2001). The experimenters used phorbol 12-myristate 13-acetate (PMA), which mimics diacylglycerol (DAG) to activate PKC (Castagna et al., 1982). Under PMA stimulated conditions tetraspanins CD9, CD53, CD81, and CD82 individually interact with PKCα and not with PI3K. Additionally, they determined that CD81 and CD151 associate with PKCβII. Moreover, in a PKCα pull-down, β1, α3, and α6 integrins were detected in complex with PKC. Therefore, it was suggested that tetraspanins serve to link PKC to integrins. In order to assess the tetraspanin domains that control PKC associations, chimeric mapping was performed by replacing portions of CD9 with portions of the non-PKC associating tetraspanin, A15/Talla1. These findings demonstrated that PKC association with tetraspanins occurs outside of the short inner loop, the large outer loop, and transmembrane 3 or 4.

A recent report also demonstrated that tetraspanin CD151 regulates skin squamous cell carcinoma through STAT3 and PKCα signaling (Li Q. et al., 2013). Utilizing wild type or CD151 ablated A431 epidermoid carcinoma cells, it was shown that the loss of CD151 reduces STAT3 activation in response to 12-O-Tetradecanoylphorbol-13-acetate (TPA) stimulation, which is another known activator of PKCα. The authors found that PKCα only associates with α6β4 upon TPA stimulation when CD151 is present. Together, these data suggest that perhaps the role for CD151 is to recruit PKCα into close proximity with the α6β4 integrin, which ultimately aids in the phosphorylation of α6β4. As such, these data build upon previous implications that tetraspanins link PKC to integrins (Zhang et al., 2001), but also provide evidence that this scaffolding is important for epidermal proliferation and STAT3 activation.

Another interesting report investigated how CD9, CD81, and CD151 expression affects PKCα association with TEMs (Gustafson-Wagner and Stipp, 2013). It was demonstrated that CD9/CD81 knockdown diminishes the ability for the α3 integrin to associate with PKCα, which delays cell spreading on laminin and directed migration. In contrast, CD151 knockdown enhanced the association of PKCα with the α3 integrin, while promoting cell migration on collagen-I. The authors propose that CD9/81 may serve as linkers of PKC to the α3 integrin subunit, or there might be an indirectly associating molecule at play. Furthermore, the authors propose that perhaps upon CD151 depletion, there is increased association between PKC and α3 due to the loss of CD151, which makes CD9/81 available to fully associate with α3, thereby promoting PKC-integrin association. This study provides substantial evidence that the roles of tetraspanins CD9, CD81, and CD151 are unique in their regulation of PKCα-integrin interactions.

Further examination into the regulatory role of tetraspanins with respect to PKC-mediated signaling has uncovered many unique cellular responses. For example, treatment of A431 cells with Calphostin C to inhibit PKCα reduced filipodia extensions as well as E-cadherin puncta formation, demonstrating the involvement of actin in tetraspanin-regulated PKC signaling (Shigeta et al., 2003). The authors suggest that CD151 directly or indirectly associates with PKCα, which they propose may activate Cdc42 to promote filipodia formation.

A more recent report from our laboratory demonstrated that CD82 regulates PKCα signaling in acute myeloid leukemia (AML) (Termini et al., 2016). Using quantitative FRET imaging and KG1a AML cell lines that overexpress wild type CD82 or a palmitoylation deficient form of CD82 (Delandre et al., 2009), we found that upon PMA stimulation, PKCα was recruited to the plasma membrane where it associates with CD82. However,

upon extended PMA stimulation, this PKCα/CD82 association is reduced in cells overexpressing the palmitoylation deficient form of CD82, demonstrating that the palmitoylation of CD82 regulates the stability of the PKCα interaction. We went on to use super-resolution imaging to examine how the scaffolding properties of CD82 regulate the macromolecular clustering of PKCα and found that upon disruption of the CD82 scaffold, there is a significant reduction in the size of PKCα clusters. Moreover, using CD82 knock-down cells, we found that while PKCα is still recruited to the membrane upon PMA stimulation, large-scale PKCα clusters are not detected. This change in PKCα clustering was then linked to alterations in downstream ERK1/2 signaling that influenced the aggressive phenotype of AML (Termini et al., 2016). Interestingly, the kinetics of PKCα oligomerization were recently quantified and modeled using HEK cells where they found that the intramolecular clustering of PKCα contributes to downstream phosphorylation (Bonny et al., 2016). Collectively, these studies illustrate that the modulation of signaling molecule clusters may serve as an important regulatory mechanism for stabilizing and/or attenuating signal transduction pathways. Moreover, our work implicates tetraspanins as critical mediators of cluster size and stability. Future super resolution imaging studies focused on identifying how the clustering of tetraspanins can modulate downstream signaling through PKC and other molecules such as Rac or Cdc42 would be valuable to help clarify how tetraspanins and PKCα mediate cytoskeleton-dependent cellular responses such as adhesion and migration.

An interesting link was also discovered between PKC and EGFR-mediated signaling that is enhanced by CD82. c-Cbl is an ubiquitin ligase recruited to EGFR where it assists with receptor down-regulation (Joazeiro et al., 1999). The authors found that PKC mediates c-Cbl phosphorylation upon EGF stimulation in CD82 expressing H2B cells (Odintsova et al., 2013). The phosphorylation of c-Cbl serves as a negative regulator of enzyme function (Ryan et al., 2006), which may be responsible for inhibiting EGFR downregulation. Therefore, without CD82 present, EGFR can be quickly downregulated as PKC is not present to regulate c-Cbl. Collectively, these studies provide substantial evidence that implicates tetraspanins as signaling scaffolds that promote the close proximity of PKC with integrins, EGFR and cytoplasmic proteins like c-Cbl.

Phosphatidylinositol 4-Kinase

Phosphatidylinositol 4-kinase (PI4K) catalyzes the conversion of phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PI4P), which is an important intermediate for lipid-mediated signaling (Clayton et al., 2013). A series of biochemical experiments demonstrated that PI4K exists within α3 integrin and CD63 containing TEMs (Berditchevski et al., 1997). The authors suggest that perhaps TEMs are responsible for linking the α3β1 integrin to PI4K. A follow up study from the same group explored this further, demonstrating that immunoprecipitation of α3 or CD151 yields similar levels of PI4K activity based upon PI4P production (Yauch et al., 1997). Additionally, using cells with diminished α3 expression, CD151 was pulled down, demonstrating that there is still PI4K associated with the complex. Conversely, immunodepletion of CD151 resulted in significantly diminished lipid kinase activity associated with α3, while CD63 and/or CD81 deletion did not have as significant of an effect. Collectively, these data implicate CD151 as a critical linker between PI4K and α3β1, which the authors suggest may support cell migration.

A subsequent follow up study demonstrated that PI4K associates with tetraspanins A15/TALLA1, CD63, CD151, CD9, and CD81, however it does not appear to associate with NAG-2, CD53, CD37, or CD82 (Yauch and Hemler, 2000). Moreover, PI3K and PI4P5K activity were not detected in CD63, CD81, and CD151 complexes, indicating that perhaps the association is specific to PI4K. Studies with CD9/CD82 chimeras were unsuccessful at determining the site of association with PI4K. Therefore, a closer examination into the structural domains within tetraspanins that contribute to their association with PI4K could provide insight into the mechanism by which tetraspanins may regulate the catalytic activity of PI4K and downstream responses.

GTPases

Rho GTPases mediate signal transduction by switching between a GTP-bound (active) and GDP-bound (inactive) state (Bishop and Hall, 2000). There are numerous effector proteins downstream of GTPases including PI3K, PI-4-P5K, MEKK1, and DAG kinase. The Rho family GTPases Rac1, RhoA, and Cdc42 as well as the Ras family of GTPases translocate to the plasma membrane upon activation (Collins, 2003), where their regulation by tetraspanins continues to be defined.

For example, CD151 was demonstrated to regulate Cdc42 for the control of cellular adhesion. Using A431 cells, CD151 antibody treatment or CD151 overexpression was found to increase Cdc42 activation, which the authors suggest controls actin reorganization, promoting filopodia-based adhesions (Shigeta et al., 2003). Another study assessed how the coexpression of CD9 and TGF-α regulates GTPase signaling, finding increased and decreased levels of activated Rac1 and RhoA respectively, with Cdc42 levels remaining unchanged upon coexpression of CD9 and TGF-α (Imhof et al., 2008). This shift in signaling was determined to be due to enhanced EGFR signaling, which ultimately contributed to enhanced stress fiber formation. Additionally, the overexpression of CD82 was shown to decrease the proportion of GTP-bound Rac1, while RhoA and Cdc42 levels remained unchanged (Liu et al., 2012).

Previous work also demonstrated that CD151 promotes the association between CD151-β1 complexes and Ras, Rac1 or Cdc42. Immunofluorescence imaging showed that CD151 regulates the translocation of Rac1 and Ras to the membrane and promoted colocalization with β1 integrins (Hong et al., 2012). Interestingly, through the use of a CD151 chimera with disrupted α3β1 integrin association, the authors showed that this mutant is unable to recruit Rac1 to the membrane. Therefore, integrins also have the capacity to link GTPases to tetraspanins in a manner similar to what was previously proposed for PKC and tetraspanins (Zhang et al., 2001; Li Q. et al., 2013). An

association between Rac1 and the C-terminal, cytoplasmic region of CD81 has also been suggested based on the use of an eight amino acid C-terminal tail peptide (Tejera et al., 2013). Future experiments that mutate or delete the CD81 C-terminal tail will be important to demonstrate that such a mutation eliminates Rac1 association, further validating the interaction. Furthermore, upon EGF stimulation, it was shown that knockdown of CD81 increases Rac activation. A more recent study identified a correlation between CD9 expression and GTP bound Rac1 expression in acute lymphoblastic leukemia patient samples (Arnaud et al., 2015). Moreover, this group also determined that the C-terminal tail of CD9 is important for regulating Rac1 activation. Interestingly, the C-terminal region of CD9 has two known palmitoylation sites (Charrin et al., 2002), and Rac can also be palmitoylated (Tsai and Philips, 2012). Therefore, it is possible that these post-translational modifications may help to anchor tetraspanins and GTPases into similar membrane compartments.

Tetraspanin regulation of RhoA signaling, which can promote changes in cytoskeletal organization, has also been characterized (Sit and Manser, 2011). Using human aortic smooth muscle cells, CD9 knockdown decreased the expression of GTP-bound RhoA, leading to defects in cellular morphology, spreading and contraction (Herr et al., 2014). The authors suggest that integrins are involved in CD9-mediated alterations in RhoA activation by possibly stabilizing integrin-ECM interactions, augmenting RhoA activation. Interestingly, a recent report demonstrates that the loss of CD151 in breast cancer cells resulted in increased RhoA activation as quantified using FRET biosensors (Novitskaya et al., 2014). These data are contrary to Hong et al. (2012), who showed no change in Rho activation upon CD151 depletion. However, the change in FRET efficiency detected was <5%, which would likely be below the detection of the small GTPase protein pull-down assays used by Hong et al. Moreover, a separate report demonstrated that the knockdown of CD151 in human dermal microvascular endothelial cells resulted in an increase in RhoA-GTP and decreased Rac1-GTP (Zhang et al., 2011). Future studies focused on the mechanism by which tetraspanins can modulate GTPase activation will be important for determining how certain tetraspanins may be targeted to control specific GTPase activities in specialized cell types.

β-Catenin

β-catenin is a component of the Wnt signaling pathway that binds to the cytosolic portion of cadherins to initiate cellular signaling (Valenta et al., 2012). Through this complex formation, β-catenin promotes the internalization and recycling of E-cadherin, thereby destabilizing the complex and ultimately reducing cell-cell adhesion. Researchers determined that ectopic CD82 expression in h1299 cells relocalizes β-catenin to E-cadherin at the cell membrane, which stabilizes complex formation (Abe et al., 2008). Furthermore, they showed that ectopic CD82 expression increased cancer cell aggregation. To assess the downstream consequences of altered β-catenin localization, the authors stimulated cells with EGF or hepatocyte growth factor (HGF), demonstrating that ectopic expression of CD82 diminished β-catenin phosphorylation. While β-catenin phosphorylation is known to destabilize the E-cadherin complex, the mechanism for tetraspanin involvement remains to be clearly defined. Based on our previous work with N-cadherin (Marjon et al., 2016), we speculate that the CD82 scaffold might contribute to cadherin clustering, which may stabilize β-catenin membrane interactions, thereby protecting β-catenin from phosphorylation and down-regulation.

More recently, CD63 was shown to stabilize β-catenin signaling. In this study, shRNA knockdown of CD63 decreased β-catenin protein expression levels, which was suggested to occur through diminished levels of inactive GSK3β, leading to increased levels of phosphorylated β-catenin (Seubert et al., 2015). Furthermore, decreased levels of the β-catenin targets, MMP-2 and PAI-1, were detected, demonstrating CD63-mediated changes in downstream β-catenin signaling. The authors went on to find that the reduced expression of CD63 diminishes the metastatic potential of lung cancer cells, while the overexpression promoted tumor aggressiveness. However, modulations in signaling induced by CD63 overexpression were not explored. A previous study provided evidence that disrupting the interaction between the α3β1 integrin and CD151 enhanced β-catenin phosphorylation (Chattopadhyay et al., 2003). Therefore, it is plausible that the combination of integrins and tetraspanins serves to stabilize β-catenin within TEMs.

TETRASPANIN POST-TRANSLATIONAL MODIFICATIONS AND SIGNALING

Palmitoylation

S-palmitoylation is the addition of a 16-carbon fatty acid chain, palmitate, to cysteine residues of either cytoplasmic or membrane proteins (Blaskovic et al., 2013). Palmitoylation of cytoplasmic proteins promotes membrane anchoring, while palmitoylation of membrane proteins facilitates trafficking and membrane organization. Palmitoylation has been confirmed for tetraspanins CD9, CD151 (Yang et al., 2002), CD81 (Delandre et al., 2009), and CD82 (Mazurov et al., 2007), however other tetraspanins also contain conserved cysteine residues that are predicted to be palmitoylated. The defined role for palmitoylation is to modulate TEM formation (Yang et al., 2004). Therefore, we took a closer examination of how tetraspanin palmitoylation contributes to the signaling that occurs downstream of TEM associated proteins.

For example, the expression of the palmitoylation deficient form of CD151 weakened its association with integrins (Berditchevski et al., 2002), resulting in diminished phosphorylation of AKT in response laminin-5 engagement. These data indicate that palmitoylation-mediated disruption of TEMs can reduce downstream signaling responses. Additionally, a palmitoylation deficient form of Tetraspanin12 was shown to have diminished association with ADAM10, resulting in decreased ADAM10 activity as assessed by APP shedding (Xu et al., 2009). Recent work from our lab has shown

that overexpression of a palmitoylation-deficient form of CD82 diminishes PKC membrane stabilization, reducing ERK1/2 activation, and downstream leukemia colony formation (Termini et al., 2016). Collectively, these studies demonstrate that tetraspanin palmitoylation contributes significantly to the regulation of downstream cellular signaling. Intracellular signaling molecules such as Ras (Eisenberg et al., 2013), Rac (Tsai and Philips, 2012), and PKC (Ford et al., 1998) can themselves be palmitoylated to assist with their membrane anchorage. As tetraspanin palmitoylation is thought to regulate lateral protein associations within TEMs, perhaps tetraspanin palmitoylation functions in concert with the palmitoylation of cytoplasmic proteins to produce stable membrane interactions critical for sustained signaling.

Glycosylation

Although the large extracellular loop of many tetraspanins has been demonstrated to have one or more potential N-linked glycosylation sites, little is known about the functional consequences of this post-translational modification. The N-glycosylation pattern of CD82 was recently identified using proteomics and glycomics, determining that there are three putative N-glycosylation sites (Wang H. et al., 2012). Previously, these sites were suggested to regulate apoptosis, however the researchers did not examine the signaling that led to these apoptotic changes (Ono et al., 1999). Interestingly, the photoreceptor-specific tetraspanin retinal degeneration slow (RDS) can also be glycosylated (Kedzierski et al., 1999; Conley et al., 2012). More recently, the function of RDS glycosylation was re-examined by expressing a glycosylation deficient version of RDS in mice, which identified differential functional outcomes in cones vs. rod photoreceptor cells (Stuck et al., 2015). Moreover, the authors determined that glycosylation regulates the formation of RDS complexes with another tetraspanin ROM-1, demonstrating that glycosylation can modulate tetraspanin complex formation. A recent report from our laboratory examined the role of CD82 glycosylation with respect to acute myeloid leukemia homing (Marjon et al., 2016). In this study, we demonstrated that mutation of the three glycosylation sites within CD82 to inhibit glycosylation resulted in increased AML cell homing to the bone marrow, which we linked to increased molecular packing of N-cadherin via super resolution imaging. Although we have yet to examine signaling deficits in cells with disrupted CD82 glycoslation, it is possible that these changes in the molecular organization of N-cadherin may modulate the activation or stability of p120 catenin or β-catenin signaling downstream of N-cadherin.

Ubiquitination

Protein ubiquitination is important for regulating cellular signaling by selectively targeting proteins for degradation. Both CD81 and CD151 were shown to interact with gene related to anergy in lymphocytes (GRAIL), which promotes tetraspanin ubiquitination, ultimately downregulating surface tetraspanin expression (Lineberry et al., 2008). Interestingly, it was determined that these tetraspanins can only be ubiquitinated at their N-terminus. Through mutational studies, it was shown that mutation of K8 and K11 diminished the ubiquitination of CD81, while mutation of K8, K11, and K17 ablated the ubiquitination of CD151. More recently it was demonstrated that TSPAN6 interacts with the adaptor mitochondrial antiviral signaling (MAVs) in 293T cells to inhibit RIG-I-like receptor (RLR) mediated signaling (Wang Y. et al., 2012). The authors went on to show that induction of RLR signaling promoted the ubiquitination of TSPAN6 at K11, K16, and K43, which are sites found within the TM1 of TSPAN6. Additionally, the authors determined that TSPAN6 ubiquitination serves to inhibit the formation of the signalosome, effectively down-regulating RLR signaling. As ubiquitination can target proteins for degradation, we suspect that tetraspanin ubiquitination will be a regulatory mechanism to allow for specific and efficient attenuation of tetraspanin-mediated signaling.

CONCLUDING REMARKS

Tetraspanins and their formation into TEMs enable the compartmentalization of membrane receptors within the plasma membrane. In this review, we focus on how tetraspanins also serve to connect these membrane-associated molecules with intracellular signaling complexes. It is now clear that tetraspanins regulate diverse cell signaling pathways that impact a breadth of biological processes. However, though numerous signaling molecules have been demonstrated to associate with tetraspanins, the mechanisms by which tetraspanins precisely modulate signal transduction remains relatively undefined. Future studies focused on how domains and motifs within tetraspanins promote or perhaps attenuate cellular signaling will help us understand the specific mechanisms used by this family of proteins to control signaling. Many laboratories are now using sophisticated imaging techniques to provide novel insight into the spatiotemporal interactions mediated by tetraspanins and TEMs. These studies will help to define how the scaffolding properties of tetraspanins contribute to the formation, stabilization and dynamics of signal transduction complexes at the plasma membrane. Moreover, these studies may provide the needed insight to establish tetraspanins and TEMs as potential therapeutic targets for the modulation of aberrant signal transduction that mediates processes such as inflammation, wound healing, and various types of cancer.

AUTHOR CONTRIBUTIONS

CT and JG wrote and edited the manuscript. CT created the figures. All authors reviewed the manuscript.

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REVIEW
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Tetraspanins and Transmembrane Adaptor Proteins As Plasma Membrane Organizers—Mast Cell Case

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The plasma membrane contains diverse and specialized membrane domains, which include tetraspanin-enriched domains (TEMs) and transmembrane adaptor protein (TRAP)-enriched domains. Recent biophysical, microscopic, and functional studies indicated that TEMs and TRAP-enriched domains are involved in compartmentalization of physicochemical events of such important processes as immunoreceptor signal transduction and chemotaxis. Moreover, there is evidence of a cross-talk between TEMs and TRAP-enriched domains. In this review we discuss the presence and function of such domains and their crosstalk using mast cells as a model. The combined data based on analysis of selected mast cell-expressed tetraspanins [cluster of differentiation (CD)9, CD53, CD63, CD81, CD151)] or TRAPs [linker for activation of T cells (LAT), non-T cell activation linker (NTAL), and phosphoprotein associated with glycosphingolipid-enriched membrane microdomains (PAG)] using knockout mice or specific antibodies point to a diversity within these two families and bring evidence of the important roles of these molecules in signaling events. An example of this diversity is physical separation of two TRAPs, LAT and NTAL, which are in many aspects similar but show plasma membrane location in different microdomains in both non-activated and activated cells. Although our understanding of TEMs and TRAP-enriched domains is far from complete, pharmaceutical applications of the knowledge about these domains are under way.

Keywords: CD9, LAT, NTAL, IgE receptor, plasma membrane, membrane microdomains, signal transduction

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INTRODUCTION

The plasma membrane, as well as other cellular membranes, is a very complex structure composed of a plethora of proteins and a variety of lipids organized into two asymmetrical leaflets. The proper function of cellular membranes depends on the composition of the leaflets and intermolecular communication of the membrane components. This is especially important in such complex processes as signal transduction from plasma membrane receptors into the cytoplasm and nucleus. Cellular membranes play a key role in signal transduction in both directions, inside-out and outside-in. For years it has been thought that the lipid composition of the membrane allows formation of signaling platforms that are critical for membrane functioning and that membrane lipids and proteins play key roles as membrane organizers (Singer and Nicolson, 1972; van Meer et al., 2008; Simons and Gerl, 2010; Treanor and Batista, 2010; Simons and Sampaio, 2011).

Many systems have been used to study the structure-function relationships of the plasma membrane components. Among them are mast cells (MCs) or MC lines. MCs are effector cells of the immune system that are able to react to external stimuli by rapid release of numerous allergy mediators from cytoplasmic granules and/or by production and secretion of a variety of cytokines and chemokines. Activation of MCs is initiated by binding of ligands [e.g., antigens (Ags)] to plasma membrane receptors or their complexes [e.g., immunoglobulin (Ig)E bound to high-affinity IgE receptor (FcεRI)]. This binding initiates cell activation events, which involve a number of signal transduction molecules forming functionally and spatially connected units, called signalosomes. Receptor-mediated responses vary in strength and duration. For example, aggregation of FcεRIs by multimeric Ag-IgE complexes leads to phosphorylation of several proteins, followed by Ca^{2+} response, in seconds after triggering, release of pre-formed granules containing various mediators, in minutes after activation, and ending by *de novo* synthesis of lipid mediators, cytokines and chemokines in tens of minutes and hours after activation (reviewed in Marshall, 2004; Galli et al., 2008; Kalesnikoff and Galli, 2008; Galli and Tsai, 2012). These signaling events could have dramatic physiological consequences, such as causing allergy disease (Galli and Tsai, 2012) or breaking down poison in snake venom (Metz et al., 2006), and therefore must be precisely regulated (Galli, 2016). There are numerous signal transduction regulators localized in the plasma membrane, membrane proximal, in the endoplasmic reticulum, as well as in the cytoplasm. This review is focused on two groups of transmembrane proteins that are involved in plasma membrane receptor regulation and functioning. The first group consists of tetraspanins, which span the plasma membrane four times and form two extracellular domains and short intracellular tails. The second group consists of transmembrane adaptor proteins (TRAPs), which possess a short extracellular domain, one transmembrane domain and a long intracellular tail with several tyrosines that once phosphorylated can serve as anchor for different proteins and in this way influence signal transduction. Although these two groups of proteins are structurally different, they share some common properties, such as palmitoylation and ability to interact with a plethora of plasma membrane-bound or intracellular proteins. Furthermore, recent studies with MCs found a cross-talk between proteins in these two groups in the regulation of plasma membrane-bound signaling events (Hálová et al., 2013).

TETRASPANINS

Tetraspanins are an evolutionarily conserved superfamily of transmembrane proteins with characteristic features. They have four transmembrane domains and two extracellular loops, a small one (SEL) and a large one (LEL). LEL possesses a conserved CCG motif and at least two other cysteine residues that form disulfide bonds inside the LEL domain (**Figure 1**). There are two main post-translational modifications occurring in tetraspanins. Most of them possess one or more N-glycosylation sites at the LEL domain with two exceptions, cluster of differentiation (CD)9, which possesses an N-glycosylation site in SEL, and CD81, which is non-glycosylated (Boucheix and Rubinstein, 2001). Interestingly, all of the so far studied tetraspanins contain a palmitoylation site (Charrin et al., 2002; Yang et al., 2002, 2004). Palmitoylation is a modification that tetraspanins share with a plethora of other transmembrane or membrane-associated proteins, such as TRAPs [including linker for activation of T cells (LAT), non-T cell activation linker (NTAL; also called LAT2), and phosphoprotein associated with glycosphingolipid-enriched membrane microdomains (PAG; also called CSK-binding proteins)] (Draber et al., 2011a; Stepanek et al., 2014), integrins (Berditchevski, 2001; Gagnoux-Palacios et al., 2003; Yang et al., 2004), SRC kinases (Kovářová et al., 2001; Gilfillan and Rivera, 2009), and others. Examples of palmitoylated proteins expressed on the MC membrane are presented in **Figure 1**. Palmitoylation is important for the protein topography and its functioning in the plasma membrane (see below).

