HHS Public Access

Author manuscript

Cancer Metastasis Rev. Author manuscript; available in PMC 2018 December 17.

Published in final edited form as:

Cancer Metastasis Rev. 2018 September ; 37(2-3): 519–544. doi:10.1007/s10555-018-9733-1.

Functional link between plasma membrane spatiotemporal dynamics, cancer biology, and dietary membrane-altering agents

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Abstract

The cell plasma membrane serves as a nexus integrating extra- and intracellular components, which together enable many of the fundamental cellular signaling processes that sustain life. In order to perform this key function, plasma membrane components assemble into well-defined domains exhibiting distinct biochemical and biophysical properties that modulate various signaling events. Dysregulation of these highly dynamic membrane domains can promote oncogenic signaling. Recently, it has been demonstrated that select membrane-targeted dietary bioactives (MTDBs) have the ability to remodel plasma membrane domains and subsequently reduce cancer risk. In this review, we focus on the importance of plasma membrane domain structural and signaling functionalities as well as how loss of membrane homeostasis can drive aberrant signaling. Additionally, we discuss the intricacies associated with the investigation of these membrane domain features and their associations with cancer biology. Lastly, we describe the current literature focusing on MTDBs, including mechanisms of chemoprevention and therapeutics in order to establish a functional link between these membrane-altering biomolecules, tuning of plasma membrane hierarchal organization, and their implications in cancer prevention.

Keywords

Ras; Wnt; n-3 PUFA; Membrane order; Nanoclustering; Membrane therapy; Cancer prevention

1 Introduction

The plasma membrane of cells acts as a barrier that controls the bidirectional movement of a plethora of molecules between the extracellular and cytosolic space of cells. This fundamental property affords protection to cells from their surroundings. In addition, the highly dynamic fluid-mosaic structure of the plasma membrane serves as a point of the first

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Conflict of interest The authors declare that they have no conflict of interest.

contact between cells and various signaling elements that allow cells to "talk to each other." Moreover, the plasma membrane is responsible for the compartmentalization of proteins, lipids, and their chemically modified counterparts into distinct assemblies that perform many of the fundamental cellular activities required for life (Fig. 1).

Highly relevant to the cancer biology field, it is now recognized that the geometry, biochemical composition, and biophysical characteristics of the plasma membrane are tightly associated with its signal processing capability [1–4]. According to this emerging picture, protein and lipid assemblies can be organized to form distinct micro- or nanodomains that facilitate signaling events [5–9]. The formation, organization, and membrane biophysical status, e.g., the degree of membrane rigidity, of these specialized signaling domains are modulated by cortical actin and the presence of specific lipids and proteins (Fig. 1) [10]. Currently, these domains are considered a predominant feature of the plasma membrane and appear to mediate critical signaling processes [11, 12], including the stabilization of β-catenin *via* stimulation of Wnt pathway-associated receptors [13–15], epidermal growth factor receptor (EGFR) signaling [16–18], and the activation of MAPK/ERK pathway components through membrane-bound Ras proteins [19–21], to name a few examples (Table 1). Interestingly, dysregulation of plasma membrane homeostasis, in part, due to the products of gene mutations as well as changes in protein and lipid localization, alters the degree of clustering and other biochemical and biophysical trademarks, thereby providing a suitable environment for the initiation of cancer-related signaling processes [22–24].

Currently, there is evidence indicating that cell *m*embranes can be "*t*argeted" by pleiotropic *d*ietary *bi*oactives (MTDBs), resulting in the remodeling of plasma membrane domains [25–27]. This suggests that select MTDBs could reduce cancer risk by attenuating oncogenic protein activity by modulating the membrane organization of essential proteins and lipids. Thus, MTDBs could potentially serve as chemoprotective agents for cancer therapy [28–30]. In this review, we describe the importance of the plasma membrane as a central cellular hub that integrates numerous cues to orchestrate the modulation of various signaling networks, which can become hyperactivated, resulting in tumorigenesis. In addition, we discuss currently available tools utilized to investigate these complexes and, sometimes, subtle processes. Lastly, we review the current literature focusing on dietary components that modulate membrane structure, including mechanisms of chemoprevention and therapeutics.

2 Plasma membrane micro- and nanodomain structure and organization

The plasma membrane is composed of a heterogeneous mixture of lipids, proteins, and carbohydrates, whose distinct organization is essential for its function [31, 32]. Lipids self-assemble into lipid bilayers, driven in great part by hydrophobic forces. Plasma membrane lipids can diffuse laterally *via* different modes and undergo phase separations to form non-homogenous nanoscopic domains (Fig. 2a, compartmentalization). Initial evidence supporting the concept of plasma membrane domain heterogeneity arose from observations that biological membranes can be separated into detergent-resistant and detergent-labile fractions [33, 34]. The presence, composition, and dynamics of these distinct plasma membrane compartments have been studied in great detail ever since. Several findings have

pointed to the existence of ordered or rigid (Lo) and disordered or fluid (Ld) phases in the plasma membrane [35–38]. It is widely accepted that the Lo phase, a highly condensed/ordered domain, is enriched in cholesterol and predominantly saturated sphingolipids, while the Ld phase, a relatively disordered domain, is enriched in unsaturated glycerophospholipids [39, 40]. From the existing body of evidence associated with these domains, the plasma membrane lipid raft model emerged [41, 42].

The current consensus describes lipid rafts as heterogeneous, highly dynamic domains that range from 10 to 200 nm in size [43]. These nanodomains are enriched in cholesterol, sphingolipids, glycolipids, and saturated glycerophospholipids as well as lipidated and glycosylphosphatidylinositol (GPI)-anchored proteins and have the ability to form higher order microdomains (> 300 nm) under specific conditions [44, 45]. The process that drives formation of the latter has been postulated to be dependent on selective lipid-lipid and protein-lipid interactions or clustering. Interestingly, although lipid rafts and raft-like domains appear small in size, they are believed to constitute a relatively large fraction of the plasma membrane, if not its majority [34, 44]. Importantly, lipid domain organization is cell type specific and variations between distinct cell types can be observed [44, 46]. The latter is driven by complex interactions between these membrane domains and the cytoskeleton as well as the relative concentrations of bioactive lipids in these domains. Plasma membrane ordered domains appear to have diverse lipid and protein compositions. Various different lipids and proteins have been shown to coalesce into specialized nanoscale proteolipid clusters (Table 1). Consequently, it has been proposed that the plasma membrane is not organized in a binary fashion, but instead contains numerous lipid raft and raft-like domains as well as non-raft domains displaying discrete compositions and features [34]. As an example, caveolin and monosialotetrahexosylganglioside (GM1), both which are considered components of rafts, are not always colocalized [47].

Highly organized domains are found both in the outer and inner leaflets of the plasma membrane (Fig. 1). In that respect, the plasma membrane is also heterogeneous across the lipid bilayer vertical axis. However, lipid raft and raft-like domains are not disconnected from one another but rather it has been postulated that they are coupled in these asymmetric lipid bilayers (Fig. 2a, transbilayer coupling) [48–50]. It has been previously shown that inner leaflet ordered domains can be induced by outer leaflet ordered domains in a reconstituted system. Additionally, experimental and simulation data have provided evidence that clustering of GPI-anchored proteins in the outer leaflet is affected by transbilayer coupling of phosphatidylserine (PS) in the inner leaflet containing a saturated fatty acyl chain [12]. Furthermore, there is evidence indicating that some raft components, e.g., cholesterol, which is found in different concentrations across lipid leaflets, can be modulated in a stimulus-dependent manner and support signal transduction in ordered domains [51].

