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Rafting on the Plasma Membrane: Lipid Rafts in Signaling and Disease

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Abstract

The plasma membrane is not a uniform phospholipid bilayer; it has specialized membrane nano- or microdomains called lipid rafts. Lipid rafts are small cholesterol and sphingolipid-rich plasma membrane islands. Although their existence was long debated, their presence in the plasma membrane of living cells is now well accepted with the advent of super-resolution imaging techniques. It is interesting to note that lipid rafts function to compartmentalize receptors and their regulators and substantially modulate cellular signaling. In this review, we will examine the role of lipid rafts and caveolae-lipid raft-like microdomains with a distinct 3D morphology—in cellular signaling. Moreover, we will investigate how raft compartmentalized signaling regulates diverse physiological processes such as proliferation, apoptosis, immune signaling, and development. Also, the deregulation of lipid raft-mediated signaling during tumorigenesis and metastasis will be explored.

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Keywords

Cancer · Caveolae · Lipid rafts · Plasma membrane compartmentalization · Signal transduction

Abbreviations

CAF	Cancer-associating fibroblasts
Cav	Caveolin
CBM	Caveolin binding motif
CSD	Caveolin scaffolding domain
DISC	Death-inducing signaling complex
DRM	Detergent-resistant membranes
DSM	Detergent-soluble membranes
ECM	Extracellular matrix
EGF Signaling	Epidermal growth factor signaling
EMT	Epithelial-mesenchymal transition
FADD	Fas-associated death domain
GPI-anchor	Glycosylphosphatidylinositol anchor
IGF Signaling	Insulin-like growth factor signaling
IRS-1	Insulin receptor substrate protein 1
LAT	Linker for activation of T cells
Ld	Liquid disordered
Lo	Liquid ordered

MAPK Signaling	Mitogen-activated protein kinase signaling
MβCD	Methyl-β-cyclodextrin
NSOM	Near-field scanning optical
PALM	Photoactivated localization microscopy
PI3K	Phosphatidylinositol-3 kinase
PM	Plasma membrane
PTEN	Phosphatase and tensin homolog
STED Microscopy	Stimulated emission depletion microscopy
STORM	Stochastic optical reconstruction microscopy
TEM	Tetraspanin-enriched membrane rafts

1 The Compartments of the Plasma Membrane as Organizers of Cellular Signaling

Plasma membrane (PM) has long been viewed as a homogenous phospholipid bilayer dispersed randomly with membrane-bound proteins (Singer and Nicolson 1972). This view was challenged by identification of many subcompartments of the plasma membrane with various compaction properties and protein composition (Yu et al. 1973). These subcompartments of PM—so-called lipid rafts—are laterally mobile, enriched in cholesterol and saturated sphingolipids. They are now recognized for their crucial roles as platforms recruiting regulatory signaling molecules and facilitating effective signal transduction.

2 Characterization of Lipid Rafts

Biochemical experiments initially revealed that extracting PM at cold temperatures by nonionic detergents, such as Triton X-100, yields two populations of PM lipids (Yu et al. 1973). These are named detergent-soluble membranes (DSMs) and detergent-resistant membranes (DRMs) (Bagatolli and Mouritsen 2013). This observation put forth the idea that the lipidic composition of PM is heterogenous rather than being a homogenous phospholipid bilayer as previously assumed (Ahmed et al. 1997; Brown and Rose 1992; Friedrichson and Kurzchalia 1998; Pralle et al. 2000; van Meer et al. 1987; Varma and Mayor 1998). Nevertheless, these findings were also challenged, as the protein composition of DRMs exhibits alterations depending on the detergent type and the detergent concentration used in lipid extraction (Mayor and Maxfield 1995; Schuck et al. 2003). The heterogeneous nature of PM lipids was explored and shown complementarily in model membranes (Feigenson and Buboltz 2001; McConnell et al. 1984; Tamm and McConnell 1985; Veatch and Keller 2003). Model membranes exhibit a phenomenon called liquid–liquid phase separation in which lipidic molecules spontaneously separate into Liquid ordered (Lo) (Hjort Ipsen et al. 1987) and Liquid disordered (Ld) (Kaiser et al. 2009; Veatch and Keller 2003) compartments. Interestingly, both Lo membranes and DRMs exhibit enrichment in terms of cholesterol and glycosylated lipids, lending credence to the lipid raft hypothesis. However, studying lipid rafts in model membranes has its handicaps. For example, sharp phase separation between compartments was observed in model membranes, yet in natural membranes, it is believed that phase separation exists as a more dynamic gradient (Sezgin et al. 2012). Also, the percentage of protein molecules incorporated into model membranes falls short compared to the protein percentage in natural membranes, which is around 25% (Dupuy and Engelman 2008). Therefore, most complex regulatory processes occurring during raft formation and cellular signal regulation through rafts are hard to examine in model membranes. More recently, a plethora of fluorescent microscopy, electron microscopy, and label-free spectroscopy techniques have been developed to study lipid rafts. And all were presented with their advantages and disadvantages (Sezgin and Schwille 2011).

However, with the advancement in super-resolution microscopy, the resolution limit of traditional fluorescence microscopy, which is