Tetraspanins are known regulators of cell migration (Boucheix and Rubinstein, 2001) and are involved in tumor progression and metastasis (Zöller, 2009). Some tetraspanins such as CD82, and often also CD9, are downregulated in advanced stages of cancer; their absence is a sign of poor prognosis in patients with several types of cancer. These tetraspanins are considered as tumor suppressors. However, some other tetraspanins, such as CD151 and TSPAN8, have been found upregulated in metastases and their upregulation was associated with poor prognosis, suggesting that they serve as tumor promotors (reviewed in Zöller, 2009). The authors speculated that the different roles of tetraspanins in tumor progression could be the result of specific tetraspanin abundance in exosomes. This could positively or negatively influence the fusion of exosomes with plasma membranes in partner cells, depending on the tetraspanin type. In this way exosomes possessing different tetraspanins could exhibit different delivery of important communicators such as mRNA and microRNA and also proteins important in cell-cell communication (Zöller, 2009).

Several tetraspanins display broad tissue expression (e.g., CD9, CD81, CD151), whereas others are restricted e.g., to leukocytes (CD37, CD53). Only a few tetraspanins (UP1a, UP1b, peripherin and ROM-1) have their distribution limited to specific tissue (Tarrant et al., 2003; Charrin et al., 2009, 2014). Although tetraspanins are abundant proteins in many cell types, animals deficient in selected tetraspanins usually do not exhibit striking phenotypes, probably due to the redundancy and functional compensation of individual tetraspanins. On the other hand, mutations in individual tetraspanin genes have been described as a cause of several life-threatening diseases (Kajiwara et al., 1993, 1994; Zemni et al., 2000; Karamatic et al., 2004; van Zelm et al., 2010). In some cases, mice lacking a particular tetraspanin mimicked the phenotype observed in humans with a defect in the same tetraspanin (Hemler, 2005).

Although several tetraspanins (CD9, CD37, CD53, CD63, CD81, CD82, CD151) are abundantly expressed on the plasma membrane of MCs and/or secretory vesicles (**Table 1**), their role in MC physiology and activation events is not completely understood. The problem is that for some tetraspanins there

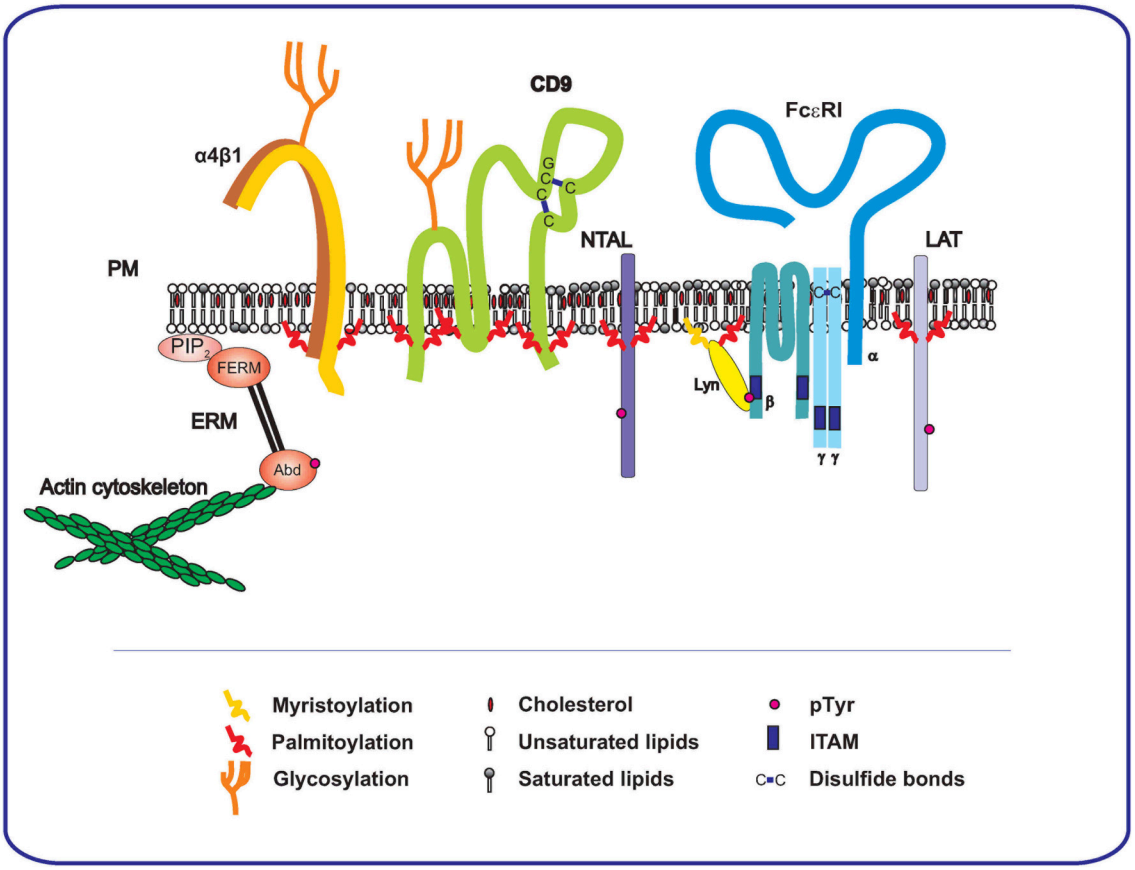

FIGURE 1 | Involvement of TEMs and TRAP-enriched domains in FcεRI signalosome. At the level of the plasma membrane, there is a cross-talk of FcεRI, consisting of four transmembrane subunits (α,β, and γγ), with tetraspanins and TRAPs. These interactions affect FcεRI signal transduction. Phosphorylated ITAMs of FcεRI bind LYN kinase, which is connected to the membrane by palmitoylation and myristoylation. Two important TRAPs, NTAL and LAT, which occupy different nanodomains, are also palmitoylated. Tetraspanins are represented by CD9, which contains six palmitoylation domains and one possible glycosylation site in a small intracellular loop, two disulfide bonds and tetraspanin conserved motif CCG are also depicted in the figure. Finally, integrins are represented by palmitoylated α4β1 that is located in close proximity of CD9. The membrane is connected with the actin cytoskeleton through the FERM (4.1,ezrin/radixin/moesin) domain and actin-binding domain (Abd) of ERM proteins. FERM binds directly to phosphatidylinositol 4 5-bisphosphate (PIP2).

are no commercially available antibodies and/or knockout (KO) animals for such studies. The role of tetraspanins in MCs was reviewed in 2012 (Köberle et al., 2012) and since then, several new studies have appeared highlighting the significance of these membrane organizers in MC physiology. The novel findings are summarized below.

CD9

Although CD9 KO mice have been prepared (Le Naour et al., 2000), they have not been used for the studies of the role of CD9 in MC physiology. Thus, our knowledge about the role of CD9 in MCs comes mainly from the studies using CD9-specific antibodies. Our recent study described a new CD9-specific monoclonal antibody (mAb), 2H9, which caused mast cell degranulation, Ca^{2+} release and tyrosine phosphorylation of several proteins including TRAP NTAL and dephosphorylation of ezrin/radixin/moesin (ERM) family proteins (Hálová et al., 2013). Phosphorylation of NTAL was brought about by complete antibody but not its $F(ab)_2$ fragment, suggesting that the Fc fragment of the antibody is involved. Further studies showed that 2H9 antibody produced NTAL phosphorylation in cooperation with FcγRs through SRC family kinase LYN, and that CD9 and NTAL co-localized in the same membrane microdomains after antibody-induced CD9 aggregation. A previous study showed that antibody-induced aggregation of human CD9 expressed in CD9-negative rat basophilic leukemia (RBL) cells, clone 2H3, also caused degranulation, but its $F(ab)_2$ fragment not (Higginbottom et al., 2000). The authors speculated that CD9 and FcεRI form complexes, and therefore crosslinking by anti-CD9 antibody induced activation by a mechanism similar to the one induced by aggregation of FcεRI. However, experiments with bone marrow-derived mast cells (BMMCs) showed that CD9 did not co-localize with FcεRI in non-activated cells, but dimerization of CD9 with bivalent antibody induced movement of CD9 into the close proximity of FcεRI (**Figure 2**). This co-localization was further strengthened by crosslinking of CD9-anti-CD9 complexes with

TABLE 1 | Selected tetraspanins expressed in mast cells and their function.

Gene symbol	Most used aliases	KO	Phenotype of KO mice, general/in mast cells	Other important functions in mast cells
CD9	BTCC-1, DRAP-27, MIC3, MRP-1, TSPAN-29, TSPAN29	Le Naour et al., 2000	Deficiency in sperm-egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Wright et al., 2004)/not studied	Antibodies against CD9 block MCs migration toward IL-16 (Qi et al., 2006) and Ag (Hálová et al., 2013) and induce Ca^{2+} release and phosphorylation of several substrates (Hálová et al., 2013)
CD37	GP52-40, TSPAN26	Knobeloch et al., 2000	Mild alteration of immune system (Knobeloch et al., 2000)/normal levels of IgE, MCs not particularly studied (Knobeloch et al., 2000)	
CD53	MOX44, Ox-44, TSPAN25			Associated with population asthma risk but not directly connected to MCs (Lee et al., 2013)
CD63	LAMP-3, ME491, MLA1, OMA81H, TSPAN30	Schröder et al., 2009	Mild defects, altered water balance (Schröder et al., 2009)/reduced degranulation, TNF-α secretion, and PCA (Kraft et al., 2013)	Antibody against CD63 suppresses degranulation and PCA (Kraft et al., 2005)
CD81	TAPA-1, CVID6, S5.7, TSPAN28	Maecker and Levy, 1997	Female infertility (Rubinstein et al., 2006), nervous system malfunctions (Geisert et al., 2002), reduced IL-4 production (Maecker et al., 1998), lower expression of CD19 on B cells (Maecker and Levy, 1997)/not studied	Antibody against CD81 suppresses degranulation and PCA (Fleming et al., 1997)
CD82	KAI1 4F9, C33, GR15, IA4, R2, SAR2, ST6, TSPAN27	Risinger et al., 2014	Mild changes in phenotype, could lead to changes in early establishment of proliferation and division when challenged with a new environment (Risinger et al., 2014)/not studied	
CD151	GP27, MER2, PETA-3, RAPH, SFA1, TSPAN24	Wright et al., 2004; Sachs et al., 2006	Bleeding (Wright et al., 2004), decreased angiogenesis (Takeda et al., 2007), kidney failure (Sachs et al., 2006)/increased late phase of PCA and production of proinflammatory cytokines (Abdala-Valencia et al., 2015)	CD151 is upregulated in MCs upon FcεRI activation (Abdala-Valencia et al., 2015)

secondary antibody (Hálová et al., 2013). Surprisingly, unlike anti-CD63 (Kraft et al., 2005) or anti-CD81 (Fleming et al., 1997), as discussed below, anti-CD9 did not modulate Ag-induced degranulation (Hálová et al., 2013).

CD9 together with CD81 have been shown to co-localize with a trimeric variant of FcεRI in human $FcεRI^{pos}$ dendritic cells isolated from the skin of patients with atopic dermatitis. In contrast, only moderate expression of CD9 and CD81 was found on $FcεRI^{neg}$ monocytes (Peng et al., 2011). Concomitant activation by FcεRI and CD9 crosslinking resulted in increased interleukin (IL)-10 production compared to crosslinking of the FcεRI alone. In contrast, co-activation of FcεRI with CD81 or activation by aggregation of CD81 alone or CD9 alone had no effect on IL-10 production (Peng et al., 1997). These data can be taken as another evidence that CD9 and FcεRI are somehow functionally cooperating.

Tetraspanins are well known regulators of chemotaxis and migration in several cell types (Berditchevski, 2001; Boucheix and Rubinstein, 2001). In MCs, IL-16 acts as a potent chemoattractant (Qi et al., 2002). Surprisingly, chemotaxis of MCs toward IL-16 was blocked by anti-CD9 antibody or by reduced expression of CD9 using RNA interference (RNAi) approach. These and other findings led to the suggestion that CD9 acts as an alternate IL-16 receptor (Qi et al., 2006). Studies with 2H9 anti-CD9 antibody showed that intact IgG or its $F(ab)_2$ fragment block chemotaxis of MCs toward Ag, whereas Fab fragments had only minimal effects on such chemotaxis. These findings suggest that inhibition of chemotaxis toward Ag is caused by events induced by aggregation of CD9 but does not require co-cross-linking of CD9 with FcγR. Decreased expression of CD9 by lentiviral-induced RNAi CD9 knockdown (KD) did not affect migration toward Ag, suggesting that CD9 is not involved in this process. However, the possibility was not excluded that residual CD9 on the cell surface is involved in the proper Ag-induced migration (Hálová et al., 2013).

CD63

CD63 was found to be located in the vicinity of FcεRI on RBL-2H3 cells (Kitani et al., 1991). In MCs and basophils, CD63 is expressed at the cell surface and at the membrane of secretory lysosomes, including serotonin-containing granules that during activation fuse with the plasma membrane. Therefore, CD63 is extensively used as an activation marker of basophils. As the response acts as "all-or-nothing" per cell, basophils either do not bind the anti-CD63 mAb at all, or they bind a maximal amount of the mAb, so that activated basophils can be easily identified (Knol et al., 1991; Hoffmann et al., 2015). Unlike basophils, a significant amount of CD63 is also expressed on non-activated human MCs of different types, but similarly to basophils it is also upregulated after activation (Valent et al.,

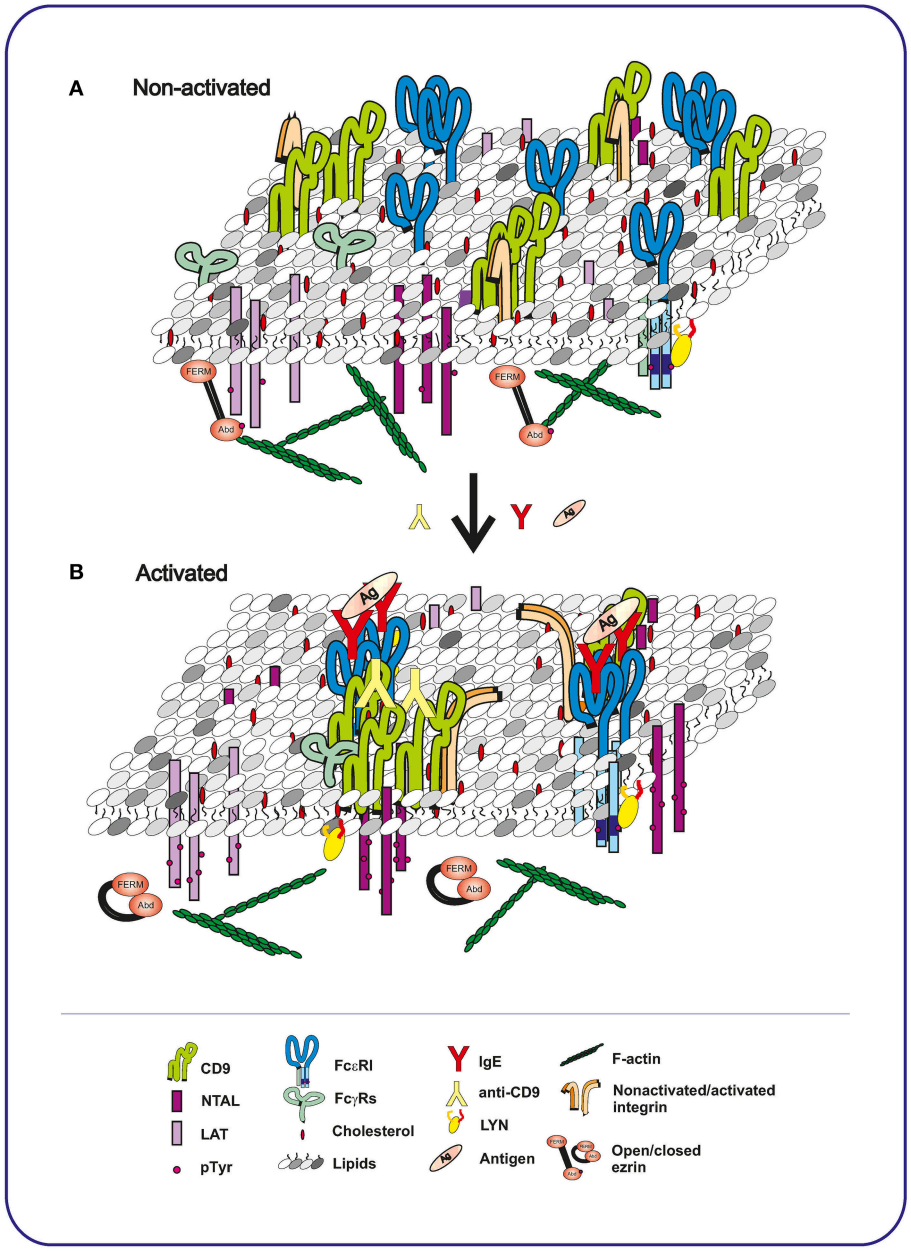

FIGURE 2 | Topography of plasma membrane components in mast cells before and after activation with Ag or CD9-specific antibody. (A) In non-activated cells, tetraspanin CD9 co-localizes with integrin in TEMs. These domains are topographically different from LAT- or NTAL-containing nanodomains, each occupying a distinct plasma membrane region. FcεRI receptor is associated with LYN kinase in another membrane nanodomains. **(B)** Binding of Ag-specific IgE to FcεRI, followed by exposure to multivalent Ag, causes multimerization of the FcεRI receptors. NTAL and LAT become phosphorylated, but are still separated in different domains and do not co-localize with FcεRI and with each other. In contrast, antibody-mediated aggregation of CD9 brings CD9 into close proximity of NTAL and FcγRs and causes NTAL phosphorylation. Activation through both FcεRI and CD9 leads to dephosphorylation of ERM and dynamic disconnection of the membrane components and actin cytoskeleton. The conclusions indicated above are based on the interpretation of previously published data (Wilson et al., 2002; Volná et al., 2004; Hálová et al., 2013).

2001). Recently, two structurally distinct isoforms of human CD63 were identified, one characteristic of vesicles and another expressed on the cell surface. Antibodies that differentiate between these two isoforms are therefore prospective diagnostic markers (Schäfer et al., 2010). Anti-CD63 mAb suppressed degranulation of adherent (but not non-adherent) RBL-2H3 cells, whereas synthesis of leukotrienes (LTs) was not affected. Initial stages of activation such as phosphorylation of signaling proteins and Ca^{2+} responses were not inhibited by the antibody. The ability of anti-CD63 mAb to suppress passive cutaneous

anaphylaxis (PCA) *in vivo* makes CD63 a possible therapeutic target (Kraft et al., 2005). As mice deficient in CD63 have been recently prepared (Schröder et al., 2009), the role of CD63 for MC development and activation could be studied using this model. Interestingly, although CD63 is highly expressed in MCs, the number of MCs and their tissue distribution was not altered in CD63 KO mice, and also MCs derived from bone marrow of CD63 KO mice developed normally. When activated with Ag, but not phorbol-12-myristate-13-acetate/ionomycin, a significant decrease in FcεRI-mediated degranulation and tumor necrosis factor (TNF)-α secretion was observed in CD63-deficient BMMCs. On the other hand, secretion of IL-6 and LTC4 that are *de novo* synthetized upon activation was unaffected in BMMCs with CD63 KO. This finding, together with the fact that TNF-α is present in preformed granules, suggested that the absence of CD63 in the secretory granules is the main cause of reduced degranulation and TNF-α secretion (Kraft et al., 2013). To test the role of CD63 *in vivo*, the authors introduced CD63 wild-type (WT) and KO MCs into mice of MC-deficient strain $Kit^{w/w-v}$ and found out that mice reconstituted with CD63 KO MCs exhibited significantly decreased degranulation and PCA. CD63-specific antibody, unlike CD9-specific antibody, was unable to inhibit MC migration toward IL-16 (Qi et al., 2006).

CD81

Similarly to antibodies directed toward CD63 (see above), mAb specific for CD81 was found to down-regulate FcεRI-mediated degranulation in RBL-2H3 cells without affecting initial tyrosine phosphorylation of signal-transduction proteins, calcium response and synthesis of LTC4. The inhibitory effect of anti-CD81 antibody was confirmed *in vivo*, where IgE-mediated PCA in rats was significantly decreased (Fleming et al., 1997). On the other hand, anti-CD81 antibody, unlike anti-CD9, was unable to inhibit MC migration toward IL-16 (Qi et al., 2006).

Despite the fact that production of CD81 KO mice was described almost 20 years ago (Maecker and Levy, 1997), the role of CD81 in MC development or activation has not been elucidated. A possible role of CD81 in MC activation was suggested based on experiments in which allergen-induced airway hyper-reactivity (AHR) was found to be diminished in CD81 KO mice. These mice, in contrast to WT mice, once challenged with ovalbumin (OVA) did not develop airway inflammation characterized by the presence of inflammatory cells, eosinophils, and synthesis of IL-4, IL-5, and IL-13 was also dramatically reduced. On the other hand, the serum levels of OVA-specific IgE were not changed (Deng et al., 2000). The authors speculated that the reduced levels of cytokines could be the reason of impaired MC activation in the absence of CD81 because anti-CD81 mAbs reduced MC activation. However, this idea has never been proved.

CD151

Unlike other tetraspanins, CD151 was found to be upregulated after activation of FcεRI in human and mouse MCs. Using CD151 KO mice, Berdnikovs and colleagues studied the role of CD151 in MC physiology (Abdala-Valencia et al., 2015). The absence of CD151, did not lead to changes in degranulation after Ag activation. On the other hand, mice with CD151 KO exhibited a significant increase in magnitude of the late phase of PCA response (24–36 h). In line with these findings, CD151-deficient BMMCs showed enhanced production of several proinflammatory cytokines, including IL-4, IL-13, and TNF-α, which was probably related to the enhanced and sustained FcεRI-induced extracellular signal-regulated kinase (ERK)1/2 and protein kinase B (PKB, also called AKT) phosphorylation in these cells (Abdala-Valencia et al., 2015).

CD53

Although CD53 is expressed in MCs, there is only one study showing association of CD53 with asthma risk via the functional promoter polymorphism. Interestingly, siRNA-mediated KD of CD53 in THP-1 human monocytic cells stimulated with house dust mite led to increased production of inflammatory cytokines as well as NFκB activity (Lee et al., 2013).

TRANSMEMBRANE ADAPTOR PROTEINS

Transmembrane adaptor proteins consist of a short extracellular domain, a single transmembrane domain and a long cytoplasmic tail. Extracellular domains are formed only by a few amino acids, and are therefore unlikely to function as receptors for extracellular ligands. The cytoplasmic tail possesses various tyrosine-containing motifs that could act, after phosphorylation, as scaffolds for anchor of multiple SRC homology (SH)2 domain-containing cytoplasmic as well as membrane-associated proteins and cytoskeletal components. As was already mentioned, most of the TRAPs possess a juxtamembrane palmitoylation motif that determines their solubility in non-ionic detergents, distribution in the plasma membrane, and some functional properties. The role of TRAPs in MC and leukocyte signaling has been recently extensively reviewed (Draber et al., 2011a, 2016; Horejsi and Hrdinka, 2014; Stepanek et al., 2014). Thus, this review is only limited to a short description of some structural properties of the most studied TRAPs in MCs that are important for the membrane organization (**Table 2**).

LAT

The cytoplasmic tail of LAT contains nine highly conserved tyrosine residues, of which five can be phosphorylated by spleen tyrosine kinase (SYK) kinase upon activation and serve as binding sites for SH2 domain-containing proteins including growth factor receptor-bound protein 2 (GRB2), phospholipase C (PLC)γ1, guanine nucleotide exchange factor VAV, ubiquitin ligase CBL, SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), and GRB2-related adaptor downstream of SHC (GADS) (reviewed in Rivera, 2002, 2005; Draber et al., 2011a). Although development of MCs was not affected in mice deficient

TABLE 2 | Selected TRAPs expressed in mast cells and their function.