Due to the great complexity of plasma membrane ordered domains, various lipid models and tools have been employed to study the factors affecting their formation and dynamics. Interestingly, due in great part to the controversies surrounding this field, the toolbox utilized to investigate these membrane domains has been consistently evolving since the initial lipid raft studies in the 1970s. As mentioned above, the first evidence of the existence of distinct domains in the plasma membrane arose from differential solubilization of these biological

structures using non-ionic detergents [33]. This allowed scientists to fractionate membranes by density centrifugation, revealing the presence of distinctive domains exhibiting different lipid-protein compositions. However, due to the high variation in the recovery of membrane components, which was partly influenced by differences in detergent types, concentration, and temperature employed during the assay, other more robust and reliable techniques have been the preferred methods of choice recently. For example, researchers studying these membrane domains have recently utilized super-resolution imaging technologies which provide improved size and temporal resolution. Experiments performed utilizing stochastic optical reconstruction microscopy (STORM), photo-activated localization microscopy (PALM), total internal reflection fluorescence microscopy (TIRFM), stimulated emission depletion (STED), immunoelectron microscopy (immuno-EM), and the combination of fluorescence lifetime imaging microscopy and Förster resonance energy transfer (FLIM-FRET) have revealed nanoscale details regarding plasma membrane morphology as well as lipid and protein composition and clustering [52–56]. Additionally, fluorescence recovery after photobleaching (FRAP) has provided evidence of proteins clustered in distinct noncholesterol-dependent signaling microdomains on the inner plasma membrane surface [56, 57]. Other techniques such as single-particle tracking (SPT) and fluorescence correlation spectroscopy (FCS) have evaluated the diffusion of membrane molecules over various length scales as well as other dynamic parameters [58–61]. Recent advances in high-resolution chemical-based imaging approaches such a nanoscale ion mass spectrometry (NanoSIMS) offer lipid resolution at the ~ 100-nm level [62–64]. Furthermore, time of flight secondary ion mass spectrometry (TOF-SIMS), which is based on event by event impacts of single clusters of particles, has achieved a resolution of ~ 10 nm [65–69]. These are very promising techniques that enhance the ability to detect co-localization of raft lipids in preserved human tissues [70]. Finally, electron microscopy (EM), owing to its high resolution, has allowed the study of the arrangement of various domain components in the outer and inner leaflet of the membrane bilayer [71, 72]. Altogether, these techniques have greatly advanced the study of plasma membrane domain organization.

Many imaging techniques rely primarily on membrane probes that can selectively distinguish/reveal distinct plasma membrane domains by exhibiting discrete emission spectra when localized in domains with different physicochemical properties, interacting with specific resident components or localizing within particular domains or by disrupting domain structure and function. Reporters that can selectively locate within rafts or bind to raft components include cholesterol-binding molecules such as filipin and the protein domain 4 of perfringolysin O; prototypical ganglioside-binding molecules such as cholera toxin B; sphingolipid reporters such as ostreolysin A, lysenin, and pleurotolysin; and raft and non-raft markers such as truncated Ras motifs and cyanine dyes (e.g., DiO, DiI, and DiD) [56, 73–79]. Most of these probes are detected utilizing fluorescence techniques. This raises some concerns since these probes are typically labeled with chromophores that are often close to the size of lipid molecules, which themselves could alter the natural state of the lipid environment under investigation. Fluorescent probe-free techniques such as mass spectrometry, Raman spectroscopy, and, as already mentioned, EM can be used to circumvent some of these issues. However, these techniques require cell fixation and longer acquisition times as well as a high degree of expertise and sophisticated equipment [73, 80,

81]. A different set of fluorescent ratiometric probes, which are sensitive to differences in physicochemical membrane properties and can distinguish between a spectrum of tightly packed raft and relatively fluid non-raft domains, include Laurdan and ANEP (e.g., di-4-ANEPPS, di-4-ANEPPDHQ, and di-8-ANEPPS) dyes. These dyes provide a fast, concentration-independent evaluation of the plasma membrane lipid environment [82–85]. On the other hand, these dyes suffer from varying rates of cell internalization accompanied by undesired staining of intracellular membranes [86]. Moreover, due to their relatively low brightness, some of these probes require high concentrations (e.g., 5 μM) for cellular imaging [87]. Finally, raft-targeting drugs have been utilized to study the various roles of membrane domains under a more focused functional context. A few examples include cholesterol-disrupting reagents such as methyl-β-cyclodextrin, mevastatin, and cholesterol oxidase and sphingolipid-disrupting molecules such as fumonisin B1, myoricin, and sphingomyelinase [88–91]. All of these molecules target essential components found in lipid rafts. However, in many cases, these molecules display pleiotropic effects beyond their intended application and require the use of high concentrations [92–96]. Moreover, these pharmacological agents often exhibit low selectivity and consequently suffer from off-target effects. Lastly, pharmacological targeting of cholesterol in the non-raft domain is possible [97]. Overall, the abovementioned reagents provide a valuable membrane-centric toolbox, capable of enhancing our understanding of plasma membrane domain structure, composition, dynamics, and function.

3 Dynamic assembly of plasma membrane signaling “hot spots”

Plasma membrane lipid rafts and raft-like domains are increasingly receiving attention as platforms that directly influence cell signaling pathways. These membrane domains are able to modulate signals received from the extracellular space, amplify these signals, multiplex them with other signals, and mediate dissemination to various cytoplasmic pathways in a two- or three-dimensional fashion. From a mechanistic perspective, an increasing body of evidence suggests that raft-like proteolipid domains coexist as a heterogeneous diversity of protein and lipid compositions in the plasma membrane (Fig. 1) [98, 99]. The organization and function of these domains are postulated to be highly dependent on membrane partitioning into distinct compartments, which in turn is driven by the actin membrane skeleton, as well as the oligomerization of proteins and lipids into large membrane-associated complexes also known as clusters (Fig. 2a, clustering). Currently, some of these domains are described as being highly dynamic, exhibiting short lifetimes (in the 1 ns–1 s range) which are thought to be stabilized and enlarged *via* protein-lipid clustering following stimulation by a ligand [100–102].

The non-random distribution of discrete organized domains exhibiting diverse biophysical and biochemical characteristics plays an essential role in modulating various signal transduction pathways (Fig. 2b). One relevant example is the canonical Wnt/β-catenin signaling pathway. Canonical Wnt/β-catenin signaling plays a central role in embryonic development as well as adult stem cell homeostasis and tumorigenesis [14, 103, 104]. This signaling pathway is initiated by the interactions between lipid-modified Wnt molecules and the integral plasma membrane receptors, low density lipoprotein receptor-related proteins 5/6 (LRP5/6), and Frizzled (Fz). Activation of these receptors by Wnt, which involves

receptor phosphorylation and endocytosis, leads to the stabilization, translocation, and accumulation of β-catenin in the nucleus. Once in the nucleus, the transcriptional co-activator nuclear β-catenin, together with transcription factors of the lymphoid enhancer-binding factor (Lef) and T cell factor (Tcf) family, will switch on various β-catenin related genes.

Recently, it has become apparent that components of the Wnt/β-catenin signaling pathway (Fig. 2b, Wnt signaling) form specialized nanoclusters in order to signal [13, 105–110]. These membrane clusters require the essential Wnt membrane receptors, LRP6 and Fz as well as the cytosolic proteins Dvl and Axin to form active clusters, i.e., Wnt signalosomes. Wnt/β-catenin pathway-related nanoclusters are small in size (< 100 nm) in the absence of Wnt ligands. However, Wnt ligands can induce nanoclustering and a shift in cluster size distribution. For example, in the presence of Wnt, large cluster patches containing LRP6 have been observed (> 200 nm). More recently, evidence from various studies suggests that select proteins, lipids, and even saccharides are involved in the formation of Wnt/β-catenin signaling-associated nanoclusters [14, 111–114]. The Wnt/β-catenin signaling pathway positive feedback regulator Ly6 family protein LY6/PLAUR domain-containing 6 (Lypd6) is required for LRP6 phosphorylation and activation in lipid rafts. This GPI-anchored protein, which preferentially localizes to lipid rafts, has been shown to form ~ 100 nm protein clusters [115, 116]. Interestingly, GPI-anchored proteins have been shown to trigger recruitment of kinases at the inner leaflet of the plasma membrane [117–119]. Additionally, casein kinase 1 gamma (Ck1γ), a kinase that phosphorylates LRP6, has been shown to be primarily present in lipid raft domains [120, 121]. Thus, clustering of Lypd6 might play an important regulatory role in the recruitment of Ck1γ and other Wnt-associated kinases, phosphorylation of Wnt receptors, and stabilization of active Wnt-related clusters in lipid rafts. Similarly, other plasma membrane components such as heparin sulfate (HS), cholesterol, and phosphatidylinositol-4,5-bisphosphate (PIP_2) might also play a role in Wnt/β-catenin pathway-related nanoclustering. Two types of HS molecules, “N-sulfo-rich HS” (N-sulfo HS) and “N-acetyl-rich HS” (N-acetyl HS), have been shown to form distinct 200-nm clusters, as measured by STED microscopy [114]. Interestingly, N-sulfo HS, not N-acetyl-HS, preferably colocalize with the Wnt receptors Fz and LRP6 as well as LRP6’s active counterpart, phosphor-LRP6 [114]. N-sulfo HS clusters have also been shown to colocalize with Wnt ligands inside cells and have been postulated to function as pre-existing cores for signalosome formation [122]. Finally, the multifunctional lipid messenger, PIP_2, whose synthesis is induced by Wnt stimulation, has been shown to be required for LRP6 aggregation, recruitment of Axin, and other effector proteins to the membrane inner leaflet, resulting in the formation of signalosomes [14, 123]. Thus, it is evident that multiple plasma membrane components such as lipids, proteins, carbohydrates, and the membrane cytoskeleton influence, in a highly dynamic yet precise manner, the plasma membrane microenvironment and organize Wnt signaling events into distinct domain platforms. These highly compartmentalized and diverse membrane domains, in turn, meticulously modulate numerous crucial cellular signaling events.

