COMMENTARY

Nanoclustering as a dominant feature of plasma membrane organization

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ABSTRACT

Early studies have revealed that some mammalian plasma membrane proteins exist in small nanoclusters. The advent of super-resolution microscopy has corroborated and extended this picture, and led to the suggestion that many, if not most, membrane proteins are clustered at the plasma membrane at nanoscale lengths. In this Commentary, we present selected examples of glycosylphosphatidyl-anchored proteins, Ras family members and several immune receptors that provide evidence for nanoclustering. We advocate the view that nanoclustering is an important part of the hierarchical organization of proteins in the plasma membrane. According to this emerging picture, nanoclusters can be organized on the mesoscale to form microdomains that are capable of supporting cell adhesion, pathogen binding and immune cell-cell recognition amongst other functions. Yet, a number of outstanding issues concerning nanoclusters remain open, including the details of their molecular composition, biogenesis, size, stability, function and regulation. Notions about these details are put forth and suggestions are made about nanocluster function and why this general feature of protein nanoclustering appears to be so prevalent.

KEY WORDS: Plasma membrane, Protein nanoclustering, Super-resolution microscopy

Introduction

Organization by compartmentalization is a general property of natural systems that efficiently facilitates and orchestrates biological events in space and time. Cells are primary examples of well-defined biological compartments within tissues. However, cells also exhibit a number of compartmentalization strategies, including membrane-delimited intracellular organelles (e.g. endosomes and lysosomes) and multi-enzyme complexes. This compartmentalization of specific cellular functions, through spatial localization, increases regulation efficiency. In addition,

the plasma membrane with its extracellular matrix and the subjacent membrane-associated cytoskeleton is also highly organized – with areas in which specific components are enriched or to which they are recruited – thereby providing specialized cellular regions, such as the basal and the apical membrane in polarized cells, the immunological synapse in interacting leukocytes or multimolecular membrane-cytoskeleton assemblies that include focal adhesions and podosomes. Once considered a relatively unstructured 'sea' of lipids and proteins that are potentially able to form aggregates (Singer and Nicolson, 1972), the plasma membrane is now widely accepted as being highly compartmentalized, thus allowing lipids and proteins to be organized in specific regions of varying size and composition (Kusumi et al., 2011; Maxfield, 2002; Nicolson, 2013).

One of the most prominent concepts for membrane compartmentalization refers to the 'lipid raft hypothesis'. The raft model originated from a study more than 25 years ago (van Meer et al., 1987) and has been extensively reviewed since then (Lingwood and Simons, 2010; Simons and Toomre, 2000). The lipid raft notion proposes the existence of small plasma membrane compartments that are enriched in cholesterol and sphingolipids, and populated by 'raftophilic' proteins such as, for example, glycosylphosphatidyl-anchored proteins (GPI-APs). Persistent controversies have led to a provisional contemporary definition that emphasizes the small and transient nature of putative lipid rafts (Pike, 2006). Yet, when considering the current, general view about the nature of lipid rafts, it must be borne in mind that membranes are rich in proteins (Jacobson et al., 2007) and that any preferential residence of membrane proteins in regions populated by raft lipids most likely occurs only on a very small spatial and temporal scale (Eggeling et al., 2009). At present, the existence of relatively large, and predominantly 'lipid raft' domains is expected to be the exception rather than the rule.

Another prominent concept for plasma membrane compartmentalization is based on the meshwork of filamentous proteins including F-actin and spectrin that immediately underlie the plasma membrane. This model originated over three decades ago on the basis of lateral diffusion studies of proteins in the membranes of red blood cells (Sheetz, 1983) and was later expanded by work from Akihiro Kusumi's group, who termed it the membrane cytoskeleton 'fence' (Kusumi et al., 2005). They also coined the term 'anchored transmembrane picket', which refers to the more or less regular array of transmembrane proteins (posts) that are anchored to the underlying membrane-associated cytoskeletal fence serving to compartmentalize the membrane. Thus, long-range diffusion of both transmembrane and lipid-anchored proteins is restricted because it is rate-limited by fluctuations in the cytoskeletal 'fence–picket' structure that

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permits the inter-compartmental barriers to be traversed by the diffusing molecule. By contrast, short-range diffusion within each compartment is much more rapid.