Gene symbol	Most used aliases	KO	Phenotype of KO mice: general/in mast cells
LAT	LAT1, pp36	Zhang et al., 1999	Defect in T-cell development—no mature T cells (Zhang et al., 1999)/reduced degranulation, Ca^{2+} release and cytokine production, PSA (Saitoh et al., 2000)
LAT2	LAB, NTAL, WBSCR15, WBSCR5	Volná et al., 2004; Zhu et al., 2004	Increased levels of natural antibodies and humoral response (Wang et al., 2005)/increased degranulation, Ca^{2+} release, cytokine production, PCA (Volná et al., 2004; Zhu et al., 2004), and chemotaxis (Tumová et al., 2010)
PAG1	PAG, CBP	Draberova et al., 2014	No visible changes in phenotype/reduced degranulation, Ca^{2+} release, cytokine production, chemotaxis, PCA (Draberova et al., 2014)
LAX1	LAX	Zhu et al., 2005a	Reduction in CD23 expression on mature B cells, spontaneous germinal center formation, hyper-responsiveness in T and B lymphocytes (Zhu et al., 2005a)/enhanced degranulation, cytokine production, cell survival (Zhu et al., 2006)
GAPT		Liu and Zhang, 2008	Increased B-cell proliferation and amount of Abs (Liu and Zhang, 2008)

in LAT (Saitoh et al., 2000), the mice exhibited reduced Ag-mediated passive systemic anaphylaxis (PSA) responses. Ag-stimulated BMMCs from LAT-deficient mice showed reduced degranulation, Ca^{2+} release, and cytokine production (Saitoh et al., 2000), whereas their chemotaxis was unchanged (Hálová et al., 2013).

NTAL

NTAL shares several structural features with LAT, including several tyrosine motifs of which five are the putative GRB2-binding sites. However, there is no major homology in amino acid sequences between these two adaptors and in contrast to LAT, NTAL lacks the PLCγ1 binding motif. Similarly to LAT, NTAL is rapidly phosphorylated upon Ag activation by SYK, and some tyrosines are also phosphorylated by LYN. In contrast to LAT, NTAL is also tyrosine phosphorylated upon c-KIT activation by stem cell factor (SCF), LYN, and c-KIT itself (Iwaki et al., 2008). Despite some similarities in features of LAT and NTAL structures, BMMCs isolated from NTAL KO mice exhibited increased degranulation, Ca^{2+} release, and cytokine production (Volná et al., 2004; Zhu et al., 2004). Chemotaxis toward Ag was also enhanced in NTAL-deficient cells (Tumová et al., 2010). In accord with these observations, PCA of NTAL-KO mice was also increased (Volná et al., 2004). Although it appeared that LAT and NTAL act as opposite regulators of MC signaling, it should be noted that the absence of both of them led to more extensive inhibition of FcεRI-induced activation than the absence of LAT alone (Volná et al., 2004; Zhu et al., 2004). Again, the only exception was chemotaxis that was increased in double KO cells compared to WT cells but still was lower than that in NTAL-deficient cells (Hálová et al., 2013). These data indicate that in the absence of NTAL, LAT acts as a negative regulator of chemotaxis.

PAG

The structure of PAG is similar to that described for LAT and NTAL, but in addition to 10 tyrosines, it possesses two proline-rich domains that serve as binding sites for SH3 domains and a C-terminal VTRL motif for interaction with the PDZ domain of cytoskeletal linker ERM-binding protein of 50 kDa (EBP50) (Brdičková et al., 2001). Upon FcεRI triggering, PAG is phosphorylated by LYN in RBL-2H3 cells (Ohtake et al., 2002). Phosphorylated tyrosine 317 (human) or 314 (mouse) is crucial for binding of CSK. In RBL cells, overexpression of PAG led to inhibition of receptor phosphorylation and subsequent decreased degranulation (Ohtake et al., 2002). When BMMCs from mice with PAG KO were analyzed, different findings were obtained. PAG deficiency led to impaired Ag-induced degranulation, extracellular Ca^{2+} uptake, tyrosine phosphorylation of several proteins (including the FcεRI), production of cytokines and chemokines, and also decreased chemotaxis (Draberova et al., 2014). PAG-KO mice also exhibited impaired PCA. On the other hand, activation through c-KIT led to increased degranulation, suggesting different regulation of this TRAP after c-KIT and FcεRI activation (Draberova et al., 2014).

LINKER FOR ACTIVATION OF X CELLS (LAX)

LAX is another TRAP with multiple GRB2-binding motifs. However, in contrast to LAT and NTAL, LAX has no palmitoylation motif and is not localized in detergent-resistant membranes (DRMs) (Zhu et al., 2002). LAX-deficient MCs exhibited enhanced degranulation, enhanced activity of p38 mitogen-activated protein kinases (MAPK; PKB), and PI3K activation after stimulation via FcεRI. Cytokine production and cell survival were also enhanced in activated LAX-deficient cells. On the other hand, the absence of LAX had no effect on calcium response *in vitro* and PCA *in vivo* (Zhu et al., 2006).

GRB2-BINDING ADAPTOR PROTEIN, TRANSMEMBRANE (GAPT)

GAPT has a short extracellular domain, a transmembrane domain, and a cytoplasmic tail with multiple Grb2-binding motifs. Similarly to LAX, GAPT does not reside in DRMs, even though it contains potential palmitoylation sites similar to LAT and NTAL. On the other hand, palmitoylation of GAPT has not

been proved. In contrast to other mentioned TRAPs, GAPT is not phosphorylated on tyrosine after FcεRI triggering (Liu and Zhang, 2008).

MEMBRANE MICRO/NANO DOMAINS OCCUPIED BY TETRASPANINS AND TRAPS

Discovery of specific membrane domains came from the studies of insoluble residues that remained after lysis of the cells in buffers containing non-ionic detergents at 4°C. These DRMs formed predominantly by sphingolipids, cholesterol and proteins, were proposed to play key roles in membrane trafficking and signaling (Simons and Ikonen, 1997). Studies of DRMs were accelerated by development of the method of their isolation by sucrose density gradient centrifugation of cells lysed in non-ionic detergents. Both tetraspanins and palmitoylated TRAPs were found to be associated with DRMs. The effect of palmitoylation on the presence of these proteins in DRMs is discussed below. Based on the differences in detergent sensitivity, TEMs, or tetraspanin web, were identified as novel structures different from glycosylphosphatidylinositol (GPI)-microdomains and caveolae. It has been proposed that TEMs are involved in the plasma membrane organization through tetraspanin-tetraspanin and tetraspanin-other protein interaction (Boucheix and Rubinstein, 2001; Claas et al., 2001). In their sensitivity to different non-ionic detergents, protein-protein and protein-lipid interactions in TEMs differ from those in TRAP-enriched domains. Whereas tetraspanin-tetraspanin interactions are preserved in nonionic detergents Brij 58, Brij 97, Brij 98, and CHAPS, Triton X-100 disrupts the majority of these interactions along with participation of tetraspanins in sucrose low-density fractions. On the other hand, lysis in Brij detergents at 37°C disrupts GPI-microdomains but not TEMs (Claas et al., 2001; Charrin et al., 2003a, 2009). Tetraspanins were found to directly interact with cholesterol (Charrin et al., 2003b). Furthermore, it has been described that tetraspanins CD82 interacts with ganglioside GM2 (Todeschini et al., 2007), tetraspanin CD9 binds to GM3 (Kawakami et al., 2002) and that these interactions are important for the association of these tetraspanins with integrins. Since the concept of TEMs was proposed, most interactions of tetraspanins with their partners (e.g., tetraspanins and integrins) were identified by co-immunoprecipitation experiments after lysis of the cells in mild detergents. Some of them were later confirmed by crosslinking experiments or Förster resonance transfer (FRET) analysis (Boucheix and Rubinstein, 2001).

The existence of specific membrane domains that are dependent on lipid composition was at first deduced on the basis of experiments with detergent solubility/insolubility of individual membrane proteins and the term DRMs was coined. Discovery and use of modern high-resolution techniques, such as (FRET; McIntosh et al., 2012), fluorescence recovery after photobleaching (FRAP; Axelrod et al., 1976), stimulated emission depletion (STED; Auksorius et al., 2008), photoactivated localization microscopy (PALM; Betzig et al., 2006), and stochastic optical reconstruction microscopy (STORM; Rust et al., 2006) contributed significantly to identifying the real interactions on the plasma membrane components under *in vivo* conditions. The first results obtained with the high-resolution microscopic techniques (Kenworthy et al., 2000, 2004; Glebov and Nichols, 2004) challenged the theories based on detergent solubility. As more studies appeared, it was apparent that the majority of the results are basically in accord with the existence of plasma membrane domains, which are, however, of smaller size than previously thought, and therefore the term nanodomains was more often used for marking small dynamic domains that vary in time and size and which are enriched in cholesterol and sphingolipids.

Regarding tetraspanins, single-molecule fluorescence microscopy of living cells revealed that tetraspanin assemblies form dynamic interaction platforms in permanent exchange with the rest of the membrane. Tracking of tetraspanin CD9 showed that most of the time it was undergoing Brownian trajectories, but it was transiently trapped in platforms enriched in CD9 and its partners. Both the mobility and partitioning in the nanodomains were dependent on palmitoylation and plasma membrane cholesterol (Espenel et al., 2008). FRET-FLIM analysis revealed homophilic (CD9-CD9) and heterophilic (CD9-CD151) tetraspanin interactions as well as their interaction with adhesion molecules, with preferential association of CD9 with intercellular adhesion molecule 1 (ICAM-1, also known as CD54) and of CD151 with vascular cell adhesion molecule 1 (VCAM-1, also known as CD106). FRAP analysis also revealed that a marker of membrane microdomains, rGPI-EGFP, diffused much faster than tetraspanins (Barreiro et al., 2008; Ley and Zhang, 2008). On the other hand, recent work analyzed TEMs by STED microscopy and showed that tetraspanins form individual nanoclusters. It was also demonstrated that CD53 and CD37 domains showed only minor overlap with clusters containing tetraspanins CD81 or CD82. It should be also noted that CD53 and CD81 reside in closer proximity to their partners, major histocompatibility complex (MHC) class II and CD19, respectively, than to other tetraspanins (Zuidscherwoude et al., 2015).

When TRAPs were examined, using cells solubilized with non-ionic detergents, LAT was found in DRMs (Zhang et al., 1998). In contrast, FcεRI was not present in such domains before activation, but once aggregated, FcεRI became associated with DRMs. It has been suggested that association of FcεRI with DRMs is a prerequisite for FcεRI activation because in DRMs, FcεRI is phosphorylated by SRC family kinase LYN, which is resident in DRMs (Field et al., 1995; Dráberová et al., 2004). However, immunogold electron microscopy studies of isolated plasma membrane sheets did not prove co-localization of aggregated FcεRI and LAT, even though both domains were increased in size (Wilson et al., 2001, 2002; Lebduška et al., 2007). Scanning electron microscopy revealed that activation of RBL cells with Ag causes redistribution of LAT in the plasma membrane, where upon activation LAT was found in bigger clusters than before activation (Veatch et al., 2012). Although the studies did not examine whether LAT clusters co-localize with FcεRI clusters the data clearly showed that aggregation of FcεRIs causes redistribution of LAT. Recent studies of LAT clustering in resting and activated T cells by

PALM (Betzig et al., 2006) and direct STORM (Rust et al., 2006) revealed that increase of LAT clusters after activation is due to the translocation of LAT from subsynaptic vesicles to cell surface and this recruitment is essential for LAT phosphorylation (Williamson et al., 2011). In another study with single and two-color PALM Sherman and collaborators showed that in resting and activated T cells LAT primarily resides in nanoscale clusters as small as dimers whose formation depended on protein-protein and protein-lipid interactions (Sherman et al., 2011). Furthermore, Lillemeier and collaborators used high-speed version of photoactivated localization microscopy (hsPALM), dual-color fluorescence cross-correlation spectroscopy (dsFCCS) and transmission electron microscopy and showed that both the T cell receptor (TCR) and LAT are preclustered into separate and spatially separated membrane domains on quiescent cells. After Ag recognition, these domains transiently concatenated into microclusters without any substantial change in the size and number of the component domains. These data suggest that partitioning immunoreceptors and their downstream signaling components into separate membrane domains, and then bringing these domains together, may be an important and general mechanism in the control of cell activation (Lillemeier et al., 2016). In another study, analysis of LAT in the plasma membrane of HeLa cells by fluorescence correlation spectroscopy (FCS) and PALM showed that LAT diffusion is retarded and its clustering in meso-scaled protein domains is decreased when associating with ordered-lipid domains in contrast to LAT associating with lipid-disordered domains (Owen et al., 2012). Importance of cholesterol for formation of DRMs and for immunoreceptor signaling has been repeatedly shown (Xavier et al., 1998; Sheets et al., 1999; Surviladze et al., 2001). It should also be noted that FcεRI-mediated activation is affected by ethanol, which seems to interfere with proper function of FcεRI-cholesterol signalosomes (Draberova et al., 2015) and that phosphatase inhibitor, pervanadate, induces FcεRI β and γ subunits tyrosine phosphorylation in the absence of FcεRI aggregation and its association with DRMs (Heneberg et al., 2010).

PALMITOYLATION OF TETRASPANINS AND TRAPS

Palmitoylation is a reversible lipid post-translational modification of juxtamembrane cysteine residues, less frequently also serine and threonine, in a variety of transmembrane or membrane-associated proteins (Resh, 1999). Palmitoylation also allows such modified proteins to float in the low-density fraction of sucrose gradient after lysis in non-ionic detergents (Charrin et al., 2002, 2009; Stepanek et al., 2014). However, loss of palmitoylation of tetraspanins and TRAPs had a different impact on their solubility in non-ionic detergents and interactions with their partners. It has been shown that palmitoylation of CD151 had minimal influence on the density of tetraspanin-protein complexes and did not promote tetraspanin localization into DRMs or its association with α3β1 integrin, but its association with other cell surface proteins, including CD9 and CD63, was reduced (Yang et al., 2002). The palmitoylation of CD9 did not influence its localization into DRMs but was necessary for its interaction with other tetraspanins, namely CD81 and CD53 (Charrin et al., 2002). Similar observations were obtained when membrane compartmentalization of integrins had been studied. It has been suggested that palmitoylation of β4 promotes its association with DRMs and SRC family kinases (Gagnoux-Palacios et al., 2003) but further studies showed that β4 palmitoylation does not increase its localization into DRMs, instead it promotes CD151–α6β4 incorporation into a network of secondary tetraspanin interactions (Yang et al., 2004).

Although importance of palmitoylation for proper function of TRAPs has been shown, it still remains in part controversial. LAT is palmitoylated at C26 and C29 and this event is crucial for LAT association with DRMs and proper immunoreceptor function (Zhang et al., 1998; Levental et al., 2010). An important question was whether palmitoylation is also important for plasma membrane localization. It has been reported that the LAT mutated in the cysteines separately or together was localized into the plasma membrane of HEK cells (Zhang et al., 1998). However in this study LAT was not examined for its co-localization with plasma membrane markers. Recent studies showed that palmitoylation at C26 is essential for transporting of LAT from Golgi to plasma membrane (Hundt et al., 2009; Chum et al., 2016). In contrast, surprisingly, NTAL and PAG did not require palmitoylation for plasma membrane localization (Chum et al., 2016).

In another study, mutant LAT was constructed, in which transmembrane domain of LAT was exchanged with transmembrane domain of LAX, another TRAP, that lacks palmitoylation motifs, but possesses LAX transmembrane signal peptide that provides localization of the LAX in plasma membrane. This LAX-LAT protein was not detected in DRMs but appeared to be fully functional in T cell activation and development (Zhu et al., 2005b). Similar results were obtained when SRC-LAT mutant was constructed and examined in similar assays. The mutant protein was localized as peripheral membrane protein through myristoylation of its SRC domain but it was excluded from DRMs and appeared to be fully functional in TCR signaling (Hundt et al., 2009). Later studies of LAX-LAT mutant protein showed that it is localized in an atypical DRMs—called "heavy" DRMs (Otáhal et al., 2010). When CD25-LAT mutant was used, it was excluded from both DRMs and "heavy" DRMs (Otáhal et al., 2010). This study also demonstrated that the level of exogenous construct expression is critical for proper interpretation of the results. At the levels of expression, corresponding to the expression level of endogenous LAT, WT LAT, present mostly in DRMs, supported signaling better than LAX–LAT mutant; the CD25-LAT mutant was the least effective in the assay (Otáhal et al., 2010). Importance of palmitoylation for association with DRMs was also shown in studies with mutant LYN kinase in RBL cells. LYN mutated in both palmitoylation and myristoylation sites, did not anchor to the plasma membrane, whereas LYN with only palmitoylation site mutated was anchored to the plasma membrane, but its localization into DRMs was markedly reduced (Kovárová et al., 2001). Interestingly, studies with RBL cells showed that

clustering of FcεRI led to co-clustering with LYN, depending on the presence of cholesterol (Veatch et al., 2012). Furthermore, FcεRI motility in the plasma membrane after Ag triggering was also dependent on cholesterol levels (Shelby et al., 2013).

TETRASPANINS AND TRAPS CROSS-TALK

Electron microscopy studies showed that LAT and NTAL, despite their structural similarities and proved association with DRMs, occupy separate membrane microdomains in MCs (Volná et al., 2004). The differences between the LAT and NTAL microdomains were confirmed in experiments in which topography of CD9 and TRAPs in the plasma membrane was examined: while NTAL co-localized with CD9, LAT did not (Hálová et al., 2013). CD9-NTAL co-localization was intensified by crosslinking CD9 with specific mAb, and this crosslinking resulted in enhanced phosphorylation of NTAL (Hálová et al., 2013). There are other examples of interaction between TRAPs and tetraspanins. Two recently identified TRAPs, SLP65/SLP76, Csk-interacting membrane protein (SCIMP) and leukocyte-specific transcript 1/A (LST1/A) were found to interact with tetraspanins (Draber et al., 2011b, 2012). SCIMP, which is expressed in B cells and other professional Ag-presenting cells, co-localized with tetraspanins CD81 and CD37 (Draber et al., 2011b), whereas LST1/A, which is expressed exclusively in the cells of myeloid origin (monocytes and granulocytes), co-localized with tetraspanins CD9 and CD81 (Draber et al., 2012).

Both tetraspanins and TRAPs were found to function as direct or indirect linkers of the plasma membrane and cytoskeleton. Co-immunoprecipitation studies showed that tetraspanins CD9 and CD81 are associated with ERM proteins, which directly interact with the cytoskeleton (Sala-Valdés et al., 2006). ERM proteins link plasma membrane phospholipids by binding their N-terminal FERM (4.1, ezrin/radixin/moesin) domain to phosphatidylinositol (4,5) bisphosphate (PIP_2) and the actin cytoskeleton through the actin-binding domain (Abd) at the C-terminus. This binding is a highly dynamic process dependent on ERM 567/564/558 threonine phosphorylation/dephosphorylation. ERMs are released from the membrane when their inhibitory threonine is dephosporylated and ERMs are transformed from "open" phosphorylated to "closed" dephosphorylated conformation (McClatchey, 2014). However, it has not been clarified whether interactions between tetraspanins and ERMs are direct or indirect through tetraspanin-interacting proteins CD9P-1 and/or EWI-2 (Sala-Valdés et al., 2006). Earlier studies showed that engaging CD81 at the surface of B cells led to phosphorylation of ezrin by the SYK kinase (Coffey et al., 2009). In MCs, crosslinking of CD9 with mAb led to dephosphorylation of ERM inhibitory threonine (Hálová et al., 2013). TRAP PAG also interacts with ezrin, but in this case through ERM-binding phosphoprotein of 50 kDa (EBP50). In this binding, the N-terminal PDZ domain of EBP50 and C-terminal domain of PAG are involved (Brdičková et al., 2001). The ezrin-EBP50-PAG complex was found to be important for the spatio-temporal control of cAMP production through the CSK-PAG inhibitory pathway in effector T cells (Cornez and Taskén, 2010).

CONCLUDING REMARKS AND PERSPECTIVES

Although knowledge about tetraspanins and TRAPs at the level of proteins and the corresponding genes and their regulators is increasing, their functioning as platforms of plasma membrane signalosomes and their mutual crosstalk are far from understood. The exact composition of TEMs and TRAPs-enriched domains is also poorly defined. This is in part due to highly dynamic nature of the membrane domains and the large number of various lipids involved. Lipids function not only as a matrix in which tetraspanins, TRAPs, and other membrane proteins are anchored, but also as modifiers of various proteins. When focusing specifically on signal transduction in MCs, there are also many poorly explored areas, which include potential roles of various post-translational modifications on topography and function of the tetraspanins and TRAPs and their interaction partners. An important area to explore is the role of miRNAs, noncoding RNAs, and other regulators of tetraspanins and TRAPs expression (Zhang et al., 2014; Rouquette-Jazdanian et al., 2015). As shown in this review focused on MCs, important discoveries concerning the role of individual members of tetraspanins and TRAPs were brought by studies based on cells deficient in selected proteins through the gene KO or KD approach. However, with the armamentarium of proteomics, lipidomics, epigenetics, super-resolution microscopy, multi-photon microscopy, etc., our pace of discoveries in the field of specific membrane nanodomains will be accelerated in the next few years. Most of the studies mentioned in this review were performed using cells cultured under *in vitro* conditions. However, one needs to understand more on how TEMs and TRAPs-enriched domains function under *in vivo* conditions. This issue involves not only the influence of various soluble factors that are absent in cell culture media, but also the interaction with other cells and extracellular matrix components that could influence functions of the tetraspanins and their interaction partners. Such type of studies in the case of MCs will deepen our understanding of the cellular and molecular mechanisms underlying allergic diseases.

AUTHOR CONTRIBUTIONS

Both authors have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Molecular Architecture of Cell Adhesion: Dynamic Remodeling Revealed by Videonanoscopy

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The plasma membrane delimits the cell, which is the basic unit of living organisms, and is also a privileged site for cell communication with the environment. Cell adhesion can occur through cell-cell and cell-matrix contacts. Adhesion proteins such as integrins and cadherins also constitute receptors for inside-out and outside-in signaling within proteolipidic platforms. Adhesion molecule targeting and stabilization relies on specific features such as preferential segregation by the sub-membrane cytoskeleton meshwork and within membrane proteolipidic microdomains. This review presents an overview of the recent insights brought by the latest developments in microscopy, to unravel the molecular remodeling occurring at cell contacts. The dynamic aspect of cell adhesion was recently highlighted by super-resolution videomicroscopy, also named videonanoscopy. By circumventing the diffraction limit of light, nanoscopy has allowed the monitoring of molecular localization and behavior at the single-molecule level, on fixed and living cells. Accessing molecular-resolution details such as quantitatively monitoring components entering and leaving cell contacts by lateral diffusion and reversible association has revealed an unexpected plasticity. Adhesion structures can be highly specialized, such as focal adhesion in motile cells, as well as immune and neuronal synapses. Spatiotemporal reorganization of adhesion molecules, receptors, and adaptors directly relates to structure/function modulation. Assembly of these supramolecular complexes is continuously balanced by dynamic events, remodeling adhesions on various timescales, notably by molecular conformation switches, lateral diffusion within the membrane and endo/exocytosis. Pathological alterations in cell adhesion are involved in cancer evolution, through cancer stem cell interaction with stromal niches, growth, extravasation, and metastasis.

Keywords: diffusion, single molecule, super-resolution, nanoscopy, membrane dynamics, focal adhesion, synapse

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INTRODUCTION

Cell junctions play a key role in the establishment and integrity of biological tissues, via protein–protein interactions at the cell surface. In multicellular animal organisms, mechanical integrity is ensured by diverse structures including *adherens* junctions, focal adhesions, desmosomes, and hemidesmosomes. Two other major functions of cell adhesion, which are not discussed here, concern epithelium and endothelium impermeability in-between cells by tight junctions, and

direct communication between adjacent cells by gap junctions. In epithelia and endothelia, cells are connected, from apical to basal side, by the stratified structures of *zonula occludens* (tight junctions), *zonula adherens* (adhesion belt), *macula adherens* (desmosomes), gap junctions, and *basal lamina*. Present among virtually all cells, apart from cells of body fluids such as blood, lymph, or sperm, contacts are subject to physiological remodeling, notably during cell division and apoptosis. Transmembrane proteins generically named *cell adhesion molecules* (CAMs) interact either among adjacent cells or with the extracellular matrix (ECM) and are connected to the cytoskeleton by specific adaptors. The main CAM families encompass:

- Cadherins, in homophilic, calcium dependent cell–cell contacts.
- Integrins, in heterophilic, calcium/magnesium-dependent cell–matrix or cell–cell contacts.
- Selectins, in heterophilic (with sugar motifs), weak cell–cell contacts.
- Members of the immunoglobulin superfamily, in homo- or hetero-philic (with integrins), cell–cell contacts.