Ras nanoclustering in the plasma membrane has also been well documented. These nanoclusters are ~ 9 nm in radius, contain ~ 6–7 Ras proteins per cluster, display a rapid turnover and short lifetime (0.1–1 s), and contain ~ 40% of the total Ras proteins [124–126].

Interestingly, nanoclusters of various Ras isoforms contain distinct lipid compositions. For example, studies have shown that HRas-GDP nanoclusters reside in cholesterol-rich domains, while HRas-GTP clusters form in a cholesterol-independent manner [127–129]. In comparison, GDP-bound and GTP-bound KRas both form discrete nanoclusters independently of cholesterol [56, 124, 125]. Similarly, other lipids, such as PIP_2, PS, and phosphatidic acid (PA), have been shown to distribute differently in certain types of Ras clusters [124, 130, 131].

With respect to membrane order, Wnt/β-catenin signaling transduction is thought to initiate from raft domains [132] and is modulated by various key lipid raft components (Fig. 2b, Wnt signaling). Early evidence suggested that caveolin, a main component of the raft subdomain known as caveolae, is necessary for the cellular internalization of active Wnt receptors and stabilization of nuclear β-catenin [133]. The localization of Wnt-associated receptors in raft domains is also believed to be essential for signal transduction and activation of downstream effectors. For example, Dickkopf 1 (Dkk1), a Wnt/β-catenin pathway antagonist, has been shown to inhibit Wnt/β-catenin signaling by removing LRP5/6 from lipid rafts, reducing its phosphorylation in these domains and inducing its internalization *via* clathrin-mediated endocytosis [120, 121]. Interestingly, even though both are necessary for β-catenin activation, receptor phosphorylation and internalization are able to occur independently of each other, suggesting that these two steps are important checkpoints in Wnt/β-catenin signaling modulation. More recently, another essential raft component was found to regulate Wnt/β-catenin signaling activity. There is now cogent evidence indicating that cholesterol selectively activates canonical Wnt signaling over non-canonical Wnt signaling [134]. Specifically, cholesterol promotes interactions of Disheveled (Dvl), a scaffold cytosolic protein associated with the Wnt/β-catenin pathway, with LRP5/6, Axin, and the anionic lipid PIP_2 in the plasma membrane, promoting activation of this pathway. Interestingly, binding of canonical Wnt to Fz and LRP5/6 has been shown to induce the local enrichment of cholesterol in the vicinity of activated receptors by bringing Fz closer to LRP6 in a cholesterol-enriched microenvironment, reminiscent of lipid rafts. This is noteworthy, because the concept that raft domains are dramatically different before and after ligand stimulation has been proposed in order to describe, in part, their highly dynamic nature and changes in membrane raft coverage. Other protein effectors known to associate with lipid rafts have been shown to regulate the Wnt/β-catenin pathway. For example, Lypd6 has been shown to physically interact with the LRP5/6, promoting LRP5/6 phosphorylation in lipid rafts and enhancing Wnt/β-catenin signaling. Interestingly, mislocalization of Lypd6 to non-raft membrane domains is sufficient to alter LRP5/6 phosphorylation and inhibit the Wnt/β-catenin pathway [115]. It is also important to note that lipid rafts and their components act as plasma membrane signaling organizers in the Wnt/β-catenin pathway *via* mediating plasma membrane lipid asymmetry. Interestingly, quantitative live-cell imaging studies have demonstrated a high correlation between the inner leaflet plasma membrane cholesterol levels and cellular Wnt/β-catenin signaling activity [51]. This suggests that stimulus-induced plasma membrane cholesterol redistribution is a key modulator of raft-dependent Wnt/β-catenin signaling.

EGF-mediated activation of ERK serves as yet another excellent paradigm for nanoscale proteolipid-based activation, recruitment, and signaling. In this example, we will describe

the binding of EGF to EGFR and the subsequent series of events that lead to the propagation of the MAPK/ERK signal (Fig. 2b, EGFR signaling). Non-active EGFR is believed to reside in a non-raft domain. The binding of EGF to EGFR results in its dimerization, activation (autophosphorylation), and the subsequent coalescence of different lipid raft domains including ganglioside GM1 and glycosylphosphatidylinositol (GPI)-anchored proteins [135, 136]. The formation of these domains may in part be driven by the generation the ionic lipids, including PA by phospholipase D2 (PLD2) [137–139] and PIP_2 by PI(4)P5 kinase [140, 141], as these lipids are known to influence the nanoscale organization of EGFR [142, 143]. Furthermore, these ionic lipids serve the role of beacons, which recruit specific effector proteins to the newly forming proteolipid complex. This includes son of sevenless 1 (SOS1) by way of its affinity for PA and PIP_2 [144, 145]. This is relevant because SOS1 functions as a guanine nucleotide exchange factor (GEF) responsible for activating Ras [138]. Not surprisingly, the inactive GDP-bound version of H- and KRas has strong affinities for PIP_2 and PA, respectively [124, 130], allowing them to preferentially interact with, and therefore be activated by, SOS1 in a predefined time and space. Newly activated GTP-bound HRas then moves into a new nanodomain spatially segregated from its inactive GDP-bound counterpart, while activated KRas remains near its original location [146, 147].

Interestingly, the formation of inactive HRas and active KRas nanoclusters is dependent on the integrity of the actin cytoskeleton, while active HRas shows no such dependency [125]. In many cases, Ras nanocluster formation is tightly correlated with ERK signaling [148], which is in agreement with the observation that actin disruption attenuates oncogenic active GTP-bound KRasG12V signaling, while having no effect on HRasG12V [125]. This is relevant because both PA and PIP_2 influence the active remodeling and stabilization of the actin cytoskeleton [149–151]. The net result of these seemingly complex interactions is the formation of a proteolipid cluster, whose heterogeneous composition is exquisitely defined both spatially and temporally, allowing the selective propagation of a signal. Collectively, this evidence establishes how critical plasma membrane domain organization is required for the controlled regulation of cellular processes.

4 Dysregulation of plasma membrane biochemical and biophysical characteristics as a cancer driver

Plasma membrane biochemical and biophysical homeostasis, e.g., precise spatial compartmentalization of signaling components, regulates intracellular signal transduction (Fig. 3a) [152]. As discussed above, multiple signaling pathways, as well as several of their effectors, have been reported to occur in lipid raft domains, including EGFR/Ras, Wnt/β-catenin, insulin receptor, and T cell and B cell receptor signaling [115, 132, 153–158]. Moreover, it has been demonstrated that multiple effectors associated with these pathways require proper membrane organization, i.e., the formation of nanoclusters, in order to signal efficiently (Fig. 3a) [13, 111, 125, 148, 159–161]. Interestingly, all of these signaling pathways and several effectors have been shown to be directly involved in signaling processes associated with cancer. This suggests that alterations in lipid raft domains may mediate tumorigenesis. In this regard, several studies have demonstrated that cancer cells exhibit higher levels of rafts/caveolae compared to normal cells and that disruption of lipid

rafts in cancer can lead to increased responsiveness to anti-cancer therapies [122, 162–166]. Additionally, some anti-cancer drugs have beneficial effects through alteration of the protein content of lipid rafts [166]. Thus, a growing number of experimental, epidemiological, and preclinical studies now support the membrane-centric hypothesis that the dysregulation of bio-chemical and biophysical factors associated with the plasma membrane actively mediates cancer risk (Fig. 3b). Consequently, the plasma membrane and these specialized membrane domains have received increased attention, due to their ability to fine-tune, stabilize, and, most importantly, mediate signaling interfaces.