200 nm, has been overcome (Hell and Wichmann 1994; Klar et al. 2001). As a result, we are now able to resolve objects smaller than 200 nm and conduct in-depth research on lipid rafts. Stimulated emission depletion (STED) (Hell and Wichmann 1994; Klar et al. 2001), photoactivated localization microscopy (PALM) (Sengupta et al. 2011; Hess et al. 2006; Rust et al. 2006), stochastic optical reconstruction microscopy (STORM) (Rust et al. 2006), and near-field scanning optical (NSOM) (Sezgin 2017) are some of the super-resolution microscopy methodologies that are utilized in order to study PM biology and composition (you may refer to Sezgin 2017, for a more detailed review on the nuances between super-resolution microscopy techniques) (Sezgin 2017). For example, STED is used to characterize the tetraspanin-enriched membrane rafts (TEMs), a high-order organization of membranous tetraspanins (Zuidscherwoude et al. 2015). Before, all proteins associated with TEMs were believed to localize to the same raft compartment. Nonetheless, this study established the existence of several different types of TEMs (Zuidscherwoude et al. 2015). Similarly, using the PALM technique, it was shown that raft-resident T-cell receptor and LAT protein locate in different raft compartments in T lymphocytes (Lillemeier et al. 2010; Rossy et al. 2013; Sherman et al. 2011). Furthermore, the STORM and PALM techniques revealed the segmented arrangement of B lymphocyte and mast cell receptors in the PM (Dustin et al. 2017; Shelby et al. 2013). Additionally, NSOM reveals raft compositions in artificial membranes (Burgos et al. 2003; Coban et al. 2007; Hollars and Dunn 1997; Hwang et al. 1998). All in all, the development of these highly sensitive microscopy tools sheds light on the previously unknown life of lipid rafts and promises more exciting times for cellular biologists.

So, what exactly are "lipid rafts"? According to the Keystone Symposia on lipid rafts in 2006, lipid rafts are PM microdomains enriched in cholesterol, sphingolipids, and glycosylated lipids and are no larger than 200 nm. Furthermore, lipid rafts can recruit smaller rafts to construct larger platform-like PM domains, which constitute complex signaling hubs within PM (Pralle et al. 2000). Lipid rafts are separated into planar lipid rafts and flask-shaped non-planar lipid rafts (caveolae) (Ludwig et al. 2016; Simons and Ikonen 1997; Yamada 1955). Planar lipid rafts exist in all cell types and are marked by the presence of Flotillin protein (Babuke and Tikkanen 2007; Staubach and Hanisch 2011; Stuermer 2010, 2011). Caveolae is present only in specific tissues like endothelium and marked by Caveolin and Cavin proteins (Hill et al. 2008; Kurzchalia et al. 1992; Rothberg et al. 1992; Way and Parton 1995). Although they differ in morphology and chemical content, both lipid rafts have been shown to significantly modulate cellular signaling. In this review, the term "lipid raft" will be used for planar lipid rafts. In the following section, the role of lipid rafts in cellular signaling will be explained in different physiological contexts. In the last section of this review, caveolae and the role of those tiny pits in cellular signaling will be reviewed.

3 Lipid Rafts in Normal Physiology

The compartmentalization of the members of the cellular signaling machinery is crucial for receiving a specific signal and generating a specific response—the goal of signal transduction. Lipid rafts are one of the tools that are utilized by cells to spatially regulate the signal. Membrane proteins are targeted to PM through their membrane-spanning and hydrophobic transmembrane domains, glycosylphosphatidylinositol (GPI)-anchors, and the covalent attachment of lipid moieties (Levental et al. 2010a; Resh 2013). Among them, GPI anchors and a subset of lipid modifications are associated with targeting proteins further in lipid rafts (Levental et al. 2010a). For example, S-palmitoylation of proteins is a well-characterized lipid modification to target proteins into rafts. The long-saturated-fatty acyl chain of palmitate (with 16 carbon atoms) is hydrophobic enough and, therefore, can integrate into lipid rafts. Also, palmitoylation is a reversible PTM, which gives targeted proteins flexibility to move in or out of the lipid rafts

(Levental et al. 2010b). Besides palmitoylation, dual acylation of proteins (consecutively adding two lipid acyl moieties) is simultaneously associated with raft-targeting, such as myristate and isoprenoid groups. However, none of those modifications alone is sufficient for lipid raft compartmentalization (Levental et al. 2010a; Resh 2013). The myristate acyl lacks the required hydrophobicity, and isoprenoid groups have unsaturated acyl chains. Another strategy used to target proteins in the rafts is the addition of cholesterol (Porter et al. 1996). Upon secretion into the extracellular space, Hedgehog proteins are directed to PM of adjacent cells in this manner (Gallet et al. 2006; Porter et al. 1996).

Once inside the lipid rafts, receptors interact with their activators or inhibitors (Simons and Toomre 2000). Hence, the molecular processes taking place in the raft or non-raft portions of the plasma membrane are of paramount significance. One other way lipid rafts might affect signaling is that the folding and therefore the functions of PM resident proteins can be altered by specific lipid-protein interactions (Laganowsky et al. 2014; Lingwood et al. 2011). Although these are two general modes of regulation in lipid rafts, they have been specifically implicated in many signaling cascades involving proliferation, apoptosis, immune responses, and development (Fig. 1).

4 Proliferation

Cellular proliferation is primarily sustained by growth factor signaling through the receptor tyrosine kinases. The activities of many receptor tyrosine kinases (RTKs) are regulated through lipid rafts (Pike 2005; Simons and Toomre 2000). Epidermal growth factor (EGF) and insulin-like growth factor (IGF) are two examples of receptor tyrosine kinases associated with lipid rafts (Simons and Toomre 2000). These receptors activate effectors such as Ras-MAPK and PI3K/Akt to sustain the proliferative state (Pollak 2008; Wee and Wang 2017). EGFR is the most studied receptor tyrosine kinase in terms of its mode of activation. For the activation of downstream signaling components, EGFR molecules must be oligomerized and autophosphorylated. Both of these activities are demonstrated to be controlled by lipid rafts (Coskun et al. 2011; Kovacs et al. 2022). Many studies have shown that disruption of lipid rafts by a cholesterol-depleting reagent—MβCD (Methyl-β-cyclodextrin)—causes aberrant activation of EGFR in the presence or absence of EGF (Jans et al. 2004; Pike et al. 2005; Roepstorff et al. 2002; Waugh et al. 1999). Therefore, to be activated appropriately, EGFR first needs to be present in raft regions; then, it needs to move to the non-raft plasma membrane after being fully activated (Kovacs et al. 2022; Lambert et al. 2006; Roepstorff et al. 2002; Turk et al. 2012; Waugh et al. 1999). Several raft-dependent regulatory mechanisms are described for IGF signaling as well.