CAMs permit outside-in signaling, similar to membrane receptors, as well as inside-out, being susceptible to variations such as activation or aggregation by intracellular signals.

DYNAMIC NANOSCOPY APPROACHES: MEASURES AT HIGH SPATIOTEMPORAL RESOLUTION

Cell contacts may be seen as static structures, through the classical representation provided by microscope images, usually obtained from fixed tissues. Yet, at the molecular scale, movements are essentially governed by thermal agitation, mostly leading to Brownian motion. This concept of dynamic molecular crowding applies to most cell constituents, including the plasma membrane, as described by the historical and still relevant fluid mosaic model (Singer and Nicolson, 1972). Molecular paths can be subjected to forces biasing Brownian motion and generating specific behaviors, like directed motion or permanent/transient immobilization (Sergé and Irla, 2013), particularly relevant for CAMs and their adaptors (**Figure 1A**). Hence cell contacts are permanently susceptible to evolve in composition and organization throughout their lifespan, from their establishment through remodeling and until disassembly.

Pioneer studies used methods such as Fluorescence Recovery After Photobleaching (FRAP), which was one of the first ways to measure the mobility of membrane components (Axelrod et al., 1976). Using antibodies or GFP as reporters, partial immobilization of CAMs such as integrins (Duband et al., 1988; Ballestrem et al., 2001) could be detected together with adhesion structures, during maturation and with associated partners such as the cytoskeleton and ECM. Other CAMs such as Junctional Adhesion Molecules (JAMs; Lamagna et al., 2005) and cadherins (Kusumi et al., 1993) were also studied by FRAP and by another technique that paved the way to single-molecule microscopy: Single-Particle Tracking (SPT) using antibodies coupled to latex or gold colloids of sub-diffraction size visualized by transmitted light. Documenting membrane events, like adhesion and endo/exocytosis, suffers from an intrinsically weak resolution along the optical axis (∼500 nm). This can be circumvented by Total Internal Reflection Fluorescence microscopy (Axelrod, 1981). This configuration generates an evanescent field restricting illumination to ∼100 nm above the coverslip, offering high axial resolution with reduced background and privileging visualization of the plasma membrane contacting the glass.

Single-Molecule Microscopy

Advances in optical microscopy over the last few decades has allowed for the detection of a single fluorescent molecule with nanometer accuracy. Imaging a point source through a microscope is limited by diffraction, generating an Airy pattern, with a diameter of $\lambda/2$ NA (∼200 nm), λ being the wavelength of light and NA the objective numerical aperture, as first determined by Abbe (1873). This pattern constitutes the *point-spread function* of the optical setup. The fluorophore localization, at the center of the Airy pattern, can be determined at high resolution providing sufficient signal-to-noise ratio, as predicted by Werner Heisenberg during the emergence of quantum theory (Heisenberg, 1927). Recent technological improvements in chemistry, optics and detectors have allowed for single-molecule detection in biological conditions. Seminal studies were first performed *in vitro*, with the pioneer observation of single enzyme activity, β-galactosidase (Rotman, 1961). An important breakthrough was later achieved by *fluorescence imaging with one-nanometer accuracy* to finely decipher myosin motion (Yildiz et al., 2003). Single-molecule observations were also reported in living cells, with pioneer works addressing transferrin (Byassee et al., 2000), epidermal growth factor (EGF; Sako et al., 2000), lipids (Schütz et al., 2000), calcium channel (Harms et al., 2001), and cadherin (Iino et al., 2001). We extended single-particle tracking by developing robust and efficient algorithms, named *multi-target tracing,* dedicated to the high probe density and low signal-to-noise ratio provoked by high acquisition rates (Sergé et al., 2008; Rouger et al., 2012). *Multi-target tracing* has been adapted to cell trajectories (Salles et al., 2013).

Emergence of Nanoscopy

The recent breakthrough of nanoscopy confirmed and further detailed previously unsuspected dynamic features of cell contacts. Single-molecule measurements require diluted enough dyes, separated on average by more than the Rayleigh criterion, $0.61\,\lambda$/NA. Two strategies were developed to surpass this limit. Stimulated-Emission-Depletion (STED; Hell and Wichmann, 1994; Hell, 2007) consists in generating an optical reduction of the *point-spread function* by using a depletion beam located around the excitation beam. Another approach has been provided by techniques such as Photo-Activated Localization Microscopy (PALM; Betzig et al., 2006; Hess et al., 2006) and Stochastic Optical Reconstruction Microscopy (STORM; Rust et al., 2006). Relying on a common scheme, these

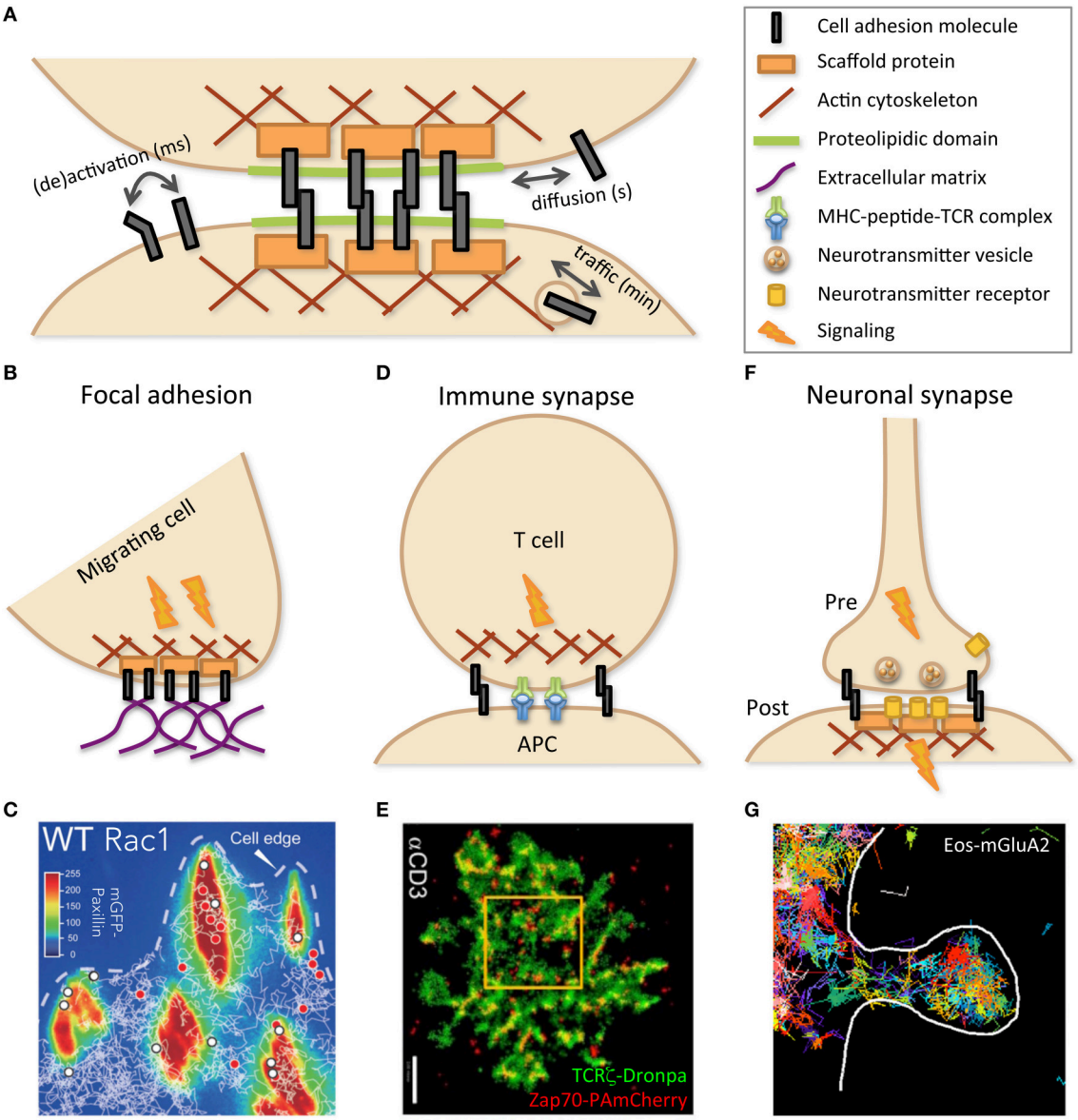

FIGURE 1 | Cell–cell adhesion is mediated by specific molecular structures. (A) Schematic representation of the building blocks involved in cell–cell contacts. Dynamic evolution, as indicated by double arrows, may occur on various time scales, through changes in molecular conformation, such as activation, and localization, both within the membrane, by diffusion, and within the cell, by vesicular traffic. As depicted by cartoons **(B,D,F)** and illustrated by experimental data **(C,E,G)**, specialized cell contacts can be implicated in structures such as focal adhesion **(B,C)**, immune (between T cell and APC; **D,E**), and neuronal (between pre- and post-synaptic neurons) synapses **(F,G)**, dealing with specific dynamics in relation with their function. **(C)** Trajectories of wild-type (WT) Rac1, tagged with Halo-tetra-methyl-rhodamin, obtained by single-particle tracking (white lines) and superimposed on mGFP-Paxillin staining (false colors identifying FAs) reveal transient (red dots) or stable (white dots) immobilization within FAs. Reprinted from Shibata et al. (2013). **(E)** PALM imaging was performed with two molecules of the TCR complex, tagged with photoactivatable fluorescent proteins, TCRζ–Dronpa and ZAP-70-PAmCherry, in an E6.1 Jurkat cell on αCD3-coated coverslip. Nanoscopy of the immune synapse reveals TCR micro- and nano-clusters (green) with ZAP-70 sub-clusters (red) associated to activated TCR. Bar: 2 μm. Reprinted from Neve-Oz et al. (2015). **(G)** Trajectories of the tagged AMPA receptor Eos-GluA2 measured by sptPALM report transient organization in nanodomains within an excitatory dendritic spine (delimited by the white line) of a rat hippocampal neuron. Reprinted from Nair et al. (2013).

methods are collectively named Single-Molecule Localization Microscopy (SMLM). High-resolution images are built by iterative photoactivation of small subpopulations of dyes, sparse enough at each time-step to deliver single-molecule accuracy. Photoactivation/deactivation uses an appropriate strategy in each approach, i.e., switching photoactivatable proteins for PALM or controlling dye blinking for STORM. The image obtained by accumulating all localized molecules with SMLM can be termed pointillism, in reference to the painting technique. The obtained subwavelength resolution is only limited by signal-to-noise ratio and can be comparable to standard electron microscopy resolution (~50 nm). These imaging techniques

allowed a technical and conceptual shift in spatial scales, from micro- to nano-scopy. In 2014, the Nobel Prize for Chemistry was awarded to Stephan Hell (Hell and Wichmann, 1994; Hell, 2007), Eric Betzig (Betzig, 1995; Betzig et al., 2006), and William Moerner (Moerner and Kador, 1989; Dickson et al., 1997) for these innovations. Nanoscopy have been extended to multicolor labeling (Bates et al., 2007; Meyer et al., 2008; Shroff et al., 2008), 3D (Huang et al., 2008; Punge et al., 2008; Vaziri et al., 2008; Shtengel et al., 2009), and living cells (Conley et al., 2008; Manley et al., 2008; Westphal et al., 2008), handling limits such as phototoxicity and artifacts putatively induced by tagging. Notably, nanoscopy revealed intense and unexpected dynamics of adhesion structures (Diez-Ahedo et al., 2009; Bakker et al., 2012; Rossier et al., 2012; Shibata et al., 2013; Ishibashi et al., 2015; Eich et al., 2016), revisiting the classical view of mostly static structures.

MOLECULAR ORGANIZATION OF CELL MEMBRANES

Several structures of the cell membrane play major roles in physiological functions through signaling and adhesion to neighbor cells and ECM. Generic features such as cytoskeleton meshwork, rafts, and protein complexes, which are subjected to thermal motion, can tailor the temporal evolution of membrane structures. Cell contacts benefit from proteolipidic domains to favor CAM aggregation, with the contribution of intracellular scaffolds and sub-membrane cytoskeleton. This leads to structures that are simultaneously elaborate and versatile, such as *focal adhesions* (FA; Rossier and Giannone, 2016), immune (Rossy et al., 2013), and neuronal (Maglione and Sigrist, 2013) synapses (**Figure 1**). Activation by fast and transient association of partners of a given signaling pathway, already localized in close proximity within narrow structures/domains, is a recurrent scheme to ensure fast and reliable signal transmission (Cebecauer et al., 2010).

Submembrane Skeleton Fences and Extracellular Matrix

The actin cytoskeleton, in association with spectrin and transmembrane proteins, exhibits a gel organization constituting a meshwork located immediately beneath the plasma membrane. This meshwork not only mechanically reinforces and controls the shape of the membrane, but also constitutes barriers. Steric hindrance consequently constrains the diffusion of membrane components within domains, according to the *fences and pickets* model (Kusumi and Sako, 1996). Cytoskeleton meshes display sub-diffraction size, as imaged by electron microscopy and as evaluated from confined trajectories obtained by single-particle tracking. Instead of strict compartmentalization, dynamic evolution of the meshwork, allowing *hop* diffusion to adjacent domains, may lead to obstructed motion/anomalous diffusion (Fujiwara et al., 2002). Accordingly, on the extracellular side, the reticulated filaments of the ECM, tightly associated to the membrane glycocalyx, constitute a meshwork analogous to the cytoskeleton. Hence, ECM–cell contacts, notably via integrins, not only mechanically support tissues, but are also expected to obstruct or confine membrane component motion.

Proteolipidic Nanodomains/Rafts

The *raft* hypothesis postulated that membrane lipids and proteins associate together according to their affinities, mostly emanating from their hydrophobicity and geometry (Simons and Ikonen, 1997). Indeed, the height of the intra-membrane part and a more or less cylindrical/conical shape, engendering a local curvature of the membrane, implicate an energetic cost. Cholesterol and saturated lipids such as sphingomyelin promote better packing within rafts. This leads to ordered and disordered phase separation co-existing within membranes, as first assessed by biochemistry. Rafts were initially proposed to contribute to protein sorting along the synthesis pathway, relying on the differential composition of the Golgi apparatus and other cellular compartments, with a key role attributed to cholesterol. They were also associated to several membrane features, including signaling platforms and adhesion structures. Rafts have been the focus of extensive research. Indeed, in contrast to the classic *floating island* metaphor, their putative sub-diffraction size and fast dynamics imply spatiotemporal characteristics just beneath the limit of most technological investigations.

Other Proteic Domains and Signaling Complexes

Membrane components may also self-organize through attractive energetic potentials, typically generated by electrostatic and Van der Waals forces, even beyond rafts. Strikingly, in the retina, rhodopsin receptors display an almost crystalline packaging on the micrometric scale (Fotiadis et al., 2003). Proteic clusters also exist at sub-diffraction size (Daumas et al., 2003), like cytoskeleton meshes and rafts, with similar roles for integrating signaling partners within platforms (Douglass and Vale, 2005). Packing together effectors can be achieved through favorable energetic interactions, as well as by connections via scaffold proteins, reinforcing functional association with physical links. For instance, signaling crosstalk between integrin and major pathways, such as EGF, are physically reinforced via scaffolds like paxillin (Legate et al., 2006).

SPECIALIZED CELL CONTACTS

Most cells are connected together to ensure proper mechanical and signaling coordination. Some contacts exhibit particularly complex dynamics and duration, as revealed by nanoscopy. We will focus on some emblematic contacts: FA, immune, and neuronal synapses (**Figure 1**). Migrating cells must establish strong though transient contacts along their path. Cell–cell contacts dedicated to information processing occur among immune and nervous cells and share the same term of *synapse*. Indeed, although the immune synapse is transient while the neuronal synapse may persist throughout life, they both contain similar features, including CAMs and signaling machinery, subjected to specific evolution over time (Dustin and Colman, 2002).

Focal Adhesion

FAs constitute a privileged site for mechanotransduction crosstalk between cells and ECM, mutually converting force sensing and signaling (Rossier and Giannone, 2016). FA nano-architecture was deciphered with 3D super-resolution using interferometric PALM. Axial position, usually poorly assessed, was determined at high resolution by analyzing the interference among the fluorescence collected by the two opposing objectives of a so-called 4π microscope (Shtengel et al., 2009). This allowed localizing FA components orthogonally to the plasma membrane, from integrins at the membrane, through adaptors such as paxillin and vinculin, to the actin cytoskeleton in the cytosol (**Figure 1B**). They notably established that talin, owing to its substantial size, crosses the whole structure (Kanchanawong et al., 2010). Comparable results were obtained for hemidesmosomes (Nahidiazar et al., 2015). Using videonanoscopy, the detailed dynamics and kinetics of integrins and their adapters were finely dissected, deciphering regulation by activation and association/dissociation to/from the cytoskeleton and ECM (Diez-Ahedo et al., 2009; Bakker et al., 2012; Rossier et al., 2012; Shibata et al., 2013; Ishibashi et al., 2015; Eich et al., 2016). This dynamic view of adhesions reveals an unsuspected plasticity in integrin number and residency time at FAs, modulated by pathophysiological conditions and extracellular signals to fine-tune ECM/cytoskeleton coupling (**Figure 1C**).

Immune Synapse

The immune synapse was initially conceptualized as the intimate contact established between a T cell and an antigen-presenting cell (Grakoui et al., 1999; Reichardt et al., 2009; **Figure 1D**). This was later extended to contacts implicating B cells and antigens (Harwood and Batista, 2010) or Natural Killer (NK) cells and target cells for delivery of lytic granules (Dustin and Long, 2010). In all cases, synapses contain specific receptors and are stabilized by CAMs. These molecules present a concentric organization, with receptors mostly at the central Supramolecular Activation Cluster (cSMAC) surrounded by CAMs such as LFA-1 at the periphery (pSMAC) and completed by distal elements with large extracellular domains (dSMAC). Immune synapse establishment (Klotzsch et al., 2015) and subsequent signaling (Salles et al., 2013) lead to antigen capture, lymphocyte activation, or target cell death. A precise choreography orchestrates co-receptors and partners associating/dissociating, as well as microtubules and their organizing center polarizing toward the cSMAC (Angus and Griffiths, 2013). STED nanoscopy revealed the intimate regulation of granule release by NK cells through the actin meshwork (Rak et al., 2011). Likewise, nanoscopy of TCR partners, Lat, and ZAP-70, documented spatiotemporal immune synapse organization, in coordination with signaling pathways, revealing patterning into micro- and nano-clusters that reorganize upon stimulation (Lillemeier et al., 2010; Sherman et al., 2011; Williamson et al., 2011; Neve-Oz et al., 2015; **Figure 1E**). Nanoscopy and single-particle tracking revealed actin reorganization upon lytic granules docking (Brown et al., 2011) and actin-mediated nano-clustering of CD1d in iNK T cells (Torreno-Pina et al., 2016). The relevance of dynamic studies performed *in vitro* that afford high resolution, like nanoscopy (Rossy et al., 2013), may be complemented by less resolved but more physiological *in vivo* measurements, notably intravital two-photon microscopy (Germain et al., 2012).

Neuronal Synapse

Like FAs and immune synapses, neuronal synapses also depict a complex sub-micrometric organization (**Figure 1F**). Single-molecule investigations have complemented neurophysiological approaches aimed at deciphering the molecular mechanisms that underlie pre- and post-synaptic plasticity. Synaptic receptors followed by single-particle tracking revealed that both inhibitory glycine and excitatory glutamate receptors reversibly aggregate together, through scaffold protein binding (Meier et al., 2001; Sergé et al., 2002). According to neuronal activity, synaptic efficiency can hence be modulated by receptor number at the post-synaptic density (Borgdorff and Choquet, 2002). However, due to their size, colloids were hampered to fully enter the synaptic cleft. This was circumvented by using smaller fluorescent labels (Dahan et al., 2003; Tardin et al., 2003). More recently, instead of monitoring a few labeled targets, nanoscopy provided a comprehensive view of the synapse at molecular resolution (Maglione and Sigrist, 2013). This further revealed dynamic spatial heterogeneity either pre- (Willig et al., 2006; Meyer et al., 2009; Ehmann et al., 2015), post- (Nair et al., 2013), or at the synaptic cleft (Perez de Arce et al., 2015). As for other cell contacts, lateral diffusion and vesicular trafficking constitute key solutions to modulate the spatiotemporal organization and function of synaptic components (**Figure 1G**).

CELL CONTACT EVOLUTION IN CANCER

Cancer primarily results from genetic alterations that lead to uncontrolled cell proliferation. Such control, critical for proper maintenance of cellularity within tissues, is achieved, at least in part, by signals emanating from contact inhibition. Epithelial–mesenchymal transition, physiologically required for embryonic development and wound healing, may also be dramatically hijacked in tumoral context, not necessarily for metastasis, but at least for chemo resistance (Fischer et al., 2015; Zheng et al., 2015). Hence, cell adhesion, in relation to signaling features such as rafts, is directly implicated in oncogenesis, often involving mutations leading to CAM up- and down-regulation (Eke and Cordes, 2015; **Figure 2**). NK cells constantly patrol organisms to detect and eliminate transformed cancer cells before massive tumor growth. As described above, super-resolution measures have improved our understanding of these cell-killing modalities (Dustin and Long, 2010; Brown et al., 2011). *In vitro* studies on fundamental cellular processes such as cell adhesion, immune interactions, as described above, as well as genome instability and cell division, are directly relevant for cancer research. Noteworthy, studies on integrin dynamics have been extended to cancer cells, revealing how the glycocalyx reinforces FAs and associated tumoral signaling (Paszek et al., 2014).

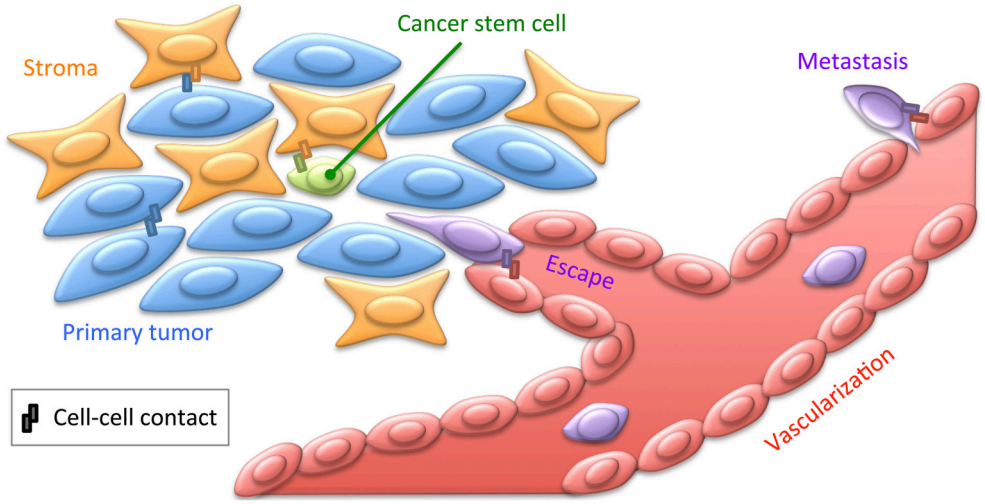

FIGURE 2 | Cell contacts in cancer. Cancer cells engage contacts with themselves as well as with their surrounding stroma, including the ECM. Tumors are believed to contain cancer stem cells, engaging privileged contacts with the stroma allowing them not only to maintain quiescence and pluripotency, but also to putatively sustain resistance to chemotherapy. Cancer cells may also engage specific contacts with the tumor neovascularization. This may lead to cancer cell escape within the blood or lymph circulation by intravasation, and subsequently to distant metastasis by extravasation (Reymond et al., 2013). These various cellular interactions implicate a broad range of CAMs, such as cadherins, integrins, or JAMs, as well as ECM and soluble factors. Most of these cancer-specific contacts thus provide privileged strategies for immunotherapeutic treatments to target tumoral cells with monoclonal antibodies directed against integrins for instance (Scott et al., 2012).

Dissemination and Metastasis by Intra- and Extra-vasation from Vascularization

Tumor neovascularization is a major step in cancer progression. Upon hypoxia, the tumor and infiltrated leukocytes release growth factors stimulating angiogenic outgrowth of endothelial cells, sprouting from pre-existing neighboring blood vessels. CAMs, and in particular integrins, play crucial roles in tumor progression, and metastasis (Desgrosellier and Cheresh, 2010; Reymond et al., 2013). Leukocyte extravasation, essentially through endothelial tight junctions, is a mandatory step for tissue entrance. Transmigration requires complex interactions involving vascular CAMs such as vascular-endothelial-cadherin and members of the immunoglobulin superfamily, platelet-endothelial-CAM-1 and JAMs. These components of endothelial junctions are also directly involved in angiogenesis. Although, molecule and cell tracking share several analytic tools (Sergé and Irla, 2013) and apart from initial studies (Gonda et al., 2010), there is still a gap between nanoscopy, mostly applied *in vitro* for molecular studies within cells, and intravital imaging, addressing cells within organisms. Indeed, intravital imaging adds several challenges, such as (i) managing animal breathing and heart beating and (ii) imaging at substantial depth within absorbing and scattering tissues, which will be challenging to reconcile with the mechanical stability and signal intensity required for nanoscopy. Data are thus essentially collected at cellular or subcellular scale, for specific molecules, although not at single-molecule resolution. Nevertheless, cancer evolution has been extensively documented regarding crucial steps such as dissemination and metastasis. Cells cultured within 3D matrix spheroids, thick tissue sections and dissected organs provide intermediate configurations from *in vitro* to *in vivo*, which are potentially better suited for nanoscopy (Ding et al., 2009; Cella Zanacchi et al., 2011). Further progress may allow *in vivo* investigations with nanometric resolution in the near future.