4.1 Cholesterol dysregulation

Unesterified free cholesterol is primarily localized to the plasma membrane, where it constitutes up to 90% of total cell cholesterol and 10–45 mol% of total plasma membrane lipids [167–169]. RNA expression and DNA mutational profiling studies have identified dysregulation of the cholesterol synthetic pathway in several cancers [170–172]. For example, data compiled by The Cancer Genome Atlas (TCGA) reveal the increased activity of genes in the cholesterol biosynthesis pathway in various cancers. Interestingly, a number of oncogenic signals, such as PI3K/AKT/mTOR, EGFR/RAS, and TP53, have been shown to stimulate the activity of the transcription factor SREBP, a major regulator of genes involved in *de novo* cholesterol biosynthesis [173]. It is also noteworthy that from a translational perspective, EGFR's association with cholesterol-rich lipid rafts underlies its resistance to EGFR tyrosine kinase inhibitors [174]. In addition, the size and number of EGFR nanoclusters is increased in lung cancer in comparison to normal lung cells [143], and both PIP_2 [143] and cholesterol [175] are necessary for the formation of EGFR nanoclusters.

Activating mutations in Wnt/β-catenin pathway effectors have been shown to upregulate genes associated with *de novo* cholesterol synthesis in various cell lines [176, 177]. Moreover, a number of epidemiologic studies have documented a positive association between elevated serum cholesterol level and risk of certain cancer types [178–182]. For example, a 10 mg/dL increase in cholesterol was associated with a 9% increase in prostate cancer relapse [178]. In this regard, several studies involving the use of HMG-CoA reductase inhibitors, e.g., statins, showed a reduced risk of melanoma, non-Hodgkin lymphoma, endometrial, breast, and colorectal cancers [183–186]. Consistent with these reports, studies have demonstrated that cholesterol levels in tumor cells are higher than those in healthy cells [187–189]. Additionally, cancer tissues display increased upregulation of HMG-CoA reductase, loss of feedback inhibition, decreased expression of the cholesterol exporter ATP binding cassette transporter A1 (ABCA1), and increased extracellular cholesterol uptake *via* LDL receptor [187, 190–194]. Collectively, these metabolic perturbations contribute to the accumulation of cellular cholesterol. Thus, it now appears that dysregulation of cholesterol homeostasis is a significant contributing factor to cancer risk.

4.2 Dysregulation of other lipids

Dysregulation of other plasma membrane lipids has also been identified in various cancers. Although overlooked in the past, changes in lipid-associated pathways in tumors are now being described more frequently. Thus, plasma membrane lipid reprogramming is an

emerging hallmark of cancer. Large-scale microarray profiling studies have revealed alterations in different metabolic pathways associated with lipid second messengers (phosphatidylinositols), lipid mediators (leukotrienes), and structural lipids (glycosphingolipids) [194]. Likewise, a relative increase in lipid levels such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), and triglycerides (TG) as well as an increase in saturated fatty acid content has also been observed in a variety of tumor types [195–199]. In addition, increased expression of lipogenic enzymes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), lipid-modifying enzymes such as phosphoinositide 3-kinase (PI3K), phospholipase C (PLC) and phospholipase D (PLD) and ATP citrate lyase (ACLY), which collectively promote cholesterol biosynthesis, represent a phenotypic alteration often observed in different cancer types and frequently predict a poor prognosis in cancer patients [199–204]. Furthermore, state-of-the-art analytical and imaging tools, such as electrospray ionization, matrix-assisted laser desorption/ionization, tandem mass spectrometry (MS/MS), and Raman scattering microscopy, have provided valuable lipidomic data that describe alterations in cellular lipid phenotype and fatty acid composition as well as specific spatial cellular distribution in cancer-related conditions [23, 196, 205–208]. It is apparent that cancer cells display a high demand for various lipids and their excess is considered a trademark of cancer. However, some important questions remain to be addressed. How does loss of membrane lipid homeostasis influence other crucial plasma membrane elements and, thus, modulate cellular signaling? How does deviation from the healthy steady-state promote cancer risk?

4.3 Dysregulation of membrane domain order and clustering and its role in signaling

Different plasma membrane components such as lipids, proteins, and the membrane skeleton influence the plasma membrane microenvironment in a direct and dynamic manner. Alternatively, plasma membrane composition and relative fluidity can regulate the organization, the activity, and, consequently, the function of several membrane effectors (Fig. 3a) [34, 209–211]. Plasma membrane organization, in part, is modulated by the preferential interaction of specific types of lipids. Due to their biochemical and biophysical properties, these lipids can form specialized discrete domains. As discussed above, cholesterol, sphingolipids, and saturated phospholipids allow the formation of relative ordered and condensed domains. In contrast, polyunsaturated and branched lipids form disordered domains [211–213]. These domains, which exhibit different biophysical and biochemical properties, are mutually excluded and coexist in the plasma membrane allowing it to display diverse properties and functionalities. Collectively, these membrane attributes give rise to a distinguishable macroscopic biophysical membrane phenotype, i.e., membrane order.

One of the most popular methods to quantify membrane order involves the use of polarity-sensitive lipophilic dyes or membrane order dyes (e.g., Laurdan and di-4-ANEPPDHQ). The vast majority of membrane order dyes are solvatochromic and display shifts in their emission maxima when found in polar *vs.* non-polar solvents [214–218]. For example, in Laurdan's case, the less-polar membrane environment of the ordered phase (often regarded as synonymous with lipid rafts) induces a 50-nm blue shift in the emission maxima. This

shift in emission profile between disordered and ordered domains allows the construction of a generalized polarization (GP) image. Hence, the calculation of a ratiometric measurement of the fluorescence intensity recorded in the two “ordered” and “disordered” channels serves as a quantitative assessment of relative plasma membrane order.

Measurements of plasma membrane order have been performed in whole organisms, intact tissue, and live cells as well as biologically derived and synthetic membranes. For example, the formation of selective, ordered lipid domains in membrane vesicles, i.e., giant plasma membrane vesicles (GPMVs), which are derived from live cells, has been documented extensively [87, 219–221]. Most importantly, these findings have served as an integral confirmation of the potential for domain formation in live cells. Not surprisingly, several studies have confirmed the presence of differences in plasma membrane order in domains with higher degrees of complexity such as live cells and tissues. As an example, membrane order experiments performed on human white blood cells showed that higher membrane rigidity resides at the immunological synapse periphery of T cells [222, 223]. Additionally, the first ever report of membrane order in an intact living organism showed high membrane order in the apical surfaces of polarized epithelial cells of zebrafish [219]. Membrane order maps have been acquired from different tissues, including the gut epithelium, muscle, kidney, retina, and neurons [219]. These and multiple other similar studies represent an important step towards understanding the physiological relevance of plasma membrane order and the role of this biophysical parameter in cellular signaling.

Plasma membrane functionality is modulated by heterogeneous lipid and protein domain composition, organization, and component interactions [211, 224, 225]. Thus, membrane order is believed to play a crucial role in plasma membrane homeostasis. Several proteins and lipids have been shown to reside in, and others to be excluded from, domains displaying distinct membrane order. Confocal, single-particle, FLIM-FRET, and FCS measurements have documented the confinement of specific effectors in distinct plasma membrane domains [142, 160, 226–229]. Moreover, membrane order has been implicated in the modulation of spatially localized signaling events. For example, large-scale membrane polarization, such as that which occurs for T cells at the immunological synapse, depends, in part, on membrane order [209, 222]. In addition, it has been established that non-activated HRas partitions into ordered domains, whereas active HRas preferably localizes in non-raft domains, which represent the hot spots of Ras activation and signaling [125, 126, 230, 231]. Lastly, as noted above, it has been recognized that effective canonical Wnt/β-catenin signaling requires the accumulation of its plasma membrane receptors, LRP6 and Fz, in cholesterol-rich domains, which in turn allows receptor phosphorylation, activation, and recruitment of effectors at the inner leaflet to transduce the Wnt-mediated signal intracellularly [51, 134, 232].