IGF signaling is initiated by insulin or IGF binding to the insulin receptor or IGFR, which results in receptor phosphorylation and activation. In 3T3-L1 adipocytes, IGFR1 is demonstrated to localize in lipid rafts (Hong et al. 2004; Huo et al. 2003). However, unlike EGFR, cholesterol depletion causes reduced IGF signal, and IGFR seems to be active only in raft regions (Huo et al. 2003). After IGFR activation, the primary effector phosphatidylinositol-3 kinase (PI3K) gets recruited to phosphorylated IGFR (therefore to PM). This recruitment occurs through the SH2 domain of the negative regulatory subunit of the PI3K-p85. On the PM, PI3K is activated after binding to IGFR (Alessi et al. 1997). Activated PI3K phosphorylates the membrane lipid PIP_2 and generates a lipidic secondary messenger PIP_3. Transiently produced PIP_3 is recognized by the PH domain, which is present within many proteins/kinases involved in cellular proliferation (Ebner et al. 2017). AKT is one of these proteins recruited to PM by its PH domain (Brazil and Hemmings 2001; Ebner et al. 2017). Upon recruitment to the PM, AKT is activated by two sequential phosphorylation events, initiates many pathways of ultimate proliferation, and inhibits apoptosis (Manning and Cantley 2007; Manning and Toker 2017). It is interesting to note that the rafts contribute to the AKT activation. Through microscopy-based methods, active AKT was demonstrated in lipid

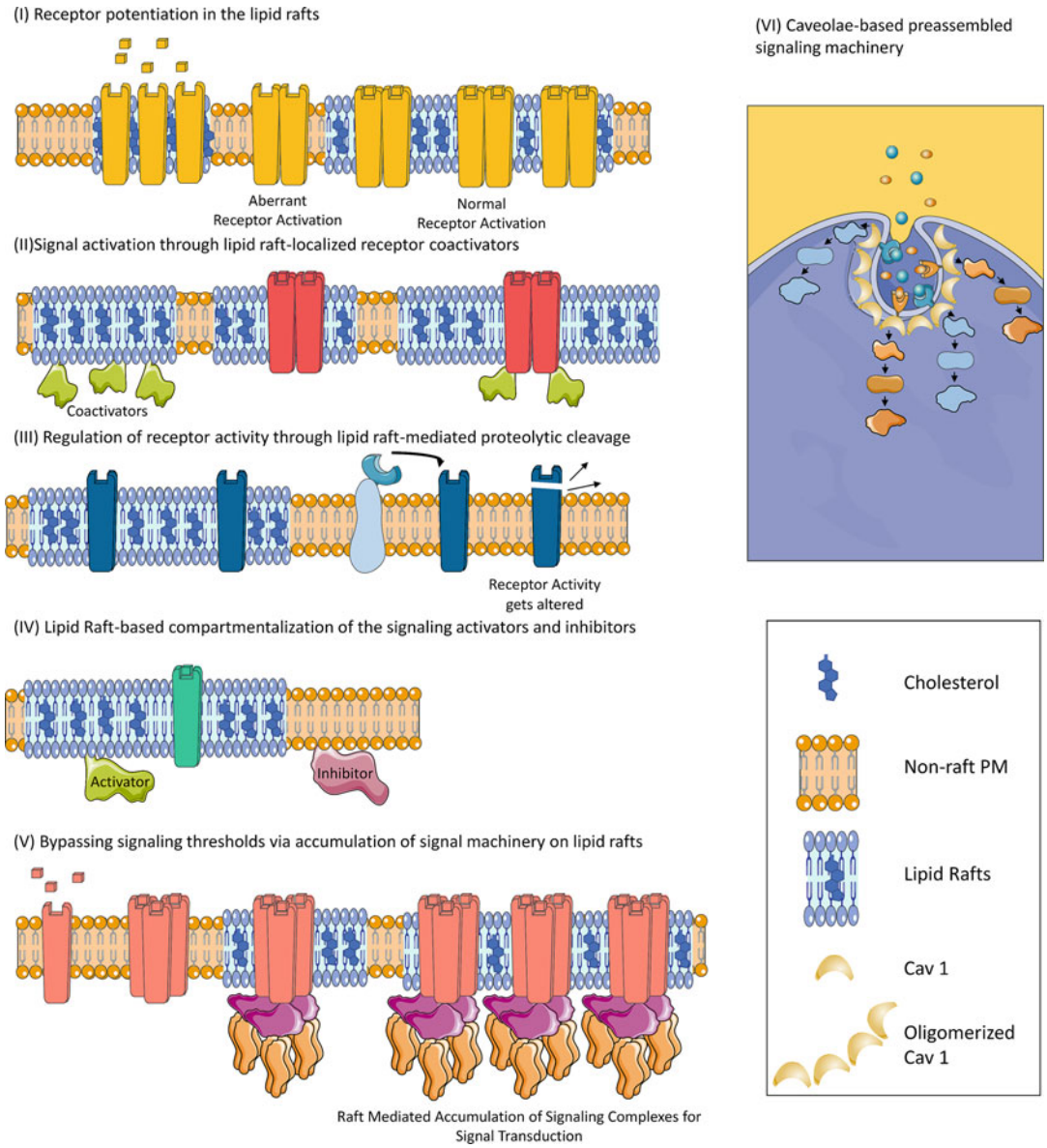

Fig. 1 Modes of signaling regulation related to lipid rafts. Receptors are potentiated within the lipid rafts. The immature association of receptors with non-raft PM causes aberrant overactivation, as in the case of EGFR (I). Both receptors and coactivators are localized to different species of lipid rafts and get associated within the same lipid raft as in the case of TCR and FcεRI signaling (II). Receptors move to non-raft PM and are proteolytically cleaved, leading EMT as in the case of CD44 signaling (III). Lipid raft-mediated differential localization of activators and inhibitors compartmentalizes signal transduction as in the case of PI3K signaling (IV). Accumulation of signaling complexes to initiate an intracellular response, as in the case of CD95/FasL signaling(V). Caveolar signal regulation occurs through caveolar coat protein Cav 1, which binds to many signaling molecules and establishes molecular machinery (VI)

rafts, and complementary studies revealed that cholesterol depletion ablates AKT activity (Gao et al. 2011; Lasserre et al. 2008). PI3K activity is reversed by phosphatase and tensin homolog (PTEN), which dephosphorylates PIP_3 lipids into PIP_2 (Song et al. 2012; Stambolic et al.