Cancer Stem Cell Interaction in Niches

It is now established that cancers are not composed of homogenous clonal cells, but contain several cell types, differentiated to various extents. This includes cells exhibiting stemness properties, which are critical for two reasons: first, being quiescent, they escape most chemotherapies that target fast dividing cells as a classical hallmark of cancer, and second, they are susceptible to lead to relapse by differentiating and proliferating after treatment. One major point responsible for disparities among cancer cells is that they express distinct CAMs and thus differentially attach to each other and to the stromal microenvironment (Weidle et al., 2016; **Figure 2**). Membrane features such as rafts are directly implicated in stem cell retention in the stromal niche (Ratajczak and Adamiak, 2015). Molecular mechanisms allowing tumor cell localization within specialized microenvironments have been identified. Cancer relapse may arise from clonal re-emergence of cells kept quiescent in privileged microenvironments (Eppert et al., 2011). In the bone marrow, interactions between hematopoietic and stromal cells allow mutual transmission of signals involved in the development and homeostasis of both cell types (García-García et al., 2015). This crosstalk involves adhesion mechanisms, with a major impact on the development, maintenance, and proliferation of hematopoietic and stromal cells. Such interactions physiologically occur between JAM-C-expressing hematopoietic stem cells and JAM-B-expressing stromal cells (Arcangeli et al., 2011; De Grandis

et al., 2016) and are extensively reorganized in leukemic context. Therefore, JAMs may provide a therapeutic target to block leukemic stem cell/stroma interactions responsible for resistance to treatment and relapse. Deciphering the *modus operandi* of JAMs in this process by nanoscopy could contribute in evaluating an adjuvant therapeutic potential for anti-JAM blocking antibodies to release leukemic cells from their niche.

CONCLUDING REMARKS

Adhesion is a common feature among nearly all cells within our organisms. CAMs are directly implicated in a broad range of physiopathological mechanisms related, for instance, to developmental defects, immunity and cancer. Increasing the resolution by one order of magnitude is a major breakthrough expected to deliver unsuspected structural and dynamic information on most cellular and cancerous processes, ranging from genomic to cell signaling mechanisms. This is also expected to aid in deciphering anti-tumoral mechanisms (Blom and Brismar, 2014), especially with respect to both spontaneous and therapeutic immunological responses. Upon examination at ever-increasing spatiotemporal resolution, subcellular structures reveal greater dynamics than previously assessed. In contrast from the static concept of CAMs and adaptors that would be definitively attached to FAs or synapses, nanoscopy offers a highly dynamic scheme of transient assemblies, emerging from stochastic motion and associations. Fast molecular reorganizations allow subtle cellular adaptions to environmental modifications. Photophysical performances, labeling specificity, and monovalency, with minimal artifacts induced by tagging, are important issues for nanoscopy, together with technological improvements in optics and sensors. Future directions will also include combining nanoscopy with complementary measures such as other imaging modalities, functional biochemical/electrical measures or single cell genomic/proteomic analyses. Microscopy modalities such as atomic-force microscopy and optical tweezers have also been considerably improved recently. Subcellular mechanical measurements allow us to include force as a new and important parameter when considering molecular interactions (Klotzsch et al., 2015). Understanding these subtle characteristics is of fundamental interest for the purpose of targeting and fine-tuning adhesion in pathologies such as cancer that profoundly implicate intercellular reorganization. Some processes, such as cancer dissemination and metastasis, intrinsically require considering a multicellular scale. Integrating super-resolution measures into whole organism or at least whole organ experiments will be another challenge. Pioneer work coupling two-photon with STED (Ding et al., 2009) or light sheet based planar illumination with SLSM (Cella Zanacchi et al., 2011), are promising steps toward intravital nanoscopy. Such experimental developments can be expected to find applications first in fundamental science before being potentially transferred to clinical use.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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METHODS
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Super-Resolution Imaging of Plasma Membrane Proteins with Click Chemistry

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Besides its function as a passive cell wall, the plasma membrane (PM) serves as a platform for different physiological processes such as signal transduction and cell adhesion, determining the ability of cells to communicate with the exterior, and form tissues. Therefore, the spatial distribution of PM components, and the molecular mechanisms underlying it, have important implications in various biological fields including cell development, neurobiology, and immunology. The existence of confined compartments in the plasma membrane that vary on many length scales from protein multimers to micrometer-size domains with different protein and lipid composition is today beyond all questions. As much as the physiology of cells is controlled by the spatial organization of PM components, the study of distribution, size, and composition remains challenging. Visualization of the molecular distribution of PM components has been impeded mainly due to two problems: the specific labeling of lipids and proteins without perturbing their native distribution and the diffraction-limit of fluorescence microscopy restricting the resolution to about half the wavelength of light. Here, we present a bioorthogonal chemical reporter strategy based on click chemistry and metabolic labeling for efficient and specific visualization of PM proteins and glycans with organic fluorophores in combination with super-resolution fluorescence imaging by *direct* stochastic optical reconstruction microscopy (*d*STORM) with single-molecule sensitivity.

Keywords: super-resolution fluorescence microscopy, localization microscopy, *d*STORM, plasma membrane organization, click chemistry, protein clusters

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INTRODUCTION

The plasma membrane in eukaryotes is involved in several cell functions such as tissue formation, signal transduction, cell adhesion, and immune response. Although much evidence suggests that the spatial arrangement of its different components, i.e., membrane proteins and lipids, determines the functionality of the PM of eukaryotic cells, the precise molecular architecture remains unclear. Our current view of the cell membrane goes beyond the "fluid mosaic model," proposed more than 40 years ago by *Singer and Nicolson*, where proteins freely diffuse in a homogeneous sea of lipids (Singer and Nicolson, 1972). In contrast, a hierarchical subcompartmentalization, where proteins are transiently trapped in lipid rafts and actin-cytoskeleton associated corrals, has been hypothesized (Kusumi et al., 2012). Dynamic data obtained by ultra-fast single particle tracking has shown reduced diffusion behavior and hoping events of different membrane proteins suggesting the presence of protein nanodomains (Kusumi et al., 2005). The predicted size of these nanoclusters is in the order of a few tens to a few hundreds of nanometers, dependent on the cell type, protein,

or lipid. However, until now two obstacles impede the exploitation of quantitative data about the architecture of membrane-associated glycoproteins: selective and efficient labeling of membrane components and the resolution limit of optical microscopy.

During the last decade, the advent of far-field super-resolution microscopy methods, such as stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006), *direct*STORM (Heilemann et al., 2008; van de Linde et al., 2011), photoactivated light microscopy (PALM) (Betzig et al., 2006), fluorescence PALM (Hess et al., 2006), stimulated emission depletion microscopy (STED) (Klar et al., 2000), ground state depletion microscopy (GSD) (Bretschneider et al., 2007), and structured illumination microscopy (SIM) (Gustafsson, 2000), has overcome this limitation. The application of these techniques revealed the existence of PM clusters with a typical size of ∼80 nm for various PM proteins (Kittel et al., 2006; Sieber et al., 2007; Williamson et al., 2011; Bar-On et al., 2012; Rossy et al., 2013). However, probing weather protein subcompartmentalization is a universal feature of PMs is still challenging. To this aim, methods devoted to stain, and visualize simultaneously a large population of PM proteins are required. Electron microscopy using immunogold labeling on isolated plasma membrane sheets revealed the existence of highly dense patches containing different membrane proteins (Lillemeier et al., 2006). More recently, the introduction of a bioorthogonal chemical reporter strategy, based on metabolic labeling and click chemistry, allowed the direct visualization of different membrane components by super-resolution microscopy (Letschert et al., 2014; Saka et al., 2014). This approach exploits the ability of the endogenous metabolic cellular machinery to recognize different metabolic surrogates containing small reactive chemical modifications ready to be conjugated with fluorophores. Non-natural methionine analogs, containing an azide, or an alkyne group, are recognized by the methionyl-tRNA synthetase and co-translationally incorporated into nascent proteins (Tom Dieck et al., 2012). On the other hand, non-native monosaccharide precursors can be used to introduce similar chemical groups into glycoproteins as post-translational modifications (Laughlin and Bertozzi, 2009a). Thus, click chemistry represents a direct labeling method for the visualization of different PM components.

Here, we report an efficient method to visualize PM proteins stained via metabolic labeling and click chemistry by super-resolution imaging with virtually molecular resolution. In particular, we present two procedures enabling quantitative super-resolution imaging of PM components on two different time-scales. First, we use L-azidohomoalanine (L-AHA), a non-natural methionine analog that is incorporated into newly synthesized proteins, typically within few hours. Second, we use peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz) as a non-native monosaccharide incorporated into membrane-associated glycoproteins during 2 days of incubation. For fluorescence labeling, we compare two click chemistry reactions, copper-catalyzed azide-alkyne cycloaddition (CuAAC), and copper-free strain-promoted azide- alkyne cycloaddition (SPAAC), with regard to labeling efficiency. For fluorescence imaging with subdiffraction-resolution, we use single-molecule localization microscopy based on photoswitching of standard fluorophores, i.e., *direct* stochastic optical reconstruction microscopy (*d*STORM) (Heilemann et al., 2008; van de Linde et al., 2011). Furthermore, we describe localization microscopy based methods to determine quantitative information on density and spatial distribution of membrane proteins such as Ripley's K function. In addition, we highlight advantages of the method and limitations that might give rise to the appearance of artificial membrane clusters. Our data indicate that high emitter densities can be achieved of both apical and basal membrane components. Inhomogeneous distributions of PM proteins or glycans are revealed, especially in two-dimensional projections of intrinsically three-dimensional (3D) structures such as filopodia and overlapping membranes. More importantly, labeled vesicles located in close proximity to the PM can be misleadingly interpreted as clusters in two-dimensional super-resolution images. A certain degree of deviation from complete spatial randomness in PM proteins was found by Ripley's K function analysis.

MATERIALS

Cell Culture and Maintenance

1. Cell line and growth media: Adherent cell line growth in appropriate culture media. In this case, we use a human osteosarcoma (U2OS) cell line in standard growth media (cDMEM: Dulbecco's modified Eagle's HAM's F12 media supplemented with 10% (v/v) fetal calf serum (FCS), 4 mM glutamine, 100 U/L penicillin, and 0.1 mg/mL streptomycin).
2. Cell culture and maintenance: T25-culture flasks (Greiner Bio-One). Cell culture incubator maintained in humidified atmosphere at 5% CO_2 and 37°C. Phosphate-buffered saline (PBS), Hank's balance salt solution (HBSS), and accutase solution.
3. Cell preparation for metabolic labeling and fluorescence imaging: 8 well Lab-Tek chamber slides (Nunc, Thermo Fisher Scientific).

Metabolic Labeling with Azido Unnatural Amino Acid AHA

1. Metabolic labeling media: Methionine free media (MFM: Dulbecco's modified Eagle's HAM's F12, with 10% FCS, 4 mM glutamine, 100 U/L penicillin, and 0.1 mg/mL streptomycin, without methionine).
2. Azido methionine analog: L-azidohomoalanine (L-AHA) (Jena Bioscience) stored as powder at 4°C.
3. Protein synthesis inhibitors: Anisomycin (Sigma-Aldrich) 10 mg/mL stock solution in dimethyl sulfoxide (DMSO)

Metabolic Labeling with Peracetylated Azido Modified Monosaccharides.

1. Metabolic labeling media: Standard growth media (cDMEM) supplemented as described in cell culture and maintenance.
2. Azido modified monosaccharides: N-azidoacetylgalactosamine (Ac_4GalNAz) (Invitrogen). Stock

solutions were prepared at 25 mM in dimethyl sulfoxide (DMSO) and stored at −20°C up to 12 months.

Alternatively N-azidoacetylmannosamine (Ac_4ManNAz) and N-azidoacetylglucosamine (Ac_4GlcNAz) can be used

Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)

1. Staining solution additives: Copper sulfate ($CuSO_4$), copper ligand Tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA), and sodium ascorbate (Sigma-Aldrich).
2. Stock solutions of 2 mM $CuSO_4$ and 10 mM THPTA in MiliQ water stored at −20°C. 100 mM sodium ascorbate in MiliQ water freshly prepared.
3. Alkyne-tagged fluorophore: 2 mM stock solution of Alexa Fluor 647 alkyne (Thermo Fischer Scientific) in DMSO stored at −20°C up to 12 months.

Strain-Promoted Azide-Alkyne Cycloaddition (Spaac)

1. DBCO-tagged fluorophore: 2 mM stock solution of Cy5 DBCO (Sigma-Aldrich) in DMSO stored at −20°C up to 12 months.

Super-Resolution Imaging with *d*STORM

1. Setup: We used a custom-made setup based on an inverted commercial microscope (IX71; Olympus) equipped with an oil-immersion objective (60x, NA 1.45; Olympus), and a nosepiece stage (IX2-NPS; Olympus) to prevent focus-drift during image acquisition. A 641-nm diode laser (Cube 640–100C; Coherent), spectrally cleaned-up with a band-pass filter (BrightLine 642/10, Semrock), was used for excitation of Cy5 and AF-647. Additionally, two lenses and a mirror, coupled to a translation stage, were used to focus the laser beam on the back focal plane of the objective and switching between different illumination modes, i.e., epi, low-angle/highly inclined and laminated light optical sheet (HILO), and total internal reflection illumination (TIR) (Sharonov and Hochstrasser, 2007; Tokunaga et al., 2008; van de Linde et al., 2011). Fluorescence emission of Cy5 and AF-647 were collected with the same objective, separated from excitation light by a dichroic beamsplitter (560/659, Semrock), filtered with appropriate band- and long-pass filters (BrightLine 697/75 and RazorEdge 647, Semrock), and projected on an EMCCD camera (Ixon DU897, Andor Technology). Additional lenses were placed into the detection path to generate a final pixel size of 134 nm.
2. Switching buffer: PBS buffer containing 100 mM β-mercaptoethylamine (MEA, Sigma-Aldrich) and an oxygen scavenger system (2% (w/v) glucose, 4 U/mL glucose oxidase and 80 U/mL catalase) adjusted to pH 7.4.
3. *d*STORM image reconstruction: Open source software for single-molecule localizations and super-resolution image reconstruction rapi*d*STORM 3.3 (Wolter et al., 2010, 2012).

Quantitative Analysis

For quantitative analysis of generated localization data based on XY coordinates lists, customized algorithms implemented with programing languages such as Python (available at http://www.python.org), and Mathematica (Wolfram Research Inc., Champaing, Il, USA) were used.

METHODS

Background

Since the development of the *Staudinger-Bertozzi ligation* between azides and phosphines in 2000 (Saxon and Bertozzi, 2000), bioorthogonal *"click chemistry"* reactions allowed the visualization of different biomolecules (e.g., proteins, glycans, lipids, and nucleic acids) in cultured cells, tissues, and living organisms (Sletten and Bertozzi, 2009). To this aim, one functional group (the label) is introduced into the biomolecule of interest followed by exogenous addition of fluorophores bearing the reactive partner (the probe). For example, unnatural amino acids and monosaccharides containing an azide group can be used as metabolic surrogates of their native counterparts to visualize proteins and glycoproteins as well as glycolipids (Laughlin and Bertozzi, 2009a; Tom Dieck et al., 2012).

Two different approaches have been used successfully to introduce amino acid analogs into proteins: (i) genetic encoding, i.e., site-specific modification, and (ii) metabolic labeling, i.e., residue-specific modification. Whereas, the first method introduces unnatural amino acids into one particular protein, the second method allows labeling of a wide part of the proteome replacing a native amino acid (e.g., methionine) by its non-natural analog (e.g., L-azidohomoalanine, L-AHA). Due to its structural similarity, L-AHA is recognized and tolerated by the methionyl-tRNA synthetase (MetRS), and incorporated into newly synthesized proteins co-translationally in a residue-specific manner. Alternatively, azido sugars (e.g., peracetylated N-azidoacetylgalactosamine Ac_4GalNAz, N-azidoacetylmanosamine Ac_4ManNAz, and N-azidoacetylglucosamine Ac_4GlcNAz), can be incorporated into different types of glycoproteins and glycolipids (Laughlin et al., 2006; Laughlin and Bertozzi, 2009a). Upon cellular uptake and deacetylation, Ac_4GalNAz, Ac_4ManNAz, and Ac_4GlcNAz are converted into activated sugars, recognized by the glycan biosynthetic machinery, and incorporated into sialic acids and mucin-type O-linked glycans, as well as into O-GlcNAc-modified proteins. After metabolic incorporation of amino acids and monosaccharide surrogates, the azide groups introduced into newly synthesized proteins and glycans can be conjugated with alkyne fluorophores via azide-alkyne cycloaddition allowing their direct visualization.

Originally, the classic reaction between terminal alkynes and azides was shown to be efficiently catalyzed by copper(I) at room temperature enabling it to proceed within minutes under physiological conditions, opening the door for biological applications (Rostovtsev et al., 2002; Tornøe et al., 2002). Since then, this reaction, now termed as the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), has been used to visualize

different metabolically labeled biomolecules (Sletten and Bertozzi, 2009). However, due to Cu(I) toxicity fluorescent staining by CuAAC has been restricted to fixed cells. To overcome this problem, two alternative strategies have been developed. In 2004, it was shown that azide-alkyne cycloaddition can be strain-promoted in the absence of copper(I) using cyclooctynes (Agard et al., 2004). Since then, different cyclooctyne molecules with enhanced efficiency have been developed for copper-free strain-promoted azide-alkyne cycloaddition (SPAAC) (Jewett and Bertozzi, 2010; Debets et al., 2011). On the other hand, the optimization of the CuAAC, by means of copper(I) ligands and further additives in the reaction buffer, preserves cell viability while live staining. For example, the use of THPTA in addition to sodium ascorbate allow efficient CuAAC bioconjugation within 5 min with low copper concentrations (e.g., 50 μM) minimizing Cu(I) toxic effects (Hong et al., 2009, 2010).

Standard fluorescence microscopy, combined with metabolic labeling and click chemistry, has been used extensively to visualize both proteins and membrane-associated glycoconjugates within different cellular contexts. For example, newly synthesized proteins have been imaged in mammalian cells and rat hippocampal neurons (Dieterich et al., 2006, 2010; Beatty and Tirrell, 2008), and different glycan populations in culture cells (Baskin et al., 2007), developing zebrafish embryos (Laughlin et al., 2008), and living *C. elegans* (Laughlin and Bertozzi, 2009b). Remarkably, these studies demonstrated the versatility of metabolic labeling for temporal profiling of dynamic changes in large protein populations and glycans. More recently, the same chemical reporter strategy has allowed direct visualization of different membrane components by super-resolution microscopy (Letschert et al., 2014; Saka et al., 2014). Stimulated emission depletion (STED) was used to image unnatural amino acids incorporated into membrane proteins in monkey kidney cell line COS-7, demonstrating protein confinement with reduced diffusion dynamics (Saka et al., 2014). On the other hand, *d*STORM was used to visualize different glycan types, including glycoproteins, after metabolic labeling with Ac_4GalNAz, Ac_4ManNAz, and Ac_4GlcNAz in human osteosarcoma (U2OS) and neuroblastome (SK-N-MC) cells (Letschert et al., 2014). Moreover, due to its ability for single-molecule detection and position determination *d*STORM measurements provided quantitative estimates of molecular densities and spatial distributions of membrane-associated glycoconjugates.

Protocols

In this section we provide protocols to combine metabolic labeling and fluorescent staining via click chemistry for super-resolution imaging with *d*STORM of membrane proteins with single-molecule sensitivity. The method comprises four steps:

Step 1. **Metabolic labeling with azido surrogates**, i.e., with L-azidohomoalanine (L-AHA) and peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz) (**Figure 1A**).

Step 2. **Click chemistry fluorescent live staining** via copper-catalyzed (CuAAC) and copper-free strain-promoted azide-alkyne cycloadditions (SPAAC) (**Figure 1B**).

Step 3. **Localization based super-resolution imaging with *d*STORM**. Image acquisition and reconstruction, identification of two-dimensional projections of three-dimensional cell structures, and labeling efficiency estimation.

Step 4. **Quantitative analysis**. Estimation of detected molecular densities using reference samples, and clustering analysis by Ripley's K function.

Step 1- Metabolic Labeling with Azido Surrogates

Protocol 1a: Metabolic Labeling with Azido Methionine Analogs (L-Azidohomoalanine, L-AHA)

1. Cell culture and maintenance: Choose an appropriate cell line, e.g., human osteosarcoma (U2OS) cells, as a model system of adherent mammalian cells. Maintain the cells at 37°C in 5% CO_2 water-saturated atmosphere in growth culture medium (cDMEM).
   For gentler detachment of cells from T25-culture flasks incubation with accutase for 5 min is preferred rather than trypsine/EDTA treatment.
2. Azido amino acid incubation: Detach cells from culture flask by incubating with accutase for 5 min, count them and seed them in LabTek 8 well chambers at 1.2×10^4 final concentration per well in cDMEM growth media, and let them grow in the cell incubator for 48–72 h at 37°C and 5% CO_2 water saturated atmosphere until 80–90% confluency. Previous to L-AHA incubation, exchange growth medium with prewarmed HBSS, and incubate cells at 37°C during 50 min to deplete the cellular reservoirs of endogenous methionine. During this time prepare a fresh solution of 4 mM L-AHA in methionine-free medium (MFM) and prewarm it. Replace HBSS with AHA solution and incubate cells at 37°C and 5% CO_2 water saturated atmosphere for the desired time, e.g., 4–5 h.
   Control samples can be prepared incubating AHA in the presence of protein synthesis inhibitor such as anisomycin at 40 μM final concentration to evaluate fluorescent background (**Figure S1**).

Protocol 1b: Metabolic Labeling with Azido Sugars (N-Azidoacetylgalactosamine, Ac_4GalNAz)

1. Cell culture and maintenance: follow the same procedure as describe above.
2. Azido sugar incubation: After accutase incubation seed the cells onto 8 wells LabTek chamber at a final concentration of 1.2×10^4 cells per well. Add Ac_4GalNAz at 25 μM final concentration. Incubate cells at 37°C and 5% CO_2 water saturated atmosphere for 48 h before fluorescence staining and fixation.
   Control cells can be prepared in absence of azido sugars to evaluate fluorescence background (**Figure S1**).