In cancer, the uncontrolled alteration of cellular plasma membrane homeostasis can lead to dramatic changes in the biochemical and biophysical characteristics of membrane domains (Fig. 3b). These changes can be triggered by various stimuli, including genetic mutations, abnormal epigenetic modifications, and carcinogenic substances [23, 233–236]. If allowed to operate unchecked, the resulting loss of homeostasis can lead to reprogramming of vital cellular features such as plasma membrane order and nanoclustering (Fig. 3b). Most

importantly, this new abnormal cellular state, which is associated with aberrant signaling events, can increase cancer risk. Although a general trend has not been established regarding the direction of membrane order dysregulation, it is clear that alterations in plasma membrane fluidity can profoundly affect several processes such as protein expression, endocytosis, apoptosis, cell proliferation, and metastasis [166]. Thus, it comes as no surprise that membrane order of cancer cells differs from that of normal cells. For example, hepatoma tumor and breast cancer cells as well as hairy cells from hairy cell leukemia have been shown to display higher membrane rigidity compared to normal cells [237, 238]. Additionally, multidrug resistant cancer cell types have been shown to exhibit an increase in membrane rigidity when compared to their non-resistant counterparts and normal cells [239, 240]. In most cases, this increase in rigidity is accompanied by an increase of membrane cholesterol and sphingomyelin. However, changes of plasma membrane order in cancer cells do not always result in higher membrane rigidity [241–243]. Fluidization of membranes in cancer cells appears to be correlated with their malignant potential and competency to metastasize. Interestingly, the ability of a cell to alter its membrane fluidity has been shown to be a key step in modulating anti-cancer drug cell penetration, transport, and responsiveness, thus leading ultimately to drug resistance [166, 238, 239]. This evidence further highlights the importance of plasma membrane order in several steps of cancer.

Similar to the case of plasma membrane order, dysregulation of protein, lipid, and saccharide nanoclustering at the plasma membrane can increase cancer risk [24, 244–247]. It has been reported that cancer-associated mutations in HRas, KRas, and NRas oncoproteins increase their activity through augmented nanoclustering of Ras on membranes [22]. Similarly, Ras clustering modulation by the overexpression of various galectins (e.g., Galectin-1 and Galectin-3), which is observed in breast, pancreatic, colorectal, and other cancers, can increase Ras nanoclustering and its oncogenic output [24, 246, 248–251]. Likewise, nanoclustering of other plasma membrane components, i.e., carbohydrates and lipids, plays an important role in cancer. For example, oligomers of galectin, the same proteins shown to increase cancer-associated Ras activity, are known to induce aggregation of carbohydrates on the surface of cells [252–255]. Several studies have revealed that the membrane of cancer cells display distinct distribution patterns of various types of saccharides relative to normal cell membranes [247]. In fact, several types of cancer cells show higher carbohydrate expression levels as well as higher membrane cluster coverage and larger carbohydrate clusters as compared to their normal cell membrane counterparts [247]. These carbohydrate alteration phenotypes are thought to promote tumorigenesis and metastasis. Finally, alterations in lipid membrane organization are also involved in clustering-associated cancer risk. As an example, analysis of large panels of tumor types shows loss of PS asymmetry and increased PS exposure on the surface of malignant cells relative to normal cells [256–259]. Cancer treatments utilizing radiotherapy and chemotherapy can exacerbate this phenotype [260–262]. Interestingly, imaging MRI studies have revealed the presence of cluster structures, stained by a PS-specific MRI-contrast agent, in tumor tissues [263–265]. As noted above, other charged lipids, involved in important cellular signaling processes, such as PIP_2 and PA have been shown to cluster in the plasma membrane and modulate cellular signaling [142, 143, 266–270]. Due to their role in several key cancer-associated processes, the modulation of these plasma membrane biochemical and

biophysical phenotypes by membrane-targeted countermeasures could serve as a cancer chemoprevention therapeutic strategy.

5 Cancer chemoprevention by membrane-targeted dietary bioactives

"Dietary bioactives" are constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs, which are responsible for changes in health status. While genetic factors contribute to cancer risk, environmental factors account for the majority of risk [271]. For example, colorectal cancer risk can be significantly reduced by dietary modification, including increased dietary fiber intake and reduced fat intake [272, 273]. Omega 3 (n-3) (e.g., α-linolenic acid, ALA) and omega 6 (n-6) (e.g., linoleic acid, LA) polyunsaturated fatty acids (PUFAs) are essential bioactive nutrients, obtained from the diet, that incorporate into cellular membranes in tissues and influence many physiological processes, including production of eicosanoids [274–277]. Two key n-3 PUFAs found in fish oil are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [278]. In humans, production of EPA and DHA from dietary precursors, e.g., ALA, is highly inefficient [279]. Additionally, there is competition with synthesis of long-chain n-6 PUFA, e.g., arachidonic acid (AA), which is produced from LA and is found in much greater abundance in a typical Western diet [280]. In general, n-6 PUFAs are associated with pro-inflammatory responses whereas n-3 PUFAs tend to cause opposing effects and are anti-inflammatory [281, 282]. Over the past few decades, it has been observed that an increase in the intake of n-6 PUFA over n-3 PUFA, e.g., high n-6 PUFA-to-n-3 PUFA ratio diet, which is associated with greater metabolism of n-6 PUFA, coincides with increases in chronic inflammatory diseases such as non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, obesity, inflammatory bowel disease (IBD), rheumatoid arthritis, and Alzheimer's disease (AD) [281]. Given that inflammation is strongly intertwined with cancer, higher intake of n-3 PUFA could provide a biological chemoprotective effect [274, 283].

With respect to clinical studies, increasing evidence suggests that the consumption of fish oil, containing EPA and DHA, may reduce cancer risk in humans [284–288]. Interestingly, EPA and DHA appear to be ideally suited to work either alone or in combination with other chemoprotective drugs, e.g., NSAIDs [289, 290]. Recently, it was demonstrated that EPA reduced rectal polyp number and size in patients with familial adenomatous polyposis (FAP), a condition associated with colorectal cancer [283, 291]. Most impressive was the fact that fish oil derived n-3 PUFA suppressed FAP to a degree similar to celecoxib, an FDA approved drug for the treatment of FAP. Additionally, in a different clinical study, it was demonstrated that EPA and DHA, contrary to LA and AA, may reduce the risk of total and advanced prostate cancer [292, 293]. Collectively, these data indicate that n-3 PUFAs hold promise as chemoprevention agents for cancer.

The consumption of n-3 PUFA in combination with other agents with complementary anti-tumor action, e.g., curcumin [294] and drugs [295], may improve their overall efficacies as cancer prevention therapies. Polyphenolic and terpenoid phytochemicals have become increasingly popular with consumers in great part because of their putative health benefits. Of these, turmeric (*Curcuma longa* Linn) extracts including curcumin (diferuloylmethane), a yellow color pigment of turmeric, have shown some promising effects in patients with

various pro-inflammatory diseases and cancer [296–303]. Completed and ongoing clinical trials (Clinical Trials.gov) examined the role of curcumin with respect to aberrant crypt foci (NCT00365209), cell proliferation and apoptosis in colonic mucosa (NCT00118989), drug combination therapy (NCT00745134), and treatment of FAP (NCT00927485, NCT00641147). Recent data from one of these phase IIa clinical trials indicates that consumption of curcumin at 4 g per day for 30 days significantly (40%) reduces the number of aberrant crypt foci (ACF) in a CRC model [304] and in men and women [305]. More recently, our laboratory examined the effect of n-3 PUFA and curcumin in a mouse colorectal cancer initiation model and found that these agents when combined synergistically reduced (75%) the number of ACF in a mouse CRC model [28]. Importantly, several studies have confirmed that curcumin is well tolerated and that the effect is potentially mediated by curcumin delivered to the target tissue, i.e., it is directly incorporated into the intestinal mucosa [28, 305, 306]. However, before a dietary combinatorial therapeutic approach can be adopted, it is critical that we fully elucidate the molecular mechanisms associated with n-3 PUFA, curcumin, and other potentially beneficial MTDB chemoprotective action. Thus, establishing a causal role of n-3 PUFA, curcumin, and other MTDBs in cancer prevention would have a major translational impact because these dietary nutrients are safe, well tolerated, relatively inexpensive and provide additional health benefits.

One of the criticisms facing the dietary chemoprevention field is the fact that dietary bioactives affect diverse physiological processes including cell membrane structure/function, eicosanoid signaling, nuclear receptor activation, gene transcription and translation, and inflammatory responses [21, 307–312]. Consequently, a huge challenge is to explain and unify these apparently disconnected signaling nodes. We propose a unifying mechanistic hypothesis to explain the function of these dietary bioactives, with an emphasis in colon cancer. We posit that n-3 PUFA and curcumin/curcuminoids fall into a unique class of MTDBs which, because of their unique amphiphilic properties, are capable of modulating plasma membrane hierarchical organization (Fig. 3c). We also believe that this ability to modulate plasma membrane biophysical properties may underlie the chemoprotective effects of other bioactives, such as cathecins and procyanidins [25, 313].