1998). Interestingly, PTEN, the negative regulator of AKT activation, has been shown to localize in non-raft membranes, whereas PDK1, the activator of AKT, has been shown to localize in raft regions. Of note, targeting PTEN to lipid rafts through molecular methods leads to PDK1 inhibition and abrogates AKT activation (Gao et al. 2011). PI3Ks have four isoforms, two of which are ubiquitously expressed (Fruman et al. 2017). However, these two isoforms—namely PI3Kα and PI3Kβ—have differential activities and regulations in cell signaling (Jia et al. 2008a, 2008b; Vanhaesebroeck et al. 2010; Zhao et al. 2006). For example, PI3Kα is the main effector and gets activated by RTK signaling (Jia et al. 2008a, 2008b; Zhao et al. 2006). On the other hand, PI3Kβ was found to signal through GPCRs (Jia et al. 2008a, 2008b). This was found to be mediated via lipid rafts. PI3Kα localizes in non-raft areas, whereas PI3Kβ interacts with Rac1 to localize to lipid rafts. This raft localization of PI3Kβ is crucial for GPCR coupling and appears to be important in a PTEN-null genetic background (Cizmecioglu et al. 2016) (Fig. 2).

The uptake of nutrients is also associated with the PI3K axis and lipid rafts. For example, in 3T3 adipocytes, when the IGF pathway is inactive, extracellular glucose transporter GluT4 is constitutively internalized. However, once activated, IGF signaling inhibits the internalization of GluT4. Uninternalized GluT4 gets colocalized with raft-resident protein flotillin or caveolae-resident protein caveolin3 and mediates glucose uptake (Cav-3) (Fecchi et al. 2006; Ribon et al. 2001). Also, long-chain fatty acid uptake can be regulated through lipid rafts. Fatty acid translocase (FAT)/CD36 was demonstrated to be extracted from the DRM fraction of PM but not from the DSM fraction (Pohl et al. 2004, 2005).

Other than receptor tyrosine kinases, some G-protein coupled receptors (GPCRs) were involved in raft-associated signaling (Ostrom and Insel 2004). Beta-adrenergic, oxytocin, serotonin, and dopamine receptors are among the GPCRs that regulate normal physiological states in the muscular and nervous systems (Björk et al. 2010; Chini and Parenti 2004; Cole and Sood 2012; Ostrom and Insel 2004). Also, several ligand-gated ion channels such as ionotropic receptors are associated with lipid rafts of neurons and muscle cells. Several glutamate receptors and GABA receptors are among them, which were demonstrated to localize to lipid rafts to relay proper excitatory signals in their target tissue

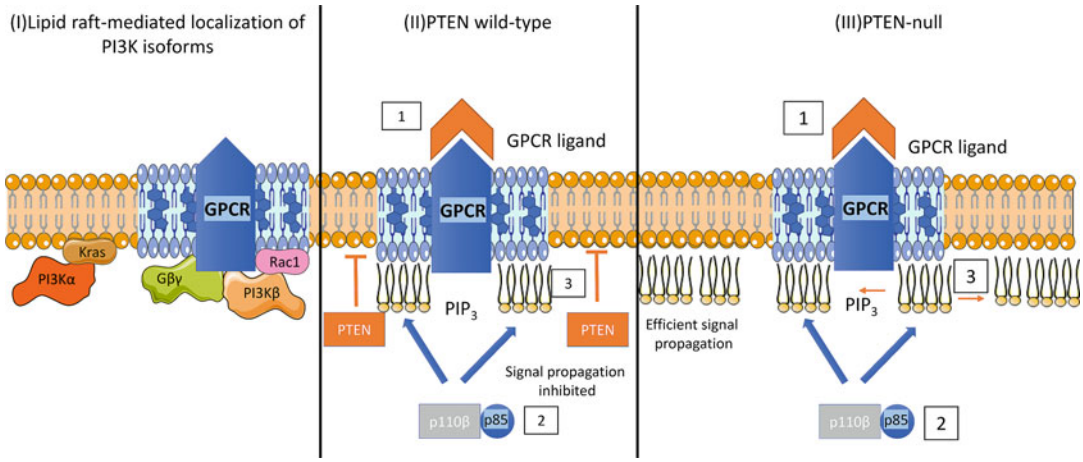

Fig. 2 Regulation of isoform-specific PI3K activity through lipid raft-based compartmentalization. Under resting conditions, PI3Kα localizes to non-raft PM, while PI3Kβ localizes to raft PM together with Gβγ and Rac1, promoting GPCR signaling (I). In PTEN wt cellular conditions, non-raft PM localized PTEN blocks propagation of PIP_3 production and signal (II). The blockage of PIP_3 production in non-raft PM is compromised in PTEN-null cells, and raft-associated GPCR/PI3Kβ signaling gets amplified (III)

(Chini and Parenti 2004; Dalskov et al. 2005; Xu et al. 2014). Aside from GPCRs, membrane compartmentalization has been shown to modulate their related heterotrimeric G proteins (Moffett et al. 2000). Gβγ subunit of heterotrimeric G associates mostly with non-raft PM, while Gα associates with lipid rafts or caveolae. Interestingly, Gα subtypes—$G\alpha_q$, $G\alpha_i$, $G\alpha_s$—also localize differentially to PM compartments. $G\alpha_i$ and $G\alpha_s$ localize to lipid rafts, while $G\alpha_q$ localizes to caveolae through its interactions with Caveolin 1 (a caveolae-resident protein) (Oh and Schnitzer 2001). Gq, on the other hand, has been shown to localize to planar lipid rafts via its interaction with the raft marker flotillin, which stimulates proliferation via the p38/MAPK axis (Sugawara et al. 2007).