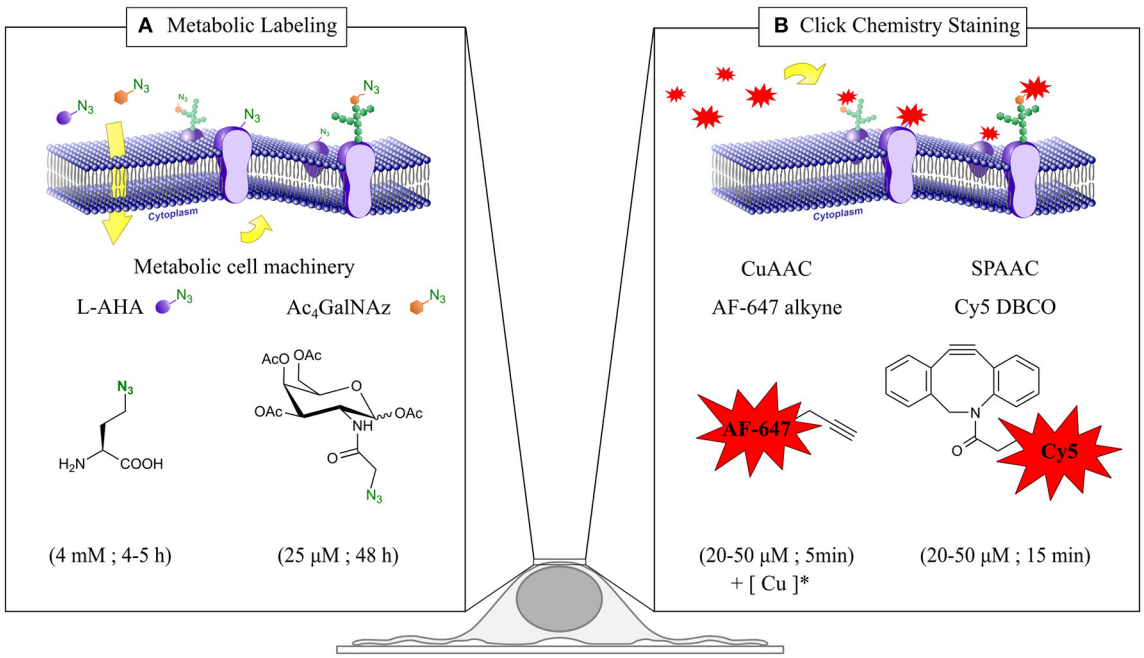

FIGURE 1 | Chemical reporter strategy based in metabolic labeling and click chemistry for *d*STORM quantitative imaging of plasma membrane (PM) proteins. (A) Incorporation of the metabolic surrogates L-azidohomoalanine (L-AHA) and N-Azidoacetylgalactosamine (Ac_4GalNAz) into newly synthesized proteins. Upon cellular uptake, L-AHA and Ac_4GalNAz are recognized by the endogenous cell machinery, and incorporated co-translationally and post-translationally into PM proteins and glycoproteins respectively. Typical incubation time and surrogate concentration for each metabolic labeling scheme are indicated at the bottom. **(B)** Click chemistry staining of PM proteins. After metabolic labeling, PM proteins bearing an azido group are stained with Alexa Fluor 647 alkyne and Cy5 DBCO via copper-catalyzed (CuAAC) and copper-free azide-alkyne cycloadditions (SPAAC) respectively. Typical incubation time and fluorophore concentration for each click chemistry reaction are indicated at the bottom. (*) Staining solution for CuAAC reaction (50 μM $CuSO_4$, 250 μM THPTA, 2.5 μM sodium ascorbate, and the desired amount of Alexa Fluor 647 alkyne in PBS).

Step 2- Fluorescence Live Staining via CuAAC and Spaac

Protocol 2a: Copper Catalyzed Azide-Alkyne Cycloaddition (CuAAC)

1. Preparation of optimal staining solution (50 μM $CuSO_4$, 250 μM THPTA, 2.5 μM sodium ascorbate, and the desired amount of Alexa Fluor 647 alkyne in PBS): For one LabTek well (final volume 200 μl). Premix 5 μl of 2 mM $CuSO_4$ with 5 μl of 10 mM THPTA stock solution. After 5 min add 5 μl of 100 mM sodium ascorbate freshly prepared stock solution in MiliQ water. Add appropriate volume of PBS and Alexa Fluor 647 depending on the desired final concentration of fluorophore. Vortex at high speed for few seconds.
   Further details in the use of copper ligands and sodium ascorbate for optimal CuAAC bioconjugation can be found elsewhere (Hong et al., 2009).
2. Fluorophore incubation: Immediately after removing the LabTek from incubator, wash cells once with prewarmed PBS and incubate them with staining solution for 5 min protected from light at room temperature. Then, wash cells three times gently with PBS and fixate them in PBS solution containing 4% formaldehyde and 0.2% glutaraldehyde respectively. Finally, wash cells three times with PBS and store them at 4°C in PBS containing sodium azide 0.2% (w/v).
   Strong fixation over long times (e.g., 1 h) in the presence of glutaraldehyde is required to minimized lateral mobility of membrane proteins (Tanaka et al., 2010).

Protocol 2b: Copper-Free Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC)

1. Staining solution: Dilute Cy5 DBCO in HBSS at desired concentration without any further additives.
   To avoid cellular stress, HBSS is preferred to PBS due to longer fluorophore incubation times.
2. Fluorophore incubation: Proceed as in point 2 of protocol 2a, i.e., wash the cells once with prewarmed PBS, exchange PBS with staining solution with desired fluorophore concentration, and incubate for 15 min instead of 5 min, wash cells three times with PBS, add fixation solution for 1 h, wash three times, and store cells at 4°C in PBS with 0.2% of sodium azide.

Step 3- Localization Based Super-Resolution Imaging with *d*storm

Protocol 3: *d*STORM Super-Resolution Imaging

1. Photoswitching buffer preparation: Prior to imaging, dissolve β-mercaptoethylamine (MEA) in PBS and keep the MEA powder reagent under argon atmosphere to avoid oxidation. Thaw stock aliquots of glucose, glucose oxidase and catalase

for the oxygen scavenger system. Mix all the reagents to final concentrations of 100 mM MEA, 2% (w/v) glucose, 4 U/mL glucose oxidase and 80 U/mL catalase. Finally adjust the pH to 7.4 with 5 M KOH solution.

2. Preparing cells for *d*STORM imaging: Exchange storing buffer with switching buffer (1.1 mL per well) and seal the LabTek with a coverslip to reduce uptake of atmospheric oxygen. Finally mount the LabTek onto the oil immersed inverted objective of the microscope.
3. Measuring *d*STORM image stack: First, localize and position cell of interest at low intensities. Then, increase the irradiation intensity, e.g., 5 kW/cm^2, to induce rapid transition of the fluorophores to their non-fluorescent off-state. Before image acquisition, exchange the illumination mode from TIRF, to epi-fluorescence and then back to TIRF to maximize the conversion of out-of-focus fluorophores to the dark state. Wait until all molecules in the field of view blink properly, typically 60 s, and start recording an image stack with the desired length and frame rate, e.g., 20,000 frames at 66 Hz (15 ms exposure time per frame).
   High irradiation intensities are crucial while measuring areas with high fluorophore densities to prevent artifacts due to overlapping of single emitter.
4. Reconstruction of super-resolution image with rapi*d*STORM: Set desired values of the minimum intensity threshold for single-molecule localization and the pixel-size of the super resolution image, e.g., 1000 photons and 10 nm respectively.
5. Identification of 2D-projections of 3D cell structures: Image consecutively the region of interest with slightly shifted (0.5–1 μm) focal planes into the cytosol.
6. Estimation of labeling efficiency: Titrate fluorophore concentration for desired fixed metabolic labeling conditions. Calculate localization density using a sliding window analysis (diameter = 1 μm, step = 100 nm). To prevent contribution from overlapping membrane structures measure localization density in regions under the nucleus.

Step 4- Quantitative Analysis of Molecular Densities and Spatial Distribution at the Nanoscale.

Protocol 4: Estimation of Detected Molecular Densities of Membrane Proteins and Glycans.

1. Preparing reference samples: To ensure detection of single and well isolated fluorophores decrease the labeling density to <20 localizations per μm^2 by adjusting the fluorophore concentration to <0.1 μM. Perform *d*STORM reference measurements using the same optical and chemical conditions, i.e., laser irradiation intensity, buffer composition and TIRF angle, as for non-diluted samples.
   Grouping localizations from isolated fluorophores: Group all localizations within a certain radius detected along the whole image stack (20,000 frames), e.g., by applying a Kalman tracking routine as implemented in rapi*d*STORM. Allow the tracking algorithm to group localizations with maximum temporal separation equal to stack length within a defined area specified by the given tracking radius. To confirm the detection of single spots vary the tracking radius from 1 to 160 nm.
2. Estimation of detected molecular densities: Plot the average track length versus the tracking radius and use the saturation level of the curve as a conversion factor reflecting the number of localizations detected per isolated fluorophore. In addition, align all the localizations within tracks with length >2 to their center of mass. Calculate the experimental precision by fitting the spatial distribution to a Gauss function.
3. Computation of Ripley's h function: We computed what we call Ripley's h function $h(d)$ as function of distance d following the standard definition for Ripley's k function (Ripley, 1977) and applying an established transformation (Kiskowski et al., 2009) allowing simple optical inspection since $h(d)$ is equal to zero for all d in the case of a spatially homogeneous point process (complete spatial randomness):

$$h(d) = \sqrt{\frac{A\sum_{i=1}^{n}\sum_{j=1}^{m}k(i,j)}{\pi m(n-1)}} - d \qquad (1)$$

where d is a distance, A is the area of the region containing all localizations, n is the total number of localizations, m is the number of test localizations in a random subset of localizations, and $k(i,j)$ is a weight defined as:

$$k(i,j) = \begin{cases} 1 \text{ if the distance between localization i and j is less than } d \\ 0 \text{ otherwise} \\ 0 \text{ if the localizations i and j are identical} \end{cases} \qquad (2)$$

For efficient computing on large datasets, we limited the number of test localizations to a subset with typically 500 localizations. For comparison with experimental data, we generated data sets with random localizations according (i) to a Poisson point process, and (ii) to a Neyman-Scott point process (Neyman and Scott, 1952). The Poisson process yields a data set of complete spatial randomness, whereas the Neyman-Scott process yields a data set with spatially Poisson-distributed parent events. Each parent event provides a set of offspring events with a Poisson distributed number of members, on average 5 (equal to the average number of localizations per fluorophore obtained experimentally from diluted reference samples). The offspring spatial coordinates are 2D Gauss distributed around each parent event with a standard deviation equal to the localization precision of 8 nm. We generated data sets with an overall localization density equal to the densities of experimental data. Simulations and statistical analysis of five cells in each data set was carried out using Wolfram Mathematica 10.4.1.

COMMENTARY

Comparison with Other Methods

During the last decades, fluorescence microscopy has allowed the direct observation of cellular processes in a relatively non-invasive fashion with high molecular specificity and temporal

resolution. However, due to the wave nature of light the spatial resolution is limited to approximately half the wavelength of the light in the imaging plane (Abbe, 1873). Recently, super-resolution microscopy methods have circumvented this problem improving the optical resolution substantially. Localization microscopy exhibits the highest spatial resolution of less than 20 nm, as compared to other super-resolution techniques such as STED (Klar et al., 2000) or structural illumination microscopy (SIM) (Gustafsson, 2000). Moreover, due to their single molecule sensitivity, localization microscopy can potentially provide quantitative information about the spatial organization of proteins, as well as the number of molecules residing inside and outside of subcellular compartments including PM nanodomains involved in different cell functions. For example, PALM and *d*STORM, in combination with genetically encoded fluorescent photactivable proteins and immunochemistry, respectively, demonstrated nanocluster organization of synaptic proteins (Bar-On et al., 2012; Ehmann et al., 2014), membrane receptors involved in cell growth, proliferation and differentiation (Gao et al., 2015), tumor necrosis (Fricke et al., 2014), or related to the immunological response (Williamson et al., 2011; Rossy et al., 2013). Comparative studies have also proven PM heterogeneity depending on protein membrane anchor types including the transmembrane protein Lat, the lipid-anchored protein Lyn, the vesicular stomatitis viral glycoprotein VSVG, and GPI anchored proteins (Sengupta et al., 2011, 2013). However, all these studies were restricted to a limited number of proteins at a given time and thus, it became obvious that a more general approach for visualizing simultaneously a large population of membrane proteins is required to inspect the global distribution of PM proteins at the nanoscale. Moreover, fluorescent staining with antibodies and genetically encoded fluorescent proteins can induce artificial clustering of membrane proteins (Tanaka et al., 2010; Magenau et al., 2015) and limit the localization precision due to their relatively large size, especially in high density labeled samples. Metabolic labeling fills both gaps by introducing small bioorthogonal chemical groups such as azides into newly synthetized proteins.

Metabolic labeling has been used during the last decade to visualize newly synthetized proteins with standard fluorescent microscopy in cultured cells, tissues, and living animals. The advantage of this staining strategy is two-fold. First, labeling proteins with small and bioorthogonal chemical handles either by co-translational incorporation of unnatural amino acids or by post-translationally modification with non-natural monosaccharides minimizes perturbation of proteins and likely resembles physiological conditions. Second, metabolic labeling constitutes a unique tool to visualize spatial patterns of wide parts of the proteome. Whereas, immunochemistry and genetically encoded fluorescent are useful to visualize one specific protein, metabolic labeling allows to stain simultaneously newly synthesized proteins in a less specific way. Because the azido amino acid L-azidohomoalanine (L-AHA) replaces endogenous methionine, all proteins containing natively at least a single methionine are prompted to be labeled. On the other hand, the peracetylated azido sugar N-azidoacetylgalactosamine (Ac_4GalNAz) is incorporated into specific subtypes of glycans such as mucin-type O-linked glycans and O-GlcNAc-modified glycoproteins (Laughlin and Bertozzi, 2009a). Further identification of which proteins incorporated successfully L-AHA or Ac_4GalNAz has been achieved using alkyne affinity-tags (e.g., biotin-FLAG-alkyne tag) instead of alkyne fluorophores, in combination with proteomics studies (Dieterich et al., 2006; Laughlin et al., 2006). It is important to remark that the incorporation of L-AHA and Ac_4GalNAz into PM proteins occurs during protein translation and post-translational glycosylation before they are delivered to the cell membrane. Therefore, different metabolic labeling conditions (e.g., changes in incubation time or concentration of the azido surrogates) can be used to study not only the spatial but also the temporal organization of newly synthesized proteins and glycans as shown previously by standard live-cell fluorescence microscopy (Baskin et al., 2007; Beatty and Tirrell, 2008; Laughlin et al., 2008; Laughlin and Bertozzi, 2009b; Dieterich et al., 2010).

When combined with super-resolution microscopy, metabolic labeling allows to inspect the overall distribution of membrane proteins at the nanoscale. This has recently been proven by STED and *d*STORM imaging of membrane proteins containing unnatural amino acids and azido sugars respectively (Letschert et al., 2014; Saka et al., 2014). Although both techniques provide images with substantially enhanced spatial resolution, due to their peculiarities, they exhibit unique advantages and limitations. For example, *d*STORM exhibits better spatial resolution than STED and has the potential to quantify molecular densities of membrane components as well as their spatial distributions. However, due to fluorophore photoswitching kinetics, the necessity of high photon yields, and slow camera frame rates, image acquisition typically requires few minutes (van de Linde et al., 2011). On the other hand, STED achieves much higher temporal resolution and therefore it is more suitable for dynamic studies. Remarkably, STED combined with fluorescence correlation spectroscopy (STED-FCS), where very small areas are scanned at frequencies in the order of a few kHz, can be used to measure diffusion dynamics of membrane proteins and lipids demonstrating molecular confinement with both high spatial and high temporal resolution (Eggeling et al., 2009; Saka et al., 2014).

Critical Parameters, Limitations, and Perspectives

The conditions presented in the given protocols constitute a robust recipe to stain and visualize large populations of PM proteins and glycans with super-resolution localization microscopy (**Figure 2**). Nevertheless, critical aspects, as well as limitations and future perspectives, with regard to obtain reliable quantitative data and avoid artifacts are shown in the next subsections. First, we highlight potential artifacts of *d*STORM as well as the inherent problem of 2D super-resolution images due to projections of 3D structures such as membrane ruffling, filopodia, overlapping membranes, and vesicles located in close proximity to the PM. Then, we compare the fluorescence staining efficiency achieved by copper-catalyzed and copper-free click chemistry reactions for fixed metabolic labeling

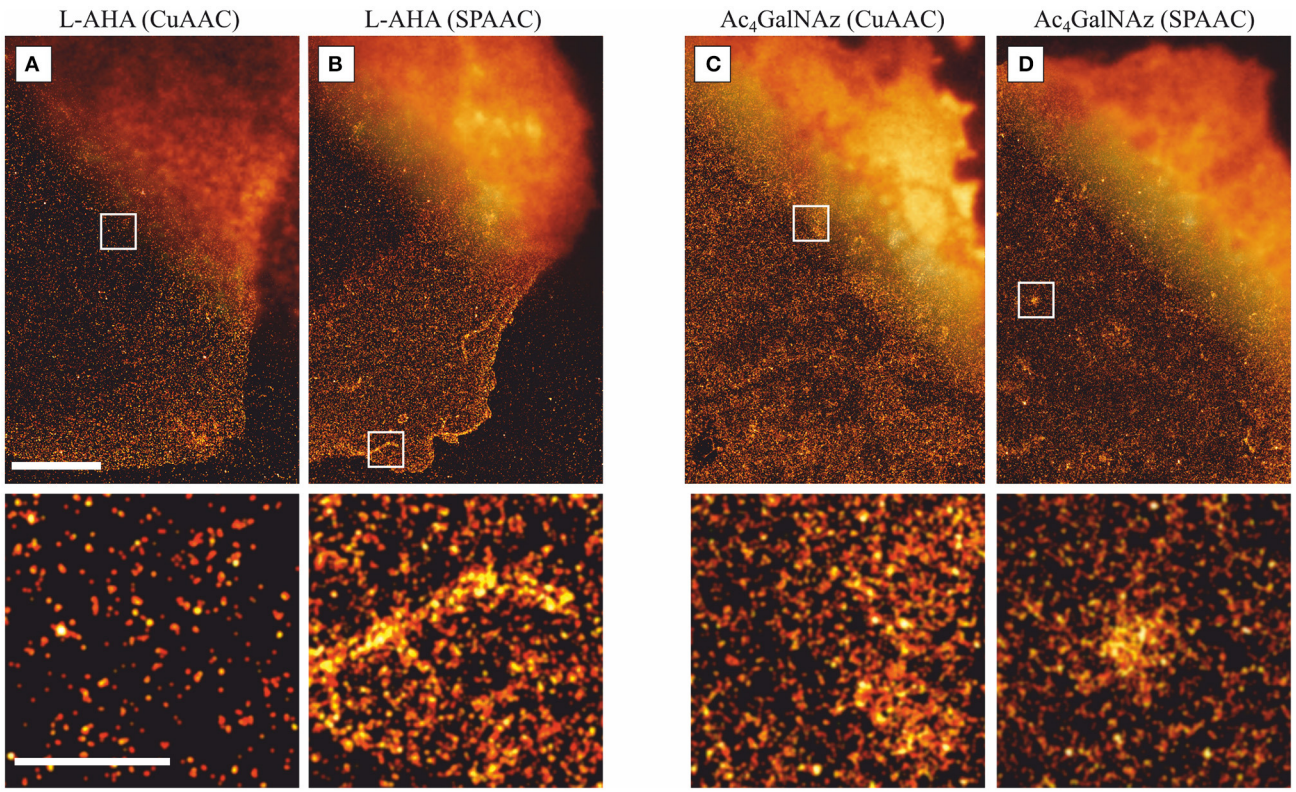

FIGURE 2 | Comparison of *d*STORM images with standard fluorescent microscopy. Representative *d*STORM and overlaid standard fluorescence images (upper right corner) of PM proteins at the basal membrane stained via **(A)** L-AHA (CuAAC), **(B)** L-AHA (SPAAC), **(C)** Ac_4GalNAz (CuAAC), and **(D)** Ac_4GalNAz (SPAAC). Comparison of L-AHA and Ac_4GalNAz stained via copper-catalyzed (CuACC) and copper-free (SPAAC) show no significant differences, indicating that the presence of copper ions or THPTA do not affect the distribution of PM components. For the four staining schemes depicted, 2D projected structures lead to spatial inhomogeneties as highlighted in the lower panels, e.g., **(A)** one fold membrane under the nucleus, **(B)** two-fold membrane structure within the lamellipodia plus one filopodia, **(C)** membrane ruffles, and **(D)** projection of a vesicle located in close proximity to the plasma membrane. All images were acquired under TIRF illumination, reconstructed with a minimum localization intensity threshold of 1000 photons, and a pixel size of 10 nm. Scale bars are 5 μm (upper panels) and 1 μm (lower panels).

conditions. Finally, we show how quantitative information about the distribution of PM components can be percolated from *d*STORM data using statistical spatial analysis approaches, such as pair-correlation and Ripley's K functions.

Artifacts and 2D Projections of 3D Structures in *d*STORM Imaging.

The intrinsic features of localization microscopy, i.e., reconstruction of super-resolution images from localization of single molecules, determine its accuracy, and reliability. The precision of position determination of single and well isolated fluorescent emitters is mainly determined by the number of collected photons, the signal-to-noise ratio, and the accuracy of the algorithm implemented in the localization software used to fit the point-spread-function (PSF) of detected fluorophores (Thompson et al., 2002; Mortensen et al., 2010; Sage et al., 2015). In contrast, other considerations must be taken into account to reconstruct reliable super-resolution images. For example, overlapping PSFs of multiple fluorophores residing in their on-state simultaneously within the same diffraction-limited area must be prevented, except specialized algorithms capable of fitting multiple emitters PSFs are used (Holden et al., 2011; Zhu et al., 2012), to avoid incorrect localizations and ensure artifact-free images reconstruction (van de Linde et al., 2010; Sauer, 2013; van de Linde and Sauer, 2014; Burgert et al., 2015). As a rule of thumb to avoid PSFs overlapping and ensure reliable spot finding and fitting, the density of fluorescent emitters has to be kept below 0.2 spots per μm^2 (Wolter et al., 2011). Therefore, appropriate measurement conditions in *d*STORM imaging such as laser irradiation intensities high enough to transfer the majority of organic dyes to long-living off states as well as suitable buffer compositions are required to guarantee good image quality.

Besides the aforementioned experimental traits of *d*STORM, inherent problems and limitations appear when studying membrane components with 2D localization microscopy. Without 3D information the ability to extract unbiased information about PM can be error prone. The existence of Z-projections of inherent cell membrane structures such as invaginations and vesicle-like structures, including fluorophore-filled endosomes in contact with or located near the PM,

as well as overlapping membranes in the lamellipodia, might distort severely the quantitative analysis and interpretation of super-resolution images. For example, a sliding window analysis applied to *d*STORM images of PM under the nucleus reveals half of the localization density compared to lamellipodia indicating a two-fold membrane structure (**Figures 3A,B**). Furthermore, circular clusters with apparent sizes ranging from a few tens to a few hundred nanometers can be visually identified from more homogeneous distributions, however it is difficult to discern weather they represent nanodomains enriched in membrane proteins or projections from fluorophore-filled vesicles in close proximity to the membrane. Whereas, a 3D-*d*STORM measurement would reduce any information bias on PM organization due to vertical projections, instrumentation, and implementation for 3D-*d*STORM is more complex and expensive compared to 2D-*d*STORM, and they usually achieve a lower axial than lateral resolution (Klein et al., 2014). In contrast, consecutive imaging of the same cell with slightly shifted focal planes above the feature of interest constitutes a fast control to determine the two-dimensional projection contribution from inherent 3D structures as shown in **Figures 3C,D** for vesicle-like structures located right above the plasma membrane (yellow circles) or further up (blue circle), and membrane ruffles (green circle) (Burgert et al., 2015).

Optimal Staining Efficiencies by Copper-Catalyzed and Copper-Free Click-Chemistry.