With respect to the molecular mechanism of action of fish oil, there is a growing body of *in vitro* and *in vivo* evidence indicating that n-3 PUFA reshape plasma membrane domains. For example, EPA and DHA are rapidly incorporated into cell primarily into membrane phospholipids at the *sn*-2 position [314, 315]. This is noteworthy, because the presence of long-chain n-3 PUFA in membrane phospholipids imparts unique biophysical properties which have been linked to alterations in plasma membrane structure and function [315–318]. DHA is known to influence membrane fluidity, ion permeability, fatty acid exchange, and resident protein function [319–321], including the inhibition of EGFR signaling in tumor-bearing mice by disassociating EGFR from lipid rafts [322]. Of particular interest is the ability of DHA to attenuate EGFR signaling. Specifically, immortalized mouse colonic cells incubated with DHA exhibited decreased GTP-bound K, H, and NRas levels following EGF stimulation [322]. Similarly, intact colonic crypts isolated from mice fed n-3 *vs.* n-6 PUFA (control)-enriched diets had reduced levels of HRas-GTP following EGF stimulation [317]. With respect to a molecular target capable of modulating Ras activation, our lab recently

identified a DHA-induced “break point” (following Grb2 recruitment to EGFR) in the Ras signaling pathway [322]. This combined with the fact that DHA can increase EGFR/SOS1 interaction, while reciprocally decreasing SOS1/Ras interaction in three cancer lines [323], suggests that plasma membrane-targeted GEFs may be modulated by DHA.

SOS1 is recruited to the plasma membrane by activated EGFR, which subsequently engages Ras [324]. This process is subject to complex regulation by the temporal and spatial production of membrane phosphoinositides through interactions with SOS1 Dbl homology (DH)/pleckstrin homology (PH) regulatory domains [325]. Two of the lipids involved in SOS1 recruitment are PA [138] and PIP_2 [144, 145, 326]. In proof-of-principle experiments, we demonstrated that n-3 PUFAs reduce the levels of PIP_2 in $CD4^+$ T cells [327], implying that MTDBs may be capable of modulating Ras activation *in vivo*. Therefore, we hypothesized that DHA reduces wild-type and oncogenic Ras signaling by disrupting SOS/Ras complex formation through altered temporal spatial production of PA and PIP_2. Importantly, the SOS/Ras interaction is a current target of pharmacological agents designed to suppress oncognenic Ras signaling [328–330]. Furthermore, oncogenic KRas-driven cancers are dependent on wild-type H- and NRas signaling, which suggests that Ras may be a target for MTDB therapy [331, 332]. This is highly relevant because approximately 30–50% of colorectal cancers (CRCs) harbor KRas mutations, and KRas-mutant CRCs exhibit resistance to standard therapy [333], thereby reducing survival [334]. Moreover, to date, targeting of mutant RAS proteins in cancers has not been possible [334]. Interestingly, the effects of n-3 PUFA are not limited to wild-type Ras, as DHA also attenuates activation of ERK and reduces the growth of mouse colonocytes expressing oncogenic HRas [322, 335]. This is consistent with work documenting the ability of n-3 PUFA to reduce cell proliferation, signaling, and anchorage-independent growth in different colon cancer cell lines (HCT116, SW480, SW620), harboring unique KRas G12V and G13D mutations [323, 336, 337]. Importantly n-3 PUFAs reduce murine pancreatic cancer development and delay progression of pancreatic ductal adenocarcinoma in mutant KRas-driven mouse models [338, 339].

High fidelity signaling of KRas is dependent on its spatial organization into defined dimers [340, 341] or nanoclusters [125, 148]. Recently, it was demonstrated that select amphiphilic agents, through direct modulation of the biophysical properties of the plasma membrane, compromise oncogenic KRas nanoclustering to modulate signal transduction [20, 124]. These findings suggest that Ras nanoclusters could be a novel therapeutic target [342]. Consistent with this hypothesis, experiments conducted by our lab using immuno-gold electron microscopy of plasma membrane sheets suggest that plasma membrane organization of inner leaflets is fundamentally altered by EPA and DHA [343, 344]. Specifically, n-3 PUFA treatment altered the nanoclustering of truncated forms of wild-type H- and KRas in cervical adenocarcinoma (HeLa) and colorectal carcinoma (HCT-116) cells [343, 344]. Our most recent work targeting the gastrointestinal tract (currently under revision) has demonstrated that dietary n-3 PUFA remodels plasma membrane phospholipids, reducing the lateral segregation of cholesterol-dependent and cholesterol-independent nanoclusters, and suppressing PA-dependent oncogenic KRas effector interactions. This results in the attenuation of oncogenic Ras-driven colonic hyperproliferation in both *Drosophila* and murine models. These findings demonstrate the

unique properties of dietary n-3 PUFA in the shaping of Ras nanoscale proteolipid complexes and support the emerging role of plasma membrane-targeted therapies.

Wnt signaling constitutes the major driving force behind the homeostatic self-renewal of the intestinal crypt [345, 346]. Dysregulation of Wnt signaling has been linked to cancer in multiple tissues [347–352]. It is noteworthy that mediators of Wnt signaling, LRP6 and Fz, are plasma membrane receptors that play a critical role in cell polarity [353, 354], stemness [355, 356], differentiation [356, 357], and neoplastic transformation [345, 355, 356, 358]. From a functional perspective, LRP6 and Fz require lipid raft localization and nanoclustering for efficient signaling and, most notably, stabilization of β-catenin [13, 110, 115, 133]. Extrinsic effectors, e.g., nystatin, which bind cholesterol and disrupt lipid rafts, alter LRP6 and Fz downstream signaling [121]. Similarly, secreted cellular effectors, e.g., Dkk1, bind and displace LRP6 from lipid rafts, leading to a reduction in LRP6 phosphorylated state and levels in lipid rafts [120]. Both distinct mechanisms of action result in the suppression of β-catenin transcriptional activity. Unfortunately, attempts to target aberrant Wnt signaling using drugs still face multiple hurdles due to poor tumor cell targeting, negative side effects associated with required long-term treatments, and a poor understanding of the mechanisms of action [359]. Consequently, there is an urgent need to develop toxicologically innocuous Wnt signaling therapeutic approaches. Nevertheless, previous studies, which have focused on suppressing β-catenin expression [360], preventing transcriptional activation of β-catenin/TCF complexes [361, 362] and inhibiting cyclooxygenase-2 (COX-2) [363, 364], have laid a strong knowledge foundation for the development of potential therapeutic tools for cancer. From a cancer prevention perspective, our laboratory examined the combined effect of n-3 PUFAs and curcumin in a mouse colorectal cancer initiation model and found that these MTDBs synergistically reduce nuclear β-catenin in aberrant crypt foci by threefold [28]. However, further studies are needed to determine precisely how MTDBs function to suppress aberrant Wnt signaling.

6 Summary and future challenges

There is an impending chronic disease crisis in our country. Thus, if the healthcare community hopes to head off the impending cancer storm, we need to get more serious about cancer prevention [365]. Unfortunately, less than 1.5% of total biomedical research is targeted to early detection and prevention of chronic disease [366]. With respect to all human malignancies, 35% are linked directly to diet and an additional 14–20% to obesity [367]. Consistent with these data, cancer risk can be lowered by 36% when humans adhere to healthy dietary principles [368]. In terms of primary cancer prevention, there is a growing body of experimental, epidemiological, and preclinical evidence indicating that diets enriched in MTDBs are protective against various types of cancers. Importantly, these diet-derived agents are toxicologically innocuous and generally free of safety problems intrinsic to drugs administered over long periods of time. However, additional mechanistic insights linking membrane therapy, diet, and cancer risk are required before this application can be used in the advancement of the chemoprevention field. Nonetheless, recent provocative work in this field has afforded us with a new mechanistic perspective in this regard. Thus, we propose that MTDBs, because of their unique properties and pleiotropic effects, are capable of modulating plasma membrane hierarchical organization, thereby suppressing various

signaling pathways that are essential for tumorigenesis (Fig. 3c). Elucidation of the role of MTDBs in cancer prevention would have a major translational impact because these dietary bioactives are well tolerated and relatively inexpensive and provide additional health benefits, such as a reduction in mortality.