5 Apoptosis

Apoptosis, or "programmed cell death," is a physiological process by which cells die during development to give rise to normal morphology or during the cell cycle when cells cannot pass the cell cycle checkpoint due to an acquired mutation. During type I apoptosis, the raft-resident CD95 (Fas) receptor needs to be bound by FasL. As a TNF family protein, CD95 possesses a cytosolic Death Domain (DD), yet the DD of CD95 functions to accumulate CD95 through protein interactions rather than induce apoptosis by itself (Ferrao and Wu 2012; Mollinedo and Gajate 2006). Liganded CD95 needs to accumulate in PM. In time, if apoptotic signals increase, accumulation of liganded CD95 reaches a threshold and recruits Fas-Associated Death Domain (FADD) (Kischkel et al. 1995; Tibbetts et al. 2003) and consecutively Caspase −8/−10 through FADD. With the recruitment of caspases, Fas/CD95, FADD, and Caspase −8/−10 form the death-inducing signaling complex (DISC) (Kischkel et al. 1995). Accumulated procaspase-8 self-cleavage occurs and initiates apoptotic signaling (Muzio et al. 1998). Following ligand interaction, CD95 travels to lipid rafts where its accumulation takes place, leading to the successful production of an apoptotic signal (Hueber et al. 2002). When cells are treated with cholesterol-depleting MβCD, it was demonstrated that apoptosis is inhibited because of the prevention of lipid raft formation, accumulation of CD95, and formation of DISC (Gajate et al. 2009; Gajate and Mollinedo 2015; Hueber et al. 2002; Wajant 2006).

6 Immune Signaling

Lipid rafts are involved in many immunogenic processes. Firstly, one of the members of the innate immune system—Toll-like receptor (TLR4), is shown to be activated by raft-resident protein-CD14 (Wright et al. 1990). CD14 is known for its role in the pathogen recognition process. For this purpose, it forms a ternary complex with bacterial lipopolysaccharide (LPS) and the host LPS binding protein to initiate the immune response (Płóciennikowska et al. 2015; Triantafilou et al. 2011; Wright et al. 1990). It is also worth mentioning that several TLRs have cholesterol-binding motifs, and cholesterol is found to regulate the responses of those TLRs (Wong et al. 2009). Aside from innate immunity, IgE signaling in mast cells is the first known lipid raft-dependent signaling. IgE ligand binds to its receptor FcεRI (Field et al. 1995). Once bound and activated, they go to DRMs to initiate the appropriate signaling (Field et al. 1995). Lymphocyte maturation appears to be through lipid rafts as well. In resting state, BCR and TCR are localized into DSM but once activated; they transfer into DRMs (Beck-García et al. 2015; Dustin et al. 2017; Sproul et al. 2000). Upon their transfer to DRMs, these receptors can colocalize with raft-resident downstream signaling elements of immune signaling such as LAT, LCK, and FYN kinases and start the immune response (Filipp et al. 2004; Levental et al. 2010b).

7 Lipid Rafts in Development and Stem Cell Biology

Embryonic development is one of the fascinating biological processes in which signaling events are regulated along the spatiotemporal dimension.

Although much is known about the signaling components involved in developmental processes, the control of these signals via lipid rafts is relatively unexplored. However, in a few studies, the presence of raft-enriched ganglioside-GM1 was demonstrated in both preimplantation mouse embryos and mouse oocytes (Comiskey and Warner 2007). Depleting cholesterol in these cells hampered raft formation (Comiskey and Warner 2007; Buschiazzo et al. 2013). Also suppressed is raft-resident c-Src, which is involved in the formation of the second polar body (Buschiazzo et al. 2013). Lipid rafts are also implicated in maintaining the identity and self-renewal of some stem cells. The existence of lipid rafts in mesenchymal stem cells and even embryonic stem cells is extensively recognized, although their regulatory roles remain largely unknown (Lee et al. 2010; Sohn et al. 2018). However, the regulation of hematopoietic stem cells (HSCs) by rafts is better characterized. It was shown that raft-resident integrin, CXCR, and CD117 receptors plays essential roles in the HSCs recognition of their stem cell niche (Alomari et al. 2019; Ratajczak and Adamiak 2015; Wysoczynski et al. 2005).

8 Lipid Rafts in Cancer and Metastasis

Cancer is uncontrollable, aberrant cellular proliferation. During carcinogenesis, mutated unhealthy cells are unable to be eliminated by apoptosis. Given the importance of lipid rafts in mitogenic signaling via RTKs and GPCRs, it is not surprising that lipid raft composition is greatly deregulated in tumor cells (Michel and Bakovic 2007; Staubach and Hanisch 2011). Of note, lipid rafts are highly abundant in tumor cells, and lipid rafts existent in tumor cells are highly enriched in cholesterol compared to their untransformed counterparts (Levin-Gromiko et al. 2014; Li et al. 2006; Owen et al. 2012). Furthermore, it has been established that raft integrity is crucial for proliferative signaling in cancer cells. For example, EGFR recruitment to the rafts was shown to be crucial for proliferation in prostate cancer (Zhuang et al. 2002; Zidovetzki and Levitan 2007), and complementarily, the disruption of lipid rafts by statins abolishes the EGF signal, leading to apoptosis (Hea et al. 2007; Hryniewicz-Jankowska et al. 2019; Zhuang et al. 2005). IGF signaling and its downstream PI3K/AKT pathway were affected by lipid raft-mediated regulation. As previously mentioned, AKT activity is controlled through lipid raft-mediated compartmentalization of its activator (PDK1) and negative regulator (PTEN) (Gao et al. 2011). However, this balance is lost in some cancers. For example, in prostate cancer LNCAP cells, raft-resident AKT and non-raft-resident AKT demonstrate different affinities to their substrates (Adam et al. 2007). These AKT pools highlight the presence of two distinct platforms for AKT signaling in the same cell population (Adam et al. 2007). Another example where alterations in raft-mediated regulation of AKT drive tumorigenesis is mantle cell lymphoma (MCL) (Reis-Sobreiro et al. 2013). In MCL cells, AKT is found to be constitutively active (Reis-Sobreiro et al. 2013; Rudelius et al. 2006), and raft-resident PDK1 and mTOR signaling components favor constant AKT activation (Reis-Sobreiro et al. 2013). Conversely, lipid raft disruption by alkyl phospholipid edelfosine displaces all AKT, PDK1, and mTOR from lipid rafts, leading to AKT inactivation and apoptosis (Reis-Sobreiro et al. 2013). Similarly, cholesterol extraction through MβCD prevents AKT activation in many transformed cell lines and promotes apoptosis (Calay et al. 2010; Motoyama et al. 2009).