The first step of any fluorescent microscopy technique is the efficient staining of the protein of interest with a fluorophore. Moreover, in localization microscopy higher staining efficiencies, reflected as higher labeling densities, affects the maximum resolution in localization microscopy (Sauer, 2013). Whereas,

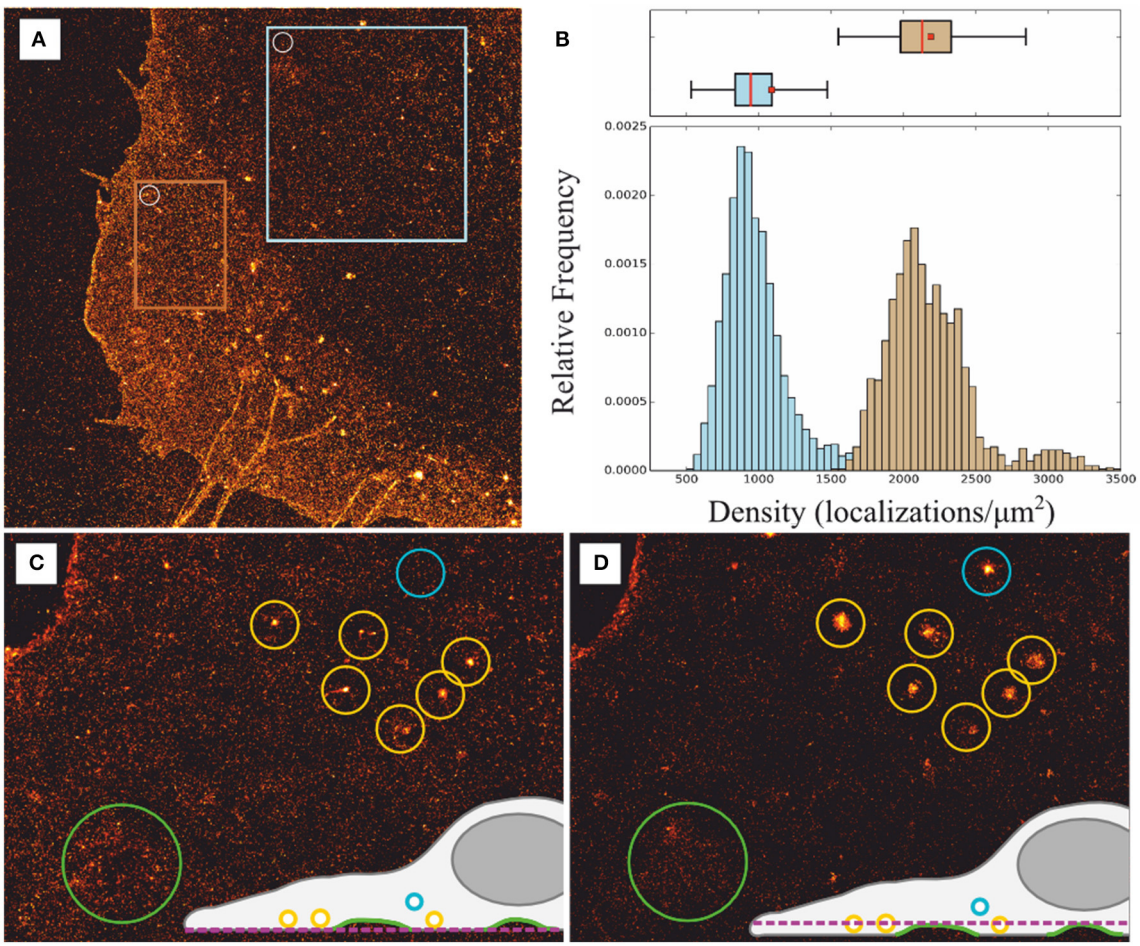

FIGURE 3 | Effect of two-dimensional projections of membrane structures. (A) *d*STORM image of PM proteins metabolically labeled with L-AHA showing overlapping membranes, vesicle-like structures, and filipodia. **(B)** Sliding window analysis to estimate PM content (white circle in **(A):** diameter = 1 μm, step = 100 nm) lead to median values of 884 localizations per μm^2 within a region under the nucleus, i.e., single membrane structure blue square in **(A)**, and 2130 localizations per μm^2 within the lamellipodia, i.e., two-fold overlapping membranes orange square in **(A)**. Box plot: red bar = median, box = 25th and 75th percentile, □ = mean. **(C,D)** Consecutive images with focal planes slightly shifted (0.5–1 μm) into the cytosol reveal artificial cluster structures generated due to vesicle-like structures located above the plasma membrane blue and yellow circles as well as inhomogeneities due to membrane ruffles green circle; adapted from Burgert et al. (2015).

imaging resolution is usually defined as the minimal resolvable distance between two emitters, the extractable structural information is also related to the sampling frequency, i.e., fluorophore labeling density, as described by the Nyquist-Shannon theorem (Shannon, 1949). In essence, the theorem states that the sampling interval, i.e., the mean distance between neighboring localized fluorophores, must be at least twice as fine as the structural details to be resolved. Therefore, higher labeling densities prevent under sampling and improve spatial resolution.

The conditions given here for click chemistry staining of membrane proteins and glycoconjugates lead to maximum labeling densities ranging from 400 to 2000 localizations per μm^2 (**Figure 4**). For the four bioconjugated systems inspected, we observed that fluorophore concentrations around 20–50 μM are required to maximize fluorescent signal. Moreover, copper-free strain-promoted azide-alkyne cycloaddition (SPAAC) is equally efficient as CuAAC to stain Ac_4GalNAz-derived glycoconjugates, and two-fold better to detect membrane proteins containing AHA. Thus, optimal conditions for click chemistry can also be achieved in absence of copper avoiding toxicity effects and simplifying the protocol.

Quantitative Analysis with *d*STORM

In *d*STORM measurements, localization densities in a certain area of the sample can be directly calculated from the coordinate lists exported by the localization software. Whereas, the number of localizations per unit area can be used to estimate the staining efficiency for different labeling conditions, it only provides relative information on the detected numbers of membrane proteins present. Since organic dyes undergo several photoswitching cycles during a *d*STORM measurement,

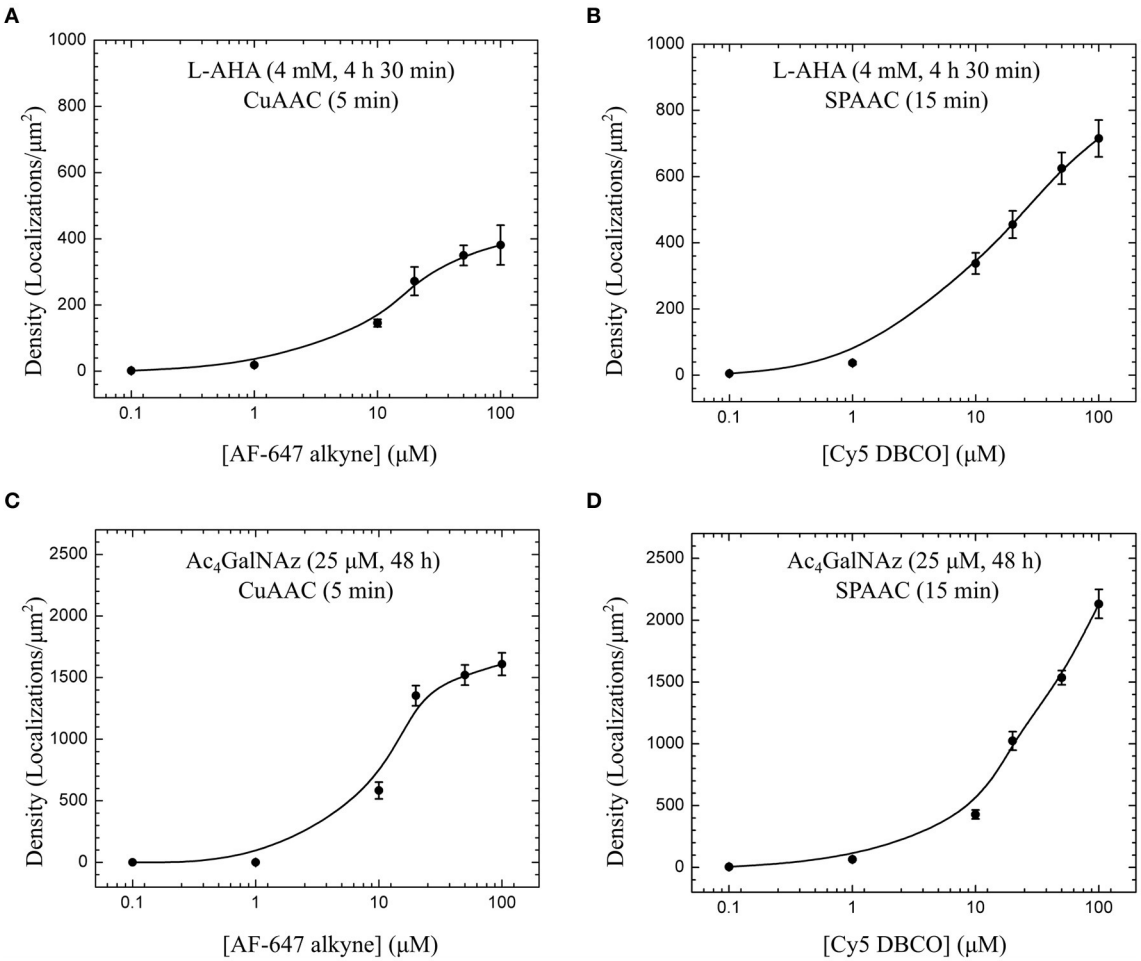

FIGURE 4 | Labeling efficiency of copper-catalyzed (CuAAC) and copper-free azide-alkyne cycloadditions (SPAAC). Fluorophore titration for the same metabolic labeling conditions, i.e., 4 mM L-AHA during 4 h 30 min **(A,B)**, and 25 μM Ac_4GalNAz during 48 h **(C,D)**, show optimal staining efficiency with AF-647 alkyne and Cy5 DBCO in the range of 20 to 50 μM for 5 min CuAAC and 15 min SPAAC reactions. For each cell, detected localizations were first obtained with a sliding window analysis (diameter = 1 μm, step = 100 nm) applied to big areas defined at bottom plasma membrane under the cell nucleus as described in **Figure 3B**. Plotted values and error bars represent median and SE of several cells imaged and analyzed for each fluorophore concentration [**(A)** 7–10 cells, **(B)** 8–15 cells, **(C)** 7–8 cells, and **(D)** 12–16 cells].

counting molecular numbers with localization microscopy requires further correction for multiple detections of the same molecule. The typical number of localizations recorded per fluorophore under the same optical and chemical conditions can be determined in diluted samples (**Figure 5**). If the blinking of isolated spots can be unequivocally assigned to single fluorophores, a conversion factor can be extracted to estimate the detected number of labeled membrane proteins (**Table 1**). For example, we estimate the density of PM proteins labeled with AHA during 4 h 30 min to be approximately ~50 μm^{-2} and ~125 μm^{-2} when stained via CuAAC and SPAAC respectively. On the other hand, we detected higher densities of glycans, in the range of ~345 μm^{-2} and ~280 μm^{-2}, metabolic labeled with Ac_4GalNAz during 48 h. It is important to mention that dividing the number of localizations in a region of interest by the average number of localizations detected per isolated fluorophore in reference experiments represents only an average correction value. To prevent over-counting effects in highly dense sample areas, more sophisticated methods based on the temporal and spatial fingerprint of single fluorophore blinking, such as off-time gap (Zhao et al., 2014) and pair correlation function analysis (PCF) (Veatch et al., 2012; Sengupta et al., 2013), can be applied.

Beyond density determination, coordinate lists obtained by localization microscopy can be used advantageously to inspect spatial distributions of membrane proteins. Analysis

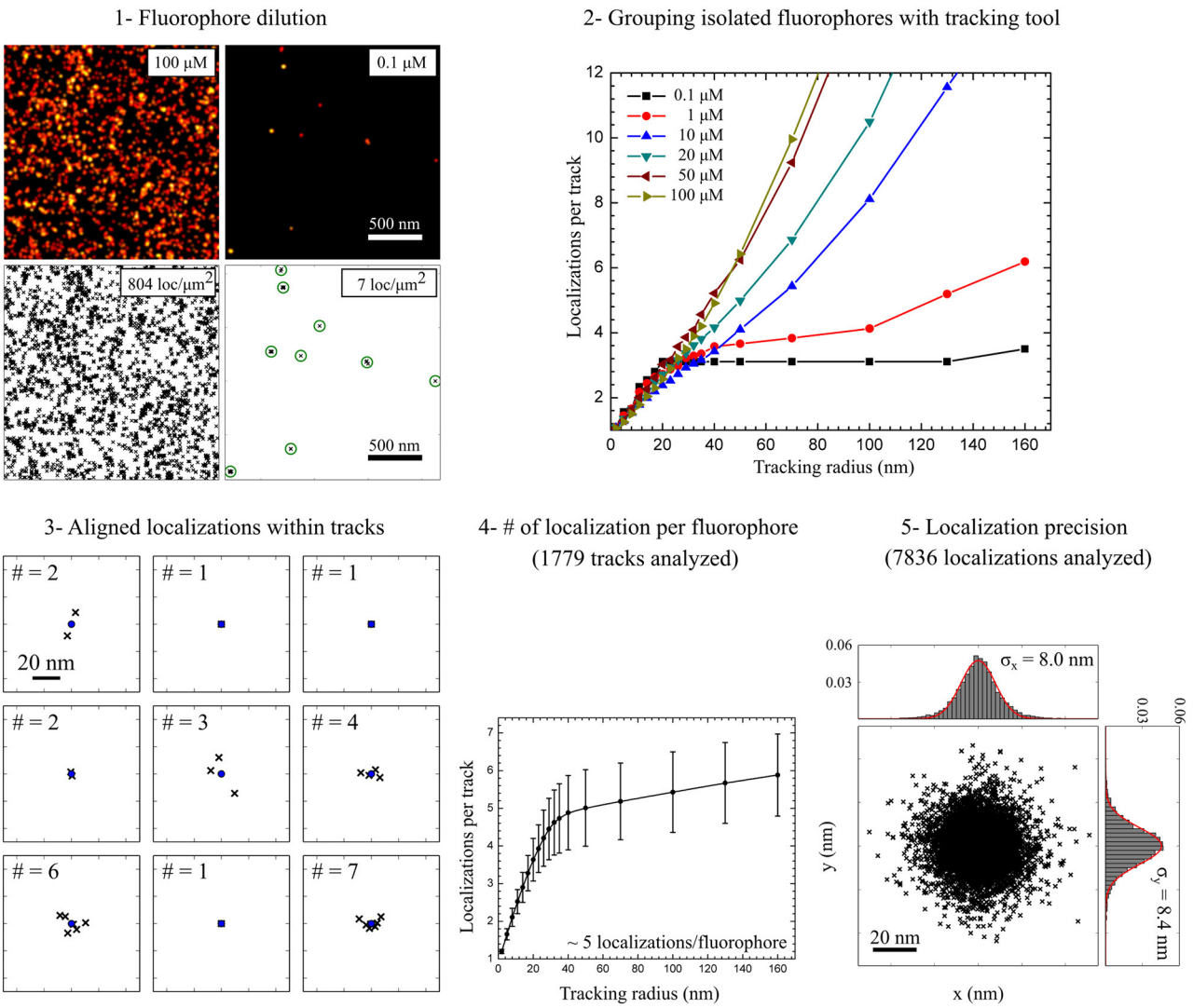

FIGURE 5 | Estimation of molecular densities and experimental localization precision. (1) Fluorophore dilution (<0.1 µM) leads to very low localization densities (<20 localizations per μm^2) allowing the detection of well isolated fluorophores. (2) Grouping localizations from isolated fluorophores was performed with a tracking algorithm. To confirm the detection of isolated fluorophores the tracking radius was varied from 1 to 160 nm for different fluorophore concentrations. (3) Localizations within tracks detected using a tracking radius = 50 nm aligned to the center of mass of each track. (4) For diluted samples the saturation level (tracking radius = 50 nm) indicates the number of localization per track, i.e., the number of localizations per isolated fluorophore. (5) Aligned localizations are used to estimate the experimental localization precision by fitting X and Y projections of the probability density function to a Gauss function.

TABLE 1 | Quantification of molecular density and experimental localization precision.

	Localization density[a] (loc/μm^2)	Conversion factor[b] (loc/fluorophore)	Molecular density (fluorophore/μm^2)[c]	σ_x (nm)[d] σ_y (nm)[e]
AHA (CuAAC)	350 ± 30	6.7 ± 1.1	52 ± 13	8.7 ± 0.1
				8.9 ± 0.1
AHA (SPAAC)	625 ± 48	5.0 ± 1.0	125 ± 35	8.0 ± 0.1
				8.4 ± 0.1
Ac_4GalNAz (CuAAC)	1520 ± 82	4.4 ± 1.4	345 ± 128	8.6 ± 0.1
				9.9 ± 0.1
Ac_4GalNAz (SPAAC)	1536 ± 58	5.5 ± 1.0	279 ± 61	8.2 ± 0.1
				8.4 ± 0.1

[a]Localization densities reflect median values calculated with a sliding window (diameter = 1 μm step = 100 nm) in regions under the cell nucleus to avoid overlapping membranes as shown in ***Figure 3****. Data presented correspond to 50 μM fluorophore concentration, i.e., AF-647 alkyne for 5 min CuACC staining and Cy5 DBCO for 15 min SPAAC staining. [b]Number of localizations per fluorophore obtained in diluted samples as described in* ***Figure 4*** *for 0.1 μM fluorophore concentrations. [c]Detected molecular densities calculated from localization densities divided by localizations per fluorophore. [d,e]Standard deviations obtained from Gauss function fits of the probability density functions calculated from aligned localizations as described in* ***Figure 4****.*

based on pair-correlation function (PCF) (Veatch et al., 2012; Sengupta et al., 2013) or nearest-neighbor based algorithms (including *Ripley's K* function) (Owen et al., 2012) can indicate weather proteins are more aggregated forming clusters or more dispersed than they were under a distribution of complete spatial randomness. All analysis routines need to take into account local self-clustering induced by single fluorophore blinking. Moreover, quantitative estimation of cluster size and densities can be difficult to extract without prior biological knowledge (Coltharp et al., 2014). Nevertheless, comparison with simulated spatial distributions mimicking experimental data can alleviate these problems and avoid miss-interpretations (Kiskowski et al., 2009; Veatch et al., 2012; Letschert et al., 2014). Finally, clustering algorithms, such as K-Means, DBSCAN, and polygon-based tessellation methods, have been used for morphological analysis of membrane proteins (Bar-On et al., 2012; Ehmann et al., 2014; Löschberger et al., 2014; Levet et al., 2015; Andronov et al., 2016). In contrast to pair-correlation and nearest-neighbor based algorithms, these methods rely on segmentation of the super-resolution image and thus the size and shape of each cluster, as well as their XY position, can be directly visualized.

To characterize the spatial distribution of PM components, we calculated Ripley's h functions from experimental data and two different sets of simulated spatial patterns. In particular, we simulated XY coordinates according to (i) a Poisson process and (ii) a Neyman-Scott process within 5 × 5 μm^2 with similar density as the number of localizations per μm^2 obtained from *d*STORM images. Whereas, a Poisson process resembles complete spatial randomness, it lacks to mimic individual fluorophore blinking inherent to *d*STORM measurements. In contrast, data sets simulated according to the Neyman-Scott process (Neyman and Scott, 1952) account photoswitching cycles from single fluorophores by including Gauss distributed offspring events around each parent position. Number of the offspring events and the standard deviation of the Gauss distribution (σ) where set from experimental data, i.e., on average ∼5 blinks per fluorophore and experimental localization precision ∼8 nm, respectively.

Ripley's k function reveals possible combinations of homogeneous distributions on large scales and clustering on small scales (e.g., due to the repeated blinking of individual labels). **Figure 6** shows direct comparison between experimental (blue line) and simulated data for a Poisson and Neyman-Scott process (black and red line respectively). For all the labeling schemes inspected, our data showed maximum clustering on a length scale similar to the estimated localization precision (i.e., d ∼20–30 nm). Therefore, clustering might reflect single fluorophore photoswitching. Since the maximum value of Ripley's h function for a simulated Neyman-Scott process is close to that of experimental data, we conclude that single fluorophore blinking is the only significant clustering process on this length scale. In addition, all the data indicate a small but significant deviation from complete spatial randomness on length scales from 30 to 800 nm. It is important to note that there is no characteristic length scale above 30 nm for any clusters of a well-defined size that can be identified. The indicated deviations from complete spatial randomness can have their origin in the various PM deformations e.g., due to the onset of vesicle formation or membrane ruffling. Whereas, it is possible to find small areas with a distribution that perfectly resemble a Neyman-Scott process (with clusters originating only from single emitter blinking), Ripley's h function for data in areas of 5 × 5 μm^2 in well-labeled cells under the nucleus (excluding double membrane contributions) typically appear as presented.

CONCLUSIONS AND REMARKS

We report a chemical reporter strategy, based on metabolic labeling and click chemistry, in combination with super-resolution imaging by *d*STORM to stain and visualize PM proteins and glycans. The labeling methodology results in staining efficiencies ranging from ∼50 to ∼350 fluorophore per μm^2 depending on the labeling scheme used. Besides the estimation of PM protein content, our data show potential

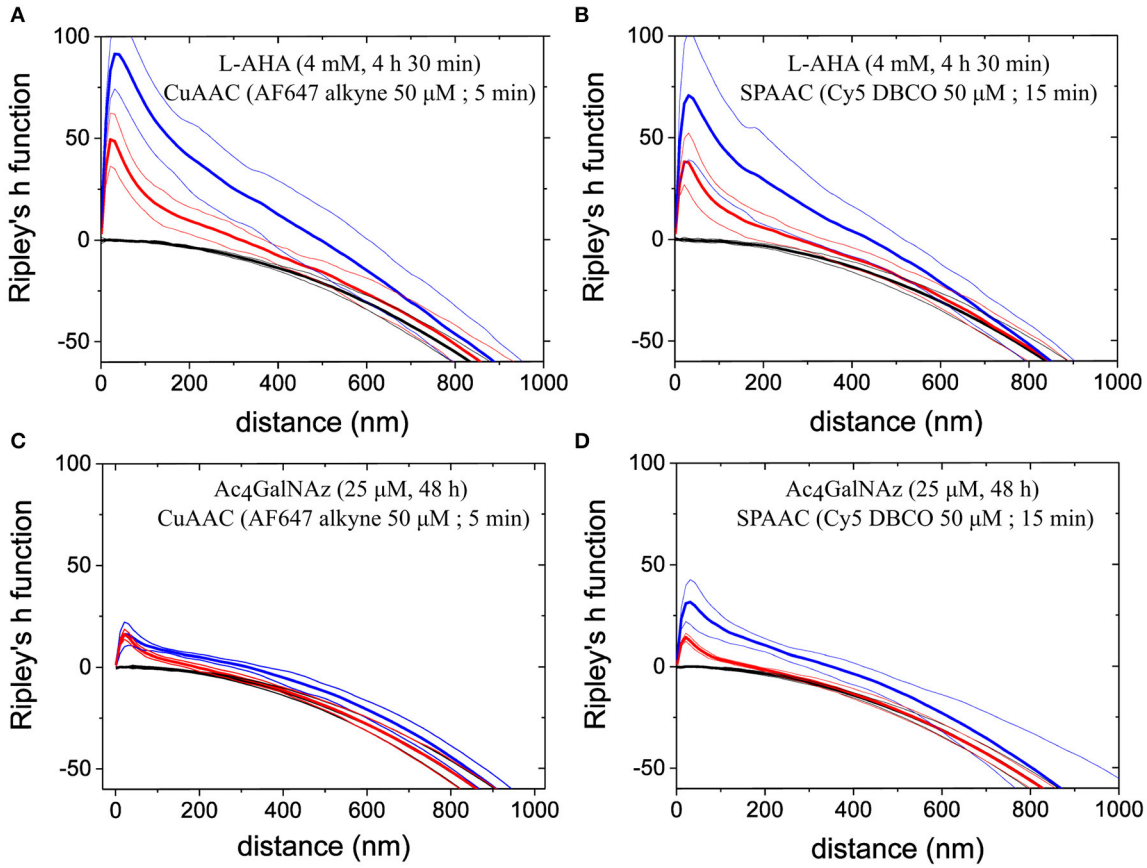

FIGURE 6 | Spatial distribution analysis by Ripley's h function. The data show Ripley's h functions computed from experimental data (blue lines) of PM proteins stained via **(A)** L-AHA (CuAAC), **(B)** L-AHA (SPAAC), **(C)** Ac_4GalNAz (CuAAC), and **(D)** Ac_4GalNAz (SPAAC). Plotted curves represent mean values (thick lines) together with 95% confidence intervals (thin) over 5 regions in total ($5 \times 5\ \mu m^2$ size) from independent cells, which appeared rather homogeneous by visual inspection. For comparison Ripley's function was computed from two simulated random point process, i.e., Neyman-Scott process (red lines) and Poisson point process (black lines). Simulation parameters, such as process intensity, average of offspring events, and spatial distribution around their parent event, where chosen to mimic localization density, photoswitching cycles, and localization precision obtained experimentally. The peak observed on short length scales for Neyman-Scott process and experimental data indicates artificial clustering due to repeated localizations from identical fluorophores within a Gauss distributions equal to localization precision, i.e., standard deviation ~8 nm. For all four staining schemes presented, Ripley's h functions show further clustering on longer length scales but more pronounced for L-AHA samples.

artifacts in super-resolution images due to 2D-projections of 3D-inherent cell structures. For example, overlapping membranes lead to overestimation of protein content, and vesicle-like structures located in closed proximity to the cell membrane appear as protein clusters and, thus, can potentially result in false interpretation of PM organization. Consecutive imaging with slightly shifted focal planes below and above the structure of interest can be used to reveal the contribution of 3D structures as two-dimensional projections. Furthermore, statistical analysis based on Ripley's function combined with point pattern simulations, can be used to identify deviations from complete spatial randomness. Our data clearly show artificial clustering due to fluorophore photoswitching at length scales related to the experimental localization precision (i.e., ~20–30 nm). Ripley's analysis also indicates a small deviation from spatial randomness at larger scales (e.g., ~30–800 nm). However, whereas these deviations from randomness might reflect some spatial organization of PM proteins at the nanoscale, their origin due to membrane modulations and ruffles, or the onset of vesicle formation cannot be completely excluded.

Finally, the examples presented here where performed at fixed metabolic conditions to incorporate azide groups in newly synthesized proteins. Experimental designs varying concentration and incubation time of metabolic surrogates combined with drug treatments can be used to study how fast proteins are delivered and trafficked from the cytosol to the plasma membrane. Reversibly, proteins can be followed after live cell staining to study membrane turnover involving different endocytic pathways. All in all, click chemistry constitutes a powerful tool to study PM composition at the molecular level as well as its dynamic organization. Moreover, the synthesis of new bioorthogonal molecules as well as their commercial availability will expand the applicability and usability of this methodology.