Acknowledgments

Funding information This work was supported by the National Institutes of Health (NIH) grants R35CA197707 and P30ES023512, the Cancer Prevention Research Institute of Texas (CPRIT), and funds from the Allen Endowed Chair in Nutrition and Chronic Disease Prevention. Natividad Fuentes is a recipient of a Predoctoral Fellowship in Pharmacology/Toxicology from the PhRMA Foundation and the National Science Foundation Texas A&M University System Louis Stokes Alliance for Minority Participation (TAMUS LSAMP) Bridge to the Doctorate Fellowship (HRD-1249272). Alfredo Erazo-Oliveras is a recipient of the 2017 Ford Foundation Postdoctoral Fellowship from The National Academies of Sciences, Engineering and Medicine and the National Institutes of Health-National Cancer Institute (NIH-NCI) Research Supplement to Promote Diversity in Health-Related Research Award (3R35CA197707-02S1).

Abbreviations

Chol	Cholesterol
EM	Electron microscopy
FLIM-FRET	Fluorescence lifetime imaging microscopy combined with fluorescence resonance energy transfer
LC	Lung cancer
NL	Normal lung
PA	Phosphatidic acid
PALM	Photo-activated localization microscopy
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PI3P	Phosphatidylinositol 3-phosphate
PI4P	Phosphatidylinositol 4-phosphate
PS	Phosphatidylserine
SPT	Single-particle tracking
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
dSTORM	Direct stochastic optical reconstruction microscopy
TIRF	Total internal reflection fluorescence

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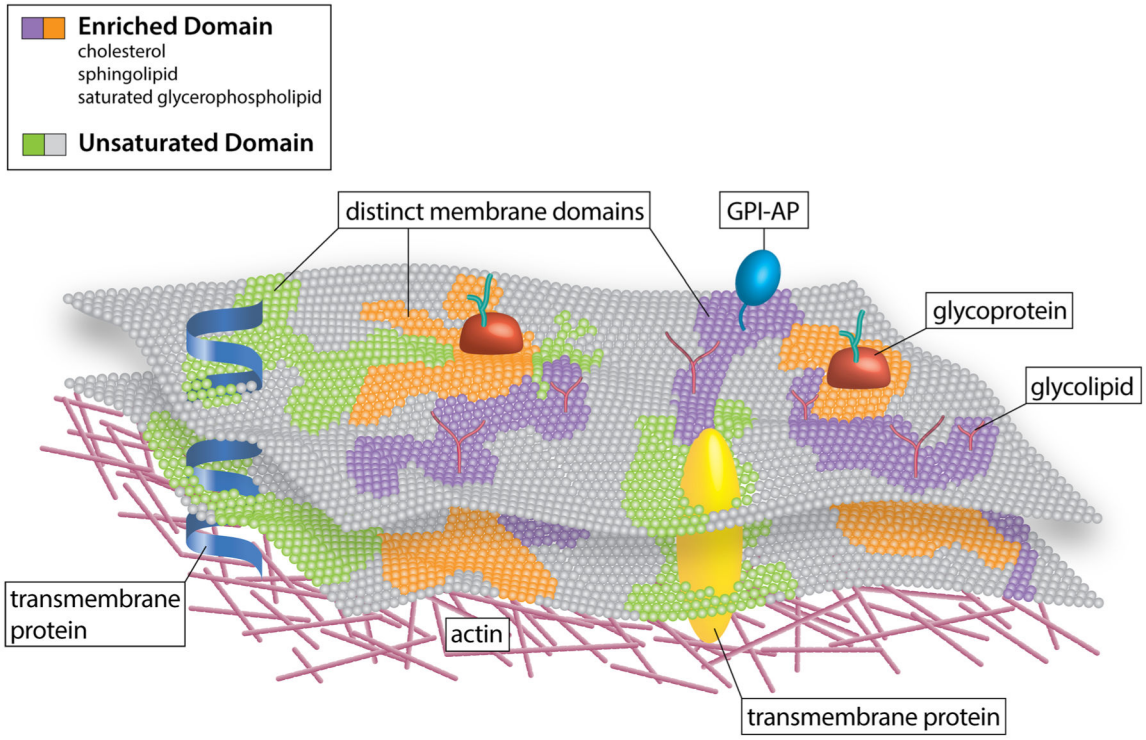

Fig. 1.

The compartmentalization of lipid-protein assemblies into specialized domains in the plasma membrane. Different lipids and proteins organized in an orchestrated manner to form distinct membrane domains, thus creating lateral heterogeneity in the plasma membrane. Moreover, plasma membrane heterogeneity is displayed across the lipid bilayer vertical axis, resulting in highly organized domains both in the plasma membrane outer and inner leaflet. The latter are not disconnected from one another rather they are coupled, bringing about bilayer crosstalk, which is essential for cellular signaling. These domains display distinct biochemical compositions and biophysical features. Some of these domains are enriched in cholesterol, sphingolipids, glycolipids, and saturated glycerophospholipids as well as lipidated and glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs), thus generating lipid raft or raft-like domains. In contrast, others domains, termed non-rafts, are enriched in polyunsaturated and branched lipids. Additionally, formation and organization of these membrane domains are believed to involve cortical actin. Finally, these diverse proteolipid domains, exhibiting varying biochemical and biophysical features, exclude one another, yet they can coexist in the plasma membrane, thus creating a multifunctional highly complex and dynamic signaling platform

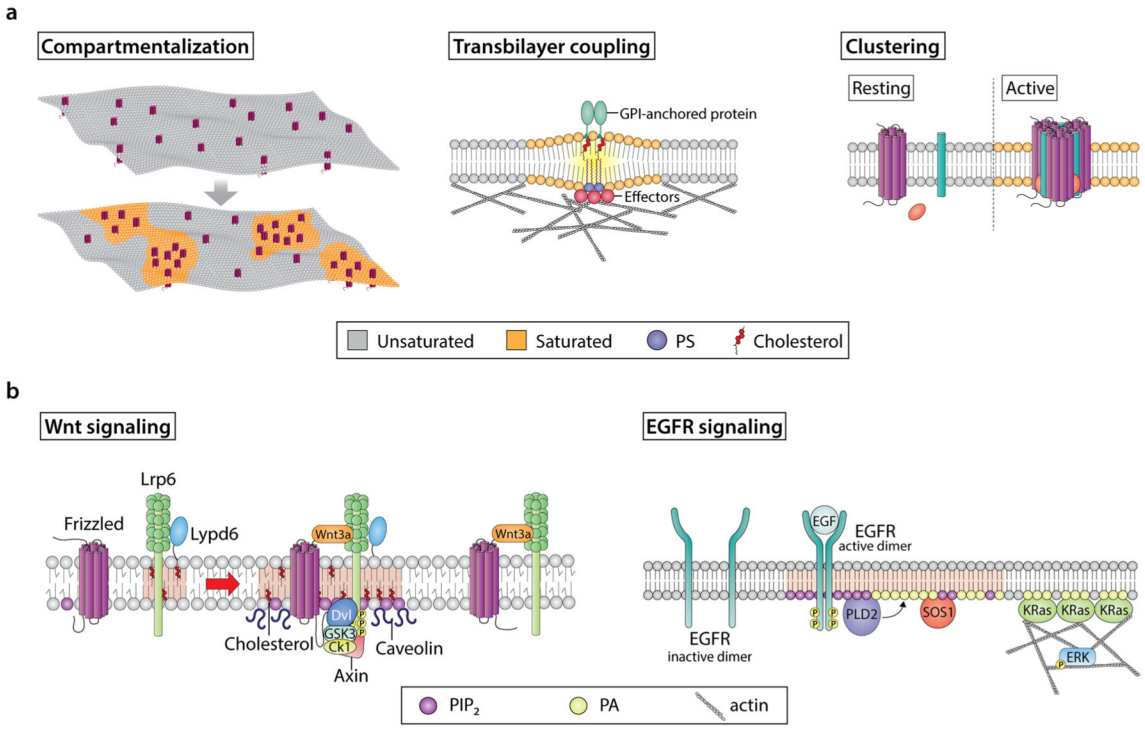

Fig. 2.