Besides being an essential constituent of PM and lipid rafts, cholesterol is the precursor of steroid hormones such as estrogen and androgen (Chimento et al. 2019). Both estrogen and androgen-mediated signaling is associated with the development of gender-specific cancers like breast cancer due to aberrant estrogen signaling and prostate cancer due to aberrant androgen signaling in males (Pelton et al. 2012; Yager et al. 2006). Amplification of estrogen receptor α (ER α) occurs in 70% of all breast cancers (Lumachi et al. 2013). Furthermore, it was demonstrated that cholesterol is a direct agonist of estrogen receptor (Casaburi et al. 2018; Wei et al. 2016). Also, a similar relationship was observed between prostate cancer and cholesterol

in prostate cancer mouse models. When fed high cholesterol diet, these mice develop more aggressive tumors compared to mice fed a regular diet (Llaverias et al. 2010). Canonically, steroid receptors belong to the nuclear receptor family and reside either in the nucleus or cytosol (Pietras and Márquez-Garbán 2007). However, in HeLa cells, a pool of Erα receptors was demonstrated to localize PM. Targeting these receptors to PM occurs through the palmitoylation modification carried out by palmityl-transferase (Acconcia et al. 2005; la Rosa et al. 2012). This pool of ERα receptors was shown to reside in lipid rafts and activate MAPK/ERK and PI3K/AKT axes (Márquez et al. 2006; Maselli et al. 2015). It is interesting to note that androgen receptors have also been shown to cluster close to lipid rafts where AKT signaling activity has been detected (Freeman et al. 2007; Pelton et al. 2012).

In addition, lipid rafts are shown to be essential for the metastatic behavior of tumor cells. CD44 is a transmembrane receptor that is involved in cell adhesion. In the clinic, it is used as a metastatic marker for some cancer types (Senbanjo and Chellaiah 2017). Through a mechanism that has been incompletely elucidated, it is believed to regulate the extracellular matrix and induce epithelial-to-mesenchymal transformation (Murai 2015). Proteolytic cleavage of CD44 is necessary for its activity in ECM (Sugahara et al. 2003). CD44 is a raft-resident protein typically, while its protease- ADAM10 resides in non-raft regions (Murai et al. 2011). However, in metastatic tissues, CD44 has been reported to migrate into non-raft areas where it is shed by ADAM10, causing migratory behavior and metastasis (Babina et al. 2014; Murai 2015).

The association of lipid rafts/cholesterol with various cancers is an active area of research. The use of drugs that inhibit cholesterol biosynthesis, like statins (Longo et al. 2020), or the use of raft-dispersing alkyl phospholipids-like edelfosine (Gajate and Mollinedo 2007; Saraiva et al. 2021; Selivanov et al. 2010; Udayakumar et al. 2016) in cancer treatment is currently being explored and discussed among cancer researchers and clinicians.

9 Caveolae

Caveolae is another compartmentalized plasma membrane microdomain described as cholesterol/sphingolipid-rich lipid rafts with cave-like invaginated morphology (Yamada 1955). The presence of caveolin proteins first molecularly characterized caveolae (Kurzchalia et al. 1992; Rothberg et al. 1992; Way and Parton 1995; Zurzolo et al. 1994). More recently, cavin proteins were discovered to be another significant constituent (Hill et al. 2008). For signal transduction, these specialized cellular morphologies present extensive importance. Several potent receptors were shown to be restricted and concentrated in caveolae, making caveolae a vital signaling hub for the cell (García-Cardeñ et al. 1996; Li et al. 1996a; Liu et al. 1996, 1997; Oh and Schnitzer 2001; Rizzo et al. 1998).

The morphological description of caveolae was first made in the gall bladder of mouse epithelium by Yamada and dates to 1955 (Yamada 1955). Later, caveolae were shown to be lipid-rich plasma membrane microdomains similar to lipid rafts, but consequent molecular characterization experiments showed that both have distinct molecular components (Liu et al. 1997; Oh and Schnitzer 2001; Zurzolo et al. 1994). The formation of functional caveolae requires the enrichment and oligomerization of a cholesterol-binding protein named Caveolin 1 (Cav1) (Fra et al. 1995; Kurzchalia et al. 1992; Monier et al. 1995; Murata et al. 1995; Rothberg et al. 1992). Cav1 was the first caveolin protein described, followed by the discovery of other homologs, Caveolin 2 (Cav2) and muscle-specific Caveolin 3 (Cav3) (Scherer et al. 1996; Way and Parton 1995). Much later than the discovery of Cav1, another essential caveolar protein-Cavin 1, was discovered (Hill et al. 2008; Liu et al. 2008). Further, Cavin 1 homologs-Cavin 2, Cavin 3, and Cavin 4 were discovered and found to have roles in caveolar function in mammals (Bastiani et al. 2009; Hansen et al. 2009; Kovtun et al. 2014; McMahon et al. 2009). Among all caveolin and cavin proteins, Cav1 and Cavin 1 were shown to be essential for the formation