AUTHOR CONTRIBUTIONS

PM and MS designed the experiments. PM and SL performed the experiments. PM and SD analyzed the data. All authors discussed results and contributed to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00098

Figure S1 | Click chemistry staining specificity. (A) To evaluate non-specific signal, control cells were incubated with AHA in the presence of 40 μM anisomycin, a protein synthesis inhibitor, and subsequently stained via CuAAC or SPAAC with 50 μM of Alexa Fluor 647 alkyne for 5 min or Cy5 DBCO for 15 min respectively. **(B)** In the case of azido sugar, control cells were incubated in absence of Ac_4GalNAz and subsequently stained via CuAAC or SPAAC with 20 μM of AF 647 alkyne for 5 min or Cy5 DBCO for 15 min respectively. All controls showed relatively low background of ∼19, 42, 10, and 20 localizations per μm^2 for L-AHA (CuAAC), L-AHA (SPAAC), Ac_4GalNAz (CuAAC), and Ac_4GalNAz (SPAAC) respectively. Values and error bars represent median and SE of localization densities obtained with sliding window analysis under the nucleus ($N = 7$ cells in all cases).

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MINI REVIEW
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Budding Yeast: An Ideal Backdrop for *In vivo* Lipid Biochemistry

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Biological membranes are non-covalent assembly of lipids and proteins. Lipids play critical role in determining membrane physical properties and regulate the function of membrane associated proteins. Budding yeast *Saccharomyces cerevisiae* offers an exceptional advantage to understand the lipid-protein interactions since lipid metabolism and homeostasis are relatively simple and well characterized as compared to other eukaryotes. In addition, a vast array of genetic and cell biological tools are available to determine and understand the role of a particular lipid in various lipid metabolic disorders. Budding yeast has been instrumental in delineating mechanisms related to lipid metabolism, trafficking and their localization in different subcellular compartments at various cell cycle stages. Further, availability of tools and enormous potential for the development of useful reagents and novel technologies to localize a particular lipid in different subcellular compartments in yeast makes it a formidable system to carry out lipid biology. Taken together, yeast provides an outstanding backdrop to characterize lipid metabolic changes under various physiological conditions.

Keywords: budding yeast, lipid-protein interactions, lipid sensors, sphingolipid, ergosterol

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INTRODUCTION

Plasma membrane outlines the boundary of a living cell by separating it from the environment, thus provide it protection and identity. Compromising membrane integrity adversely affects the cellular function leading to release of calcium and local accumulation of vesicles (Krause et al., 1994). It has been proposed that increased membrane tearing results in cell death due to overwhelming repair process (Petrof et al., 1993; McNeil and Steinhardt, 1997). Plasma membrane acts as a selective barrier as well as means of communication with extracellular environment through signal transduction in a cell (Harder, 2012; Astro and de Curtis, 2015). In addition, membranes compartmentalize eukaryotic cell into different subcellular structures and act as scaffold for certain enzymatic reactions that allow reactions to be spatially confined inside a cell and 3-D cytosol to 2D membrane (Dislich and Lichtenthaler, 2012), respectively.

Biological membranes are non-covalent assembly of phospholipids, sterols and proteins. About 20–30% of eukaryotic genome has been estimated to encode for the membrane proteins (Krogh et al., 2001; Almen et al., 2009). Phospholipid species could be categorized into thousands types in eukaryotic cells based on their head group, acyl chain length and number and position of double bonds in it (Fahy et al., 2009). Because of their small size and hydrophobicity, lipids exhibit constant lateral and transverse movements in membrane bilayer providing it fluid-like characteristics whereas mobility of membrane proteins is somewhat restricted. Membrane proteins transduce information across the bilayer, thus establish communication to external environment. Transmembrane domain of proteins are embedded in membrane and thus interact with lipids in

bilayer. Lipids have been shown to regulate function of different membrane associated proteins either directly through modulating their functions (Contreras et al., 2011; Laganowsky et al., 2014) or indirectly by altering physical properties of membrane bilayer (Lee, 2004; Lundbaek et al., 2010). Numerous membrane proteins have been found to exhibit specificity toward certain lipid for their organization and function (Contreras et al., 2011; Laganowsky et al., 2014). A detailed information of lipid environment around a protein is therefore required to understand the function of cell membranes and membrane proteins (Coskun and Simons, 2011).

Lipids and proteins self-assemble through non-covalent interactions in biological membranes where certain lipids such as cholesterol and sphingolipids are known to exhibit higher mutual affinity leading to ordered domain formation termed as "lipid raft." Such membrane domains are believed to be important for various cellular processes such as signal transduction, membrane trafficking in mammalian cells (Simons and Ikonen, 1997; Simons and Toomre, 2000; Simons and Vaz, 2004; Simons and Sampaio, 2011). In order to understand the organization of lipids and proteins and lipids' structural specificity in determining protein function in such domains, model membranes such as small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), giant unilamellar vesicles (GUVs), supported lipid bilayers (SLBs) and biochemical approaches were employed (Smith, 2012; Zhao and Lappalainen, 2012; Lagny and Bassereau, 2015). However, studies in these systems are challenging due to limited success in membrane protein purification and their poor reconstitution in desired lipid environment (Seddon et al., 2004). Moreover, they do not provide systems-level understanding of highly-complex biological membranes owing to presence of limited diversity in lipid composition.

Biochemical approaches were invented to understand the organization and distribution of lipids and proteins in biological membranes based on differential detergent solubility (Brown and Rose, 1992; Lichtenberg et al., 2005) and differential fractionation on sucrose density gradient (Yao et al., 2009) of membrane domains. Detergent resistant membranes (DRMs) were speculated to be equivalent to "lipid raft." However, lipid-protein composition of such domains varied depending on the method of isolation (Lichtenberg et al., 2005; Babiychuk and Draeger, 2006; Williamson et al., 2010). In fact, detergents itself were shown to induce domain formation rather isolation of naturally existing membrane domains (Heerklotz, 2002). Knowledge gained from these systems therefore remain dubious and do not provide the real picture of lipid-protein distribution and function in a cell. To gain systems-level understanding of the aforementioned issue, a biological system, carrying adequate complexity, yet amenable for lipid composition manipulation is required.

YEAST: AN IDEAL SYSTEM FOR LIPID-PROTEIN INTERACTIONS

Budding yeast *Saccharomyces cerevisiae* is a powerful and convenient model organism for research in cell and membrane biology. It offers a unique advantage to understand the lipid-protein interactions due to availability of a vast array of genetic and cell biological tools. *S. cerevisiae* is genetically tractable and has benefitted almost every discipline of biology in general and cell biology in particular. A large collection of tools e.g., genome-wide yeast strain libraries carrying open reading frame (ORF) deletions (Winzeler et al., 1999; Giaever et al., 2002), genes tagged with high-affinity epitope for biochemical protein purification (Puig et al., 2001; Ghaemmaghami et al., 2003) or GFP (Huh et al., 2003) are available for budding yeast. In addition, synthetic genetic array (SGA) strategies (Baryshnikova et al., 2010a,b; Costanzo et al., 2010; Wagih et al., 2013; Chong et al., 2015), allow to study potential genetic interactions among genes in different pathways. These genomic collections are very useful for characterization of genes and proteins involved in lipid metabolism. Importantly, genome of *S. cerevisiae* is annotated thoroughly (Goffeau et al., 1996) that allows the identification of gene/protein homologs in human and other eukaryotes (Zhang and Bilsland, 2011), therefore enabling the application of knowledge gained in yeast to higher mammals including human.

As a model system, yeast offers several additional advantages for comprehensive understanding of lipid biology. Yeast can be cultured in completely defined media under simple and controlled growth conditions allowing an accurate interpretation of lipid associated phenotype as opposed to mammalian cells which are generally grown in serum containing medium. Serum is the rich source of lipids and fatty acids besides growth factors and other nutrients, therefore interpretation of lipid associated defects is difficult in mammalian cells under such conditions. Importantly, lipid metabolic pathways are well conserved between yeast and other eukaryotes (Lykidis, 2007; Hannich et al., 2011). Yeast has relatively simple repertoire of lipids in the range of several hundred (Guan and Wenk, 2006; Ejsing et al., 2009) compared to thousands of lipid species in mammalian cells (Yetukuri et al., 2008; Sampaio et al., 2011). Taken together, robust information can be generated in greater detail in budding yeast in a relatively short span of time due to its shorter doubling time, simple lipid metabolic pathways and well characterized genome.

LIPID HOMEOSTASIS IN YEAST

Biosynthesis and metabolism of glycerophospholipids, sphingolipid, and sterols in yeast have been discussed extensively in literature (Dickson, 2008; Carman and Han, 2009; Hannich et al., 2011). Lipid metabolism pathways in yeast are simpler as compared to mammalian cells, given the higher number of genes with multiple paralogs as suggested by complexity of mammalian lipidome (Quehenberger and Dennis, 2011; Sampaio et al., 2011) yet core lipid biosynthetic pathways are conserved from yeast to human (Kurat et al., 2006; Nielsen, 2009). Yeast has been instrumental in the discovery and characterization of many genes involved in lipid metabolism. Yeast deletion collections has been employed in number of high-throughput screens to investigate the phenotype of gene deletion and its interactions with other genes in lipid metabolism. For example, systematic analysis of yeast strains revealed genes that cause defect in lipid

metabolism (Daum et al., 1999). In addition, a genome wide screen helped reveal the role of sphingolipids and ergosterol to cell surface delivery, identification of inositol auxotrophic phenotypes (Hancock et al., 2006; Villa-Garcia et al., 2011) and genes responsible for lipid droplet formation (Szymanski et al., 2007; Fei et al., 2008; Bozaquel-Morais et al., 2010). In order to gain insights about the spatial localization of lipid biosynthesis, green fluorescent protein (GFP) collection of yeast strains was harnessed. By surveying localization of GFP tagged enzymes of lipid biosynthesis, it was observed that ER is the main organelle for lipid synthesis. In addition, significant number of these enzymes were observed in mitochondria, Golgi vacuoles, and vesicles (Natter et al., 2005). Genetic approaches have tremendously helped discovery and functional characterization of genes involved in lipid metabolism.

ORGANIZATION AND DYNAMICS OF LIPIDS AND PROTEINS IN YEAST PLASMA MEMBRANE

Plasma membrane in mammalian cell usually contains, ∼30–40% cholesterol and ∼10–20% sphingolipid of total plasma membrane lipids (Lange et al., 1989; van Meer, 1989). Budding yeast does not have cholesterol and sphingomyelin instead contains inositol phosphoceramide (IPC) and ergosterol, an equivalent of mammalian sphingolipid and cholesterol (Montefusco et al., 2013, 2014; Aguilera-Romero et al., 2014). As mentioned earlier, cholesterol and sphingolipids are known to form ordered domains which are believed to be important for various cellular processes (Simons and Ikonen, 1997; Simons and Toomre, 2000; Simons and Vaz, 2004; Simons and Sampaio, 2011). Similar domains are observed in budding yeast where they are enriched in ergosterol and complex sphingolipids (Kubler et al., 1996; Bagnat et al., 2000) including IPC, mannose-inositol-phosphoceramide (MIPC), and mannose (inositol phosphate) 2-ceramide (M(IP)2C) (Dickson et al., 2006; Dickson, 2008). Such ordered domains are also known as membrane compartment of Can1 (MCC) and membrane compartment of Pma1 (MCP, Malinska et al., 2004; Grossmann et al., 2007), eisosomes (Walther et al., 2006) in yeast. Later studies revealed that yeast plasma membrane is rather domainized (Spira et al., 2012) probably due to inherent slow diffusion of lipids (Greenberg and Axelrod, 1993) and proteins (Valdez-Taubas and Pelham, 2003) in yeast plasma membrane. Interestingly, yeast plasma membrane were observed to slow down the lateral diffusion of heterologous expressed human serotonin$_{1A}$ receptor as compared to that in mammalian cells (Ganguly et al., 2009).

LIPID VISUALIZATION METHODS

Research of decades has enhanced our understanding about lipids' functions and establish them as active membrane components (Watkins et al., 2011). Presence of specialized membrane domains such as lipid rafts are proposed to be hub for cellular signaling, membrane sorting, and endocytosis reviewed in (Simons and Ikonen, 1997; Simons and Toomre, 2000; Simons and Vaz, 2004; Simons and Sampaio, 2011). However, existence of lipid rafts still remains a matter of debate in cell biology. In addition, organization and dynamics of lipids in membranes of different subcellular structures have not been probed accurately. Interestingly, dynamics of lipid metabolism is altered during cell cycle progression in mammalian fibroblast cells (Singh et al., 2013) and aging (Choi et al., 2015). For example, about 40% increase was observed in cholesterol content in rat fibroblast cells (Singh et al., 2013) while sphingolipid levels are dysregulated during aging rats and mice (Sacket et al., 2009; Babenko and Shakhova, 2014; Mc Auley and Mooney, 2015).

Visualization of lipids in native environment has been challenging due to limited availability of appropriate probes to recognize naturally occurring lipids in live cell. Novel tools are being developed to investigate the localization and dynamics of lipids in different subcellular compartments in live cell (Maekawa and Fairn, 2014). Based on their mode of incorporation in cell membrane, lipid probes can be categorized as exogenous and endogenous. Exogenous lipid probes are fluorescently tagged lipid analogs, antibodies and lipid binding protein domains that get incorporated in cell membrane upon exogenous supplementation. Lipid probes used to study lipid domains comprise analogs of sterols such as cholestatrienol (Nystrom et al., 2010), dehydroergosterol (DHE) and 25-NBD-cholesterol (Wustner, 2007), phospholipids (Eggeling et al., 2009) and fluorescent tagged proteins in mammalian cells (Wustner, 2007). High resolution microscopy using fluorescence correlation with fluorescently labeled lipids has demonstrated that lipid diffusion is restricted in certain domains of the plasma membrane (Eggeling et al., 2009). Exogenous lipid probes are useful and get readily incorporated in the membrane, but may not be a good mimic of naturally occurring lipids, as observed for the cholesterol fluorescent analog dehydroergosterol (DHE) and 25-NBD-cholesterol. DHE preferentially localizes in liquid ordered domain whereas 25-NBD-cholesterol majorly partitions in disordered domains (Wustner, 2007).

Lipid-binding proteins, such as lysenin (Ishitsuka and Kobayashi, 2004), cholera toxin (Heyningen, 1974), S.V. equinatoxin (Barlic et al., 2004; Yachi et al., 2012) have been useful in studying membrane domains in plasma membrane. These motifs are part of different amphitropic proteins where they help proteins to associate with membranes by binding to unique lipid. Lipid binding toxins need to be improved and evolved to circumvent their harmful effects as they are shown to kill cells by forming pore in plasma membrane. Antibody against lipids is an effective novel approach to visualize lipids in live cell. For example, antibodies against the lyso (bis) phosphatidic acid (LBPA, Kobayashi et al., 1998) phosphatidylglucoside (Murate et al., 2010), ceramides (Cowart et al., 2002), and even an antibody that recognizes ceramide/cholesterol enriched domain have been described (Scheffer et al., 2006). However, generating antibody of high specificity against a lipid is challenging due to their poor antigenicity and highly similar structures.

Further, presence of cell wall around yeast poses a limitation for the usefulness of exogenous lipid probes as opposed to higher eukaryotes. Labeling of plasma membrane can be probably

achieved by shaving off cell wall enzymatically. However, it might affect the organization of lipids and proteins in plasma membrane as cell wall interacts with plasma membrane through embedded proteins in it. Importantly, cells are fixed to freeze distribution of lipids and protein and later labeled with fluorescent tagged lipid binding antibodies and toxins. However, fixation methods like formaldehyde and glutaraldehyde does not entirely preserve the localization of integral proteins and lipids (Hammond et al., 2009; Tanaka et al., 2010). This creates a concern for their applicability in determining proper localization of lipids in a cell.

Endogenous probes are genetically encoded fluorescent protein domains of amphitropic protein and toxins that specifically bind to a lipid. They can access lipids in plasma membrane as well as in membranes of subcellular structures therefore provide information about localization and dynamics of different lipids. These probes can be expressed as an epitope-tagged fusion to allow for lipid visualization using a plasmid-based biosensor. Examples of this strategy include the use of the pleckstrin homology (PH) domain from phospholipase C for phosphatidylinositol 4,5-bisphosphate (PIP2) and the Lact-C2 domain for phosphatidylserine (PS) in *S. cerevisiae* (Fairn et al., 2011; Das et al., 2012). In particular, these probes have been successfully used to monitor the localization and distribution of PS and PIP2 in *S. cerevisiae* where PS gets concentrated at polar cortex during cell polarization (Das et al., 2012) NCB whereas distribution of PIP2 remains uniform all over the cell cortex. Endogenous lipid probes provide advantage over exogenous probe as no staining procedure is required. In addition, localization of lipids could be fixed using formaldehyde under these conditions as that can fix protein domain of these probes. However, endogenous lipid probes recognize "free" lipids and may sequester these lipids if overexpressed in a cell thus making lipid molecule unavailable for their physiological function. Budding yeast offers tremendous potential for the development of these probes because of its facile genetics. Employing yeast, new tools are being developed to probe the distribution of native lipids in the cells by developing specific antibodies against different lipids. For example, attempts are in place to develop bicyclic peptides to specifically bind sphingolipids (Heinis et al., 2009; Takahashi-Umebayashi et al., 2011). A systematic screen was targeted to reveal lipid-protein interactions in *S. cerevisiae* (Gallego et al., 2010). For which, nitrocellulose arrays containing different sets of lipids were used to determine the binding profiles of different soluble proteins. They reported several novel surprising interactions indicating that there is a still huge gap in our understanding of lipid-proteins interactions. Such studies provide a starting point for validation of these interactions with newly developed tools.

UNIQUE REAGENTS IN YEAST FOR LIPID-PROTEIN INTERACTIONS

In addition to number of available libraries, novel strategies and tools have been developed to study structural importance of different lipids by tweaking structure of lipids through metabolic engineering in yeast. For example, acyl chain remodeling of phospholipids in cardiolipin, phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine can be achieved by deletion and overexpression of certain enzymes in yeast reviewed in Renne et al. (2015). These strategies could help in understanding the function of acyl chain remodeling in yeast physiology such as growth, mating and aging. In addition, trafficking of a subset of yeast plasma membrane proteins is known to be dependent on phospholipid and sterol biosynthesis reviewed in Bankaitis et al. (2012). In this regard, engineered yeast strains that produce lipids carrying specific alterations would be useful tool. For example, yeast lipidome has ergosterol and sphingolipids as two major lipids which considerably differ from their equivalents in mammalian cells. Recently, strains have been engineered that produce sphingolipids of shorter chain C18 (Cerantola et al., 2007; Epstein et al., 2012) instead of C26 and cholesterol instead of ergosterol (Souza et al., 2011). These strains would be valuable tool to study the lipid specificity in trafficking and function of membrane proteins and would help in delineating the functional differences between cholesterol and ergosterol on yeast membrane proteins. Lipid homeostasis in yeast is maintained by lipid synthesis and lipid storage. Excess amount of lipids are stored in form of lipid droplets (LDs) thus LDs act as reservoir for membrane components and source of energy during adverse condition. Yeast strains have been constructed that are devoid of lipid storage (lipid droplets) (Sandager et al., 2002). They would therefore be an asset in gaining comprehensive understanding about the role of LDs in cell survival under various physiological and stress conditions and would provide mechanistic details about lipid homeostasis and metabolism.

CONCLUSION AND FUTURE PERSPECTIVE

Research in yeast have made important contributions to the study of lipid homeostasis and function, and provided significant insights into fundamental pathways in lipid metabolism that could be extended to more complex organisms (Nielsen, 2009). Employing genetic screens and quantification of lipids under different environmental conditions, yeast could help uncover many unsuspected and novel molecular interactions among proteins and lipids. Yeast has potential for the development of new technologies that could help us understand the lipid distribution, interaction and their involvement in biogenesis of different cellular structures and as signaling molecule in cellular signaling events as depicted in **Figure 1**. Integration of new technologies in budding yeast could help us understand the fundamental questions of aging and diseases. For example, microfluidic chips are being developed to follow the replicative life span in yeast (Zhang and Bilsland, 2011; Jo et al., 2015; Liu et al., 2015), localization of proteins and lipids in yeast could establish the link between dynamics of lipid metabolism with aging. In addition, lipid disorders observed in humans can be

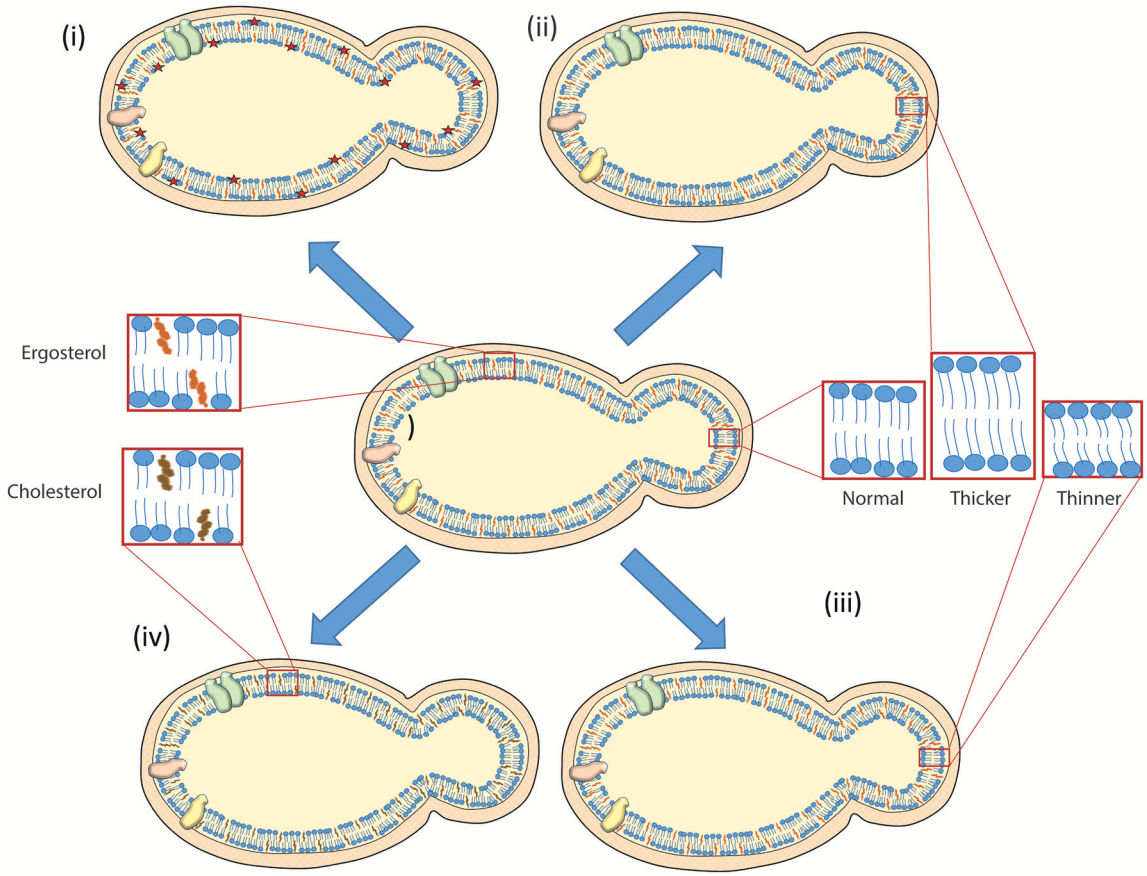

FIGURE 1 | Scheme depicting different lipid modulations achieved in budding yeast. A thick cell wall is present around the plasma membrane carrying proteins (shown in cyan, orange, and yellow just to distinguish them as different) in budding yeast. Cell wall and lipids are depicted in orange and blue color, respectively. A handful of examples are presented (i) visualizing lipids with development of fluorescent biosensors as red star (Fairn et al., 2011; Das et al., 2012) (ii) remodeling membrane to be thicker by acyl chain lengthening (Dickson et al., 1990; Gaigg et al., 2006; Renne et al., 2015) (iii) producing thinner membrane by acyl chain shortening (Cerantola et al., 2007; Epstein et al., 2012; Renne et al., 2015) (iv) converting ergosterol to cholesterol in budding yeast (Souza et al., 2011). See text for more details.

replicated in yeast to gain better and robust understanding about their molecular mechanisms that help in development of the treatment against such disorders. Yeast has been employed as model system to find cure for Parkinson's disease (Khurana and Lindquist, 2010). Taken together, yeast would be an ideal system for making advancement in these areas thus providing details regarding the localization of lipids in their native environment under different cellular processes, and enhancing our understanding about the lipid distribution, dynamics and trafficking under different environmental conditions.

AUTHOR CONTRIBUTIONS

PS collected the material and wrote it.

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