Modulation of lipid and protein organization in plasma membrane domains and their functions. **a** Examples of various membrane domain features. Highly dynamic interactions between lipid and protein molecules shape many of the features display by specialized plasma membrane domains. For example, the preferential interactions between specific proteins and cholesterol, sphingolipids, and, in multiple cases, charged signaling lipids can induce precise spatial compartmentalization of key membrane components, thus creating molecularly well-defined domains. This, in turn, regulates cellular signaling by mediating the recruitment of specific signaling effectors at an exact location and time. Another fundamental feature of membrane domains is proteolipid clustering. In many cases, assembly of clusters requires a stimulus to initiate the movement of cluster forming molecules between different membrane domains, resulting in the activation and oligomerization of these effectors. Plasma membrane clusters contain a variety of protein functionalities that can originate from both the plasma membrane and cytosol. Clustering of multiple functionalities at the membrane modulates high specificity and low membrane molecule diffusion, which in turn enhances signaling robustness. Lastly, proteins and lipids (e.g., cholesterol, GPI-anchored proteins, PS) are organized in plasma membrane domains within a bilayer with distinctive outer exoplasmic and inner cytoplasmic leaflets. These leaflets differ in terms of their lipid and protein compositions. Accordingly, specific membrane domains can induce the formation of proteolipid assemblies in the opposing leaflet. Inner leaflet effector organization is regulated by complex interactions between actin, lipids, and other proteins. These inner leaflet proteolipid assemblies can influence other effectors located in the outer leaflet and engage in transbilayer coupling. This is important, because transbilayer coupling is a mechanism by which membrane domain components are brought together at the two sides of the plasma membrane to efficiently signal. **b** Examples

of the role of membrane domains in signaling events associated with cancer. Wnt signaling receptors, i.e., LRP6 and Fz, localize in both raft and non-raft domains. In the presence of Wnt, cholesterol bilayer asymmetry is triggered, which leads to enrichment of cholesterol in the inner leaflet. Moreover, Wnt-bound Fz-LRP6 complexes preferably localize to cholesterol-enriched membrane domains containing caveolin. This, in turn, leads to the recruitment of the Wnt signaling effector Dvl. The ability of Dvl to oligomerize promotes Fz and LRP6 clustering and recruitment of Axin, leading to LRP6 phosphorylation by GSK3 and CK1 in lipid rafts. Simultaneously, lipid kinases (e.g., PI4KII and PIP5KI, not shown) are recruited to these sites and promote production of PIP_2, which in turn promotes LRP6 and Fz clustering and phosphorylation. Importantly, although Wnt-bound LRP6-Fz complexes can localize to non-raft domains, Lypd6, a GPI-anchored protein that localizes specifically in lipid rafts, ensures that LRP6 phosphorylation and thus receptor activation and efficient signaling occur in lipid raft domains. EGFR signaling is initiated from highly organized nanoscale proteolipid domains, driven by the spatiotemporal production of specific lipids. For example, EGFR activation by EGF initiates the formation of lipid domains, which are enriched in PIP_2 and PA. The PA generated by PLD2 acts as a beacon to recruit KRas and SOS1, and along with PIP_2, acts as a cofactor for SOS1-mediated activation of KRas. Furthermore, this pool of PA stabilizes the actin cytoskeleton, of which KRas nanoclustering is dependent. Together, these steps contribute to efficient ERK activation and downstream signaling

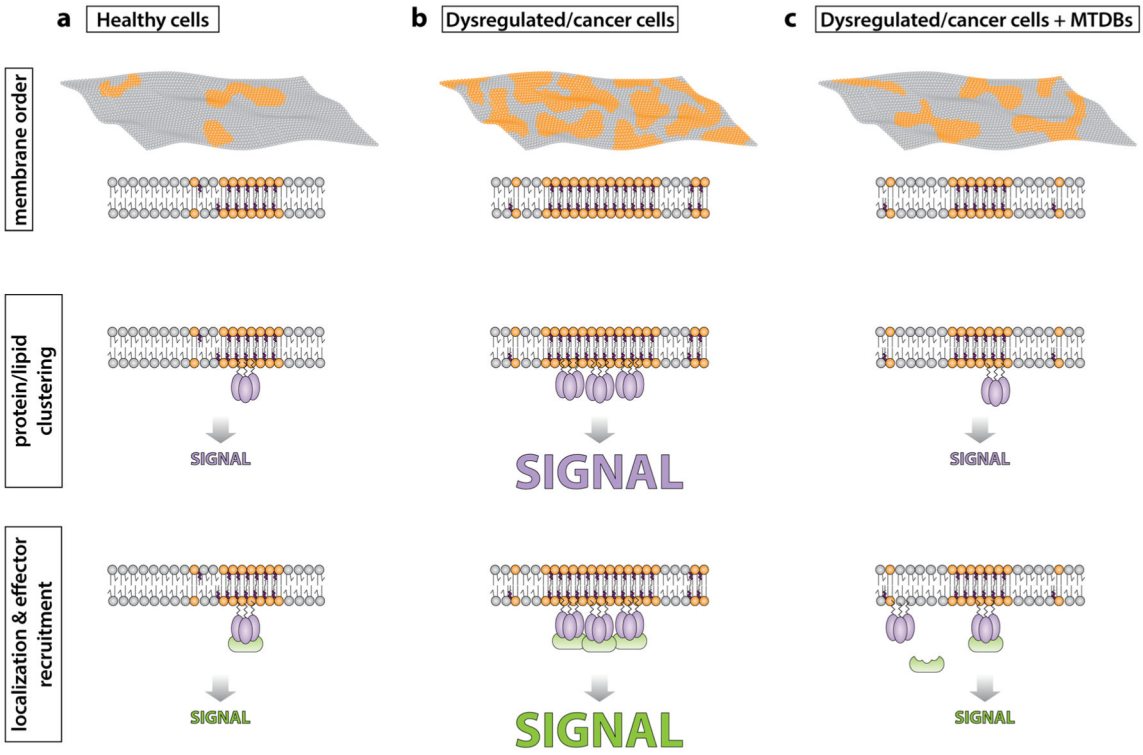

Fig. 3.

Dysregulation of plasma membrane domains and the role of MTDBs as countermeasures for loss of membrane homeostasis. Plasma membrane domain biochemical and biophysical homeostasis, e.g., membrane order, proteolipid clustering, and precise spatial compartmentalization of signaling components, regulates intracellular signaling. **a** In healthy cells, membrane domains display “normal” levels of order and proteolipid clustering and signaling-associated effectors localize at the right place in the right time. Together, this ensures canonical cell signaling. **b** During cancer, alterations of membrane domain features are observed such as increased membrane order, clustering, and effector recruitment as well as mislocalization of domain effectors. Loss of membrane domain homeostasis leads to an atypical cellular state, thus driving aberrant signaling. **c** Interestingly, MTDBs have the ability to target and reshape plasma membrane domains. In some cases, MTDBs have been shown to alter membrane fluidity, proteolipid complexes, and effector localization and suppress oncogenic signaling. In this way, MTDBs are able to restore, to a significant extent, healthy cell signaling

Table 1

Nanoscale proteolipid clusters relevant to cancer biology

Main protein	Lipids	Effectors	Size (~)	Technique	Function	References (PMID)
EGFR	PA, PIP_2	Grb2, PI3K, SOS1	20–30 nm, 200 NL, 300 LC	EM, dSTORM	Proliferation, migration	20516214, 25001389
KRas-GTP	PA, PIP_3, PS	PI3K, Raf	6 nm	EM, FLIM-FRET	Proliferation, survival	25234412, 19115142
HRas-GDP	PIP_2, PIP_3, PI3P, PI4P, PS, Chol		11 nm	EM, FLIM-FRET		25234412, 19115142
HRas-GTP	PIP_3, PI4P, PS	PI3K, Raf	6 nm	EM, FLIM-FRET	Proliferation, survival, migration	25234412, 19115142
Rac1	PIP_2, PIP_3	Tiam1, WAVE	200 nm	STORM, TIRF SPT-PALM	Cytoskeleton, migration	29141223
Frizzled	chol	Dvl, LRP5/6			β-Catenin, stemness	25024088, 28377511, 15459103, 19619488
Lrp5/6	PIP_2	Axin, Ck1, Frizzled, GSK3	100 nm, +WNT 200 nm	STORM	β-Catenin, stemness	23400998, 19619488
Lypd6		LRP5/6	100 nm	STED	β-Catenin, stemness	28404749
Dvl	PA, PIP_2, PS, Chol	Axin, Frizzled	> 500 nm	EM, FRAP, FLIP, TIRF	β-Catenin, stemness	25024088, 11101902, 24606934, 19234454, 17529994, 16263762, 19619488