of a functional caveolae (Hill et al. 2008; Liu et al. 2008). Caveolae formation starts with binding caveolin proteins to cholesterol in a 1:1 ratio and budding of caveolin-rich vesicles from Golgi network membrane domains (Hayer et al. 2010; Murata et al. 1995). After caveolin accumulation around the cholesterol-rich lipid species, caveolin proteins self-assemble themselves into homo- and hetero-oligomers (Monier et al. 1995). Oligomerized caveolins recruit important lipid species such as phosphatidylserine (PS), phosphatidylinositol phosphate PIP_2, sphingomyelin, and gangliosides (Sohn et al. 2018). Caveolin-rich lipid rafts are further tightened and fixated by the recruitment and the binding of Cavin proteins. By following those steps, caveolae reach their well-known striated disc-like appearance under the electron microscope (Monier et al. 1995; Parton et al. 2021; Rothberg et al. 1992).

10 Compartmentalized Signaling Through Caveolae

Caveolae is a critical regulatory plasma membrane compartment for signal transduction. The regulation of cellular signaling gets affected by caveolae at two levels. First, preassembled signaling complexes are compartmentalized and enriched in the caveolar membrane. Some of the most studied signaling cascades get initiated from this membrane microdomain, and their receptors are restricted at the caveolar membrane (Martinez-Outschoorn et al. 2015; Okamoto et al. 1998). Insulin, EGF, PDGF, GPCR, and eNOS are some of the well-studied cellular signals that are relayed into the cells through caveolae (Couet et al. 1997b; Liu et al. 1996, 1997; Nystrom et al. 2013; Oh and Schnitzer 2001). Second, the activity of these signaling complexes is regulated by caveolae or, more specifically, caveolar protein Cav1. Over the years of research, it was shown that the receptors of the enlisted signaling pathways above, as well as H-Ras, K-Ras, Src-tyrosine kinase, and heme oxygenase, precipitate together with Cav1 in co-immunoprecipitation experiments (Li et al. 1996b; Song et al. 1996; Taira et al. 2011). Further molecular characterization of the interaction of Cav1 with the members of these signaling pathways revealed the Caveolin scaffolding domain (CSD), which is mapped to 82–101 residues of Cav1 and regulates all the Cav1-associated signaling. CSD was shown to have a negative regulatory role on Cav1-associated signaling. For this inhibitory regulation to occur, CSD binds to the caveolin binding motif (CBM), which is present in many Cav1-associated signaling components (Bernatchez et al. 2005; Kirkham et al. 2008; Nystrom et al. 2013; Song et al. 1996; Taira et al. 2011). Although Cav1 interaction was shown to inhibit the basal activation signaling elements such as Src and K-ras (Couet et al. 1997a; Li et al. 1996b), the inhibitory activity of CSD is best characterized in eNOS signaling. First, eNOS hydrophobic pocket, therefore catalytic activity, was shown to be blocked and inhibited by the side-chain extension of phenylalanine 92 (F92) of CSD (Bernatchez et al. 2005; Trane et al. 2014). Moreover, the presence of a peptide identical to CSD was shown to inhibit the eNOS signal. The mutated version of the CSD peptide, which lacks all the aromatic residues (including F92), was found to activate the eNOS signal due to a probable competition between the mutated peptide and Cav1 (Bernatchez et al. 2011). Although CSD is largely acknowledged as the caveolae's signal regulatory component, there is still some dispute in the caveolae field. The main cause of this contention is CSD itself, which may be buried inside the plasma membrane (Kirkham et al. 2008). Recent studies indicated that Cav1 has a more dynamic structure than initially contemplated and that CSD can exist in multiple states, which might support signaling switches in different contexts (Liu et al. 2016; Sinha et al. 2011).

Caveolar proteins other than Cav1 were also found to affect several cellular signals. For example, Cav2 fatty acylation and phosphorylation have a role in regulating insulin signaling pathway. These two post-translational modifications

inhibit the insulin receptor-SOCS3 phosphatase interaction, leading to IRS-1 activation and activated Stat3 translocation to the nucleus (Kwon et al. 2009, 2015; Kwon and Pak 2010). Other studies indicated that Cav3 might function in carrying the membranal signal to the nucleus. For example, C-terminal 154–156 residues of Cav3 were shown to regulate insulin-induced phospho-ERK translocation to the nucleus (Kwon et al. 2011). Interestingly, Cav3 was also found to be crucial for activating the estrogen receptor by the hormone 17β-estradiol (Totta et al. 2016). Cavins as well were found to affect cellular signaling. Firstly, the essential Cavin component of caveolae-Cavin 1 determines the fully functional caveolae pool and, as a result, dictates the location of activated receptors signaling through caveolae (Li et al. 2014; Moon et al. 2014a). Also, Cavin-3 was found to be necessary for ERK activation over AKT activation by keeping caveolae anchored to the cortical cytoskeletal elements through myosin-1c (Hernandez et al. 2013).

Caveolae can also affect cellular signaling by regulating the actin cytoskeleton. As a result, it plays a role in lipid sorting and delivery (Echarri and del Pozo 2015). Consequently, caveolae can function in the organization of nanoscale membrane domains, which ultimately control the activation of signaling receptors (Blouin et al. 2016). Mechanical forces can as well affect caveolae dynamics, contributing to the organization of the plasma membrane by the caveolae (Nassoy and Lamaze 2012; Sinha et al. 2011). As an example, c-Src gets activated when caveolar disassembly occurs due to the distribution of Cav1 and membrane lipids like sphingolipids due to membranal stretch (Gervásio et al. 2011). More interestingly, the absence of Cav1 leads to the disorganization of Ras protein, lipid, and phosphatidylserine distribution (Ariotti et al. 2014). Finally, caveolar disassembly was shown to affect changes in Gaq-Cav1 interaction and result in reduced Ca^{+2} signaling, concomitant with perturbations in the localization of calcium pumps (Fujimoto 1993; Gervásio et al. 2011; Guo et al. 2015).

11 Caveolae in Cancer

Caveolar composition and signaling have long been related to cancer. First, it was observed that Cav1 levels decreased and caveolae got lost during the cellular transformation of NIH 3T3 cells after the expression of oncogenes (Koleske et al. 1995). Cav1 expression levels are inversely correlated with the colony size of the transformed cells (Koleske et al. 1995). Also, Cav1 knock-out mice exhibit hyperproliferation in the lung and vascular tissues (Drab et al. 2001; Razani et al. 2001). Next, a role for Cav1 in breast cancer is described. Cav1 deletion in MMTV-PyMT model of breast cancer in mice leads to a delayed initiation of tumor growth, an increase in tumor burden, ERK1 hyperactivation, and cyclinD1 overexpression (Williams et al. 2004). However, cancer progression and survival studies revealed that the main predictor of survival is the Cav1 level in stromal cells rather than epithelia (Sloan et al. 2009; Witkiewicz et al. 2009). The lower the expression of Cav1 in stromal cells, the faster the cancer progresses (Sloan et al. 2009; Witkiewicz et al. 2009). Lisanti and colleagues isolated breast stromal cells (cancer-associating fibroblasts (CAFs)) from patients and compared their metabolism to normal fibroblasts of the matched patient (Martinez-Outschoorn et al. 2015; Pavlides et al. 2009). CAFs represent significant metabolic/expressional differences compared to normal fibroblasts. Then, they devise a physiological mechanism named the "Reverse Warburg Effect" in which transformed cells induce a differentiation program in stromal cells (Martinez-Outschoorn et al. 2015; Pavlides et al. 2009) Therefore, the idea is that cancer cells induce tumor stromal cells to undergo a myofibroblast differentiation. Differentiated tumor stroma activates TGFβ signaling and goes through a metabolic transformation yielding a Warburg metabolism in stromal cells, although present in normoxic conditions. Metabolically altered stroma then sustains the tumor growth and progression by representing metabolites like lactate or pyruvate (Pavlides et al. 2009). Besides Cav1,

caveolar protein cavin1 is considered a prognostic marker for prostate cancer (Moon et al. 2014b), while caveolar CD36 protein level is associated with breast cancer (DeFilippis et al. 2012).

12 Concluding Remarks

After its initial postulation in 1997 (Simons and Ikonen 1997), lipid rafts became a generally accepted concept in recent years. Advances in microscopy techniques revealed that PM is not only "not homogenous" but much more heterogenous than we thought before. There are probably different subtypes of lipid rafts. For example, a two-color PALM study has shown that TCR, Zap70, and LAT proteins are destined to different membrane microdomains upon activating TCR signaling (Sherman et al. 2011). The concept of distinct lipid raft types brings some provoking questions as "How can we differentially mark these raft species molecularly?", "How proteomics of distinct raft types differ?", "How can specific proteins are targeted to different raft species?", "Are these different subcompartments preassembled or co-assembled?" Another outstanding question is, "What would we encounter if we increase lipid raft resolution into atomic levels?". In other words, "Can we crystalize these solid-like microdomains and determine their structure by X-ray crystallography?". The crystallization of lipid rafts has been speculated earlier (de Almeida and Joly 2014). Moreover, there were few attempts to crystalize lipid rafts in model membranes, but the data acquired in these studies were restricted (de Almeida and Joly 2014; Park et al. 2020; Ziblat et al. 2012). However, recently developed co-Mesh X-ray crystallography could be a promising method to unravel the structures of lipid rafts. With this technique, serial X-ray images of multiple crystals are acquired and assembled so that they can present information on the dynamic nature of the molecules within the crystals (Sierra et al. 2015). As a result, this approach might be useful for determining the structure of lipid rafts.

Now, it is clear that planar lipid rafts and caveolae are both cholesterol-sphingolipid-rich membrane domains, one favoring the presence of flotillin and the other caveolin. However, we are unaware of how they are specifically localized to the PM, meaning if planar/non-planar rafts favor particular dimensional positioning within the cell. In those terms, we need more precise knowledge of the interactions between rafts and cytoskeleton. Another interesting and not thoroughly answered question is, "Within the same organism, why do some cells acquire caveolae and why do others not?" It is now known that the presence of both Cav 1 and Cavin 1 is a prerequisite for caveolae formation (Parton et al. 2021). However, we are still far from understanding how both proteins integrate at the membrane and enable the typical membrane curvature of caveolae.

In some disease conditions, it is now well characterized that lipid raft-mediated signaling gets perturbed. Mainly in many cancer types, it was observed that raft-mediated signaling is deregulated. It can be reflected by the increased cholesterol levels within the whole cells and cholesterol within the rafts of cancers compared to their untransformed counterparts. It is yet unclear what kind of dysregulations take place in the rafts of those cancer cells. For example, we know that when cells are depleted from cholesterol by MβCD, EGFR moves into non-raft regions, and tumorigenic signaling is activated. Nevertheless, quite the opposite effect is observed for other RTKs. For example, raft blockage results in the inhibition of proliferative signaling of IGFR. So, "What specific raft-mediated mechanisms distinguish these akin signaling molecules?"

Another topic open to discussion is the disruption of lipid rafts as a therapy against cancer. First, the use of raft inhibiting—alkyl phospholipid-like edelfosine—is being investigated as an anticancer drug (Mollinedo 2014). The anticancer effects of edelfosine are demonstrated in many studies in the cell culture or xenografted animals of several types of tumors (Mollinedo 2014). Also, another edelfosine analog, perifosine, is under investigation for its anticancer effects on hematological cancers and solid tumors (Richardson et al. 2012). A more dramatic approach is using cholesterol synthesis inhibitors-statins for cancer

treatment. Like perifosine, statins are also investigated in numerous clinical trials (di Bello et al. 2020). Given the critical functions of lipid rafts and cholesterol, the value of inhibiting them will become evident in the coming decade.

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