Other notions have also been put forth with regard to factors that govern plasma membrane compartmentalization on different length and time scales, such as lipid shells (Anderson and Jacobson, 2002), tetraspanin-enriched microdomains (Yáñez-Mó et al., 2009) and galectin lattices (Lajoie et al., 2009). Moreover, recent evidence suggests that these possible local organizers are not independent from each other but, rather, act in a synergistic manner that remains to be precisely defined (Delaguillaumie et al., 2004; Lajoie et al., 2009).

The term 'microdomain' is generally employed to describe membrane compartmentalization that occurs either naturally or is ligand induced. However, the size of such domains can vary from a few nanometers to several micrometers, most probably owing to their different molecular composition and different capacities to coalesce with or segregate from each other. A plausible hypothesis is that plasma membrane compartmentalization is based on a hierarchical organization of molecules that ranges from classic protein oligomerization to nanometer-sized or micrometer-sized clusters, which may provide multi-scale regulation of membrane protein function. However, in this Commentary, we will focus on organization at the smallest scale, namely, the association of membrane proteins into clusters of nanometer dimensions into which other proteins or lipids may be incorporated.

The rapid development of super-resolution optical microscopy (Table 1) and single-particle tracking approaches in the past few years has provided – and is continuing to provide – indisputable evidence for nanoclustering of many, if not most, proteins on mammalian cell plasma membranes – even in the absence of any activation or ligand binding. Whereas examples of nanoclustering in neurobiology can be found in a recent review (Willig and Barrantes, 2014), we will focus here on some other salient examples of receptor nanoclustering, including those involved in pathogen binding, cell adhesion and the immune response.

The diversity of membrane protein nanoclustering
Lipid-anchored proteins
Some of the smallest documented nanoclusters are those that contain GPI-APs. These extracellular, lipid-linked proteins constitute a diverse set of proteins tethered to the outer leaflet of the plasma membrane that exhibits specific sorting and signaling properties. GPI-APs are regulated by alterations in cholesterol and sphingolipid levels in cell membranes and, as such, have been considered to be classic lipid-raft markers (Mayor and Riezman, 2004). The first studies discussing the unusual nature of GPI-AP nanoclustering were reported by two different groups using complementary approaches; i.e. chemical crosslinking (Friedrichson and Kurzchalia, 1998) and fluorescence resonance energy transfer (FRET) (Varma and Mayor, 1998). Later, using a combination of homo- and hetero-FRET techniques, it was shown that GPI-APs co-exist on the cell membrane as mixtures of monomers together with a fraction (20–40%) that includes cholesterol-sensitive oligomers (<5 nm) composed of at most four molecules; this was shown for a diverse set of GPI-AP species (Sharma et al., 2004). Surprisingly, the relationship between monomers and nanoclusters did not obey the mass-action law that would be expected for a classic chemical equilibrium, because the percentages of GPI-APs nanoclusters
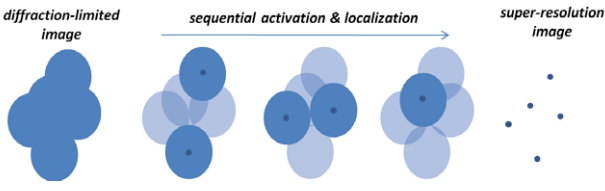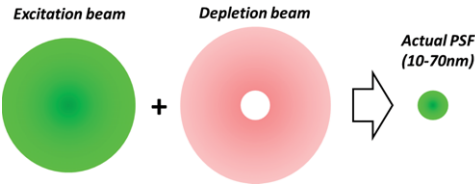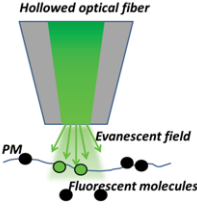
and their molecular density were concentration-independent (Mayor and Rao, 2004). Importantly, GPI-AP nanoclustering has been more recently confirmed by super-resolution microscopy techniques (Table 1), including near-field scanning optical microscopy (NSOM) (van Zanten et al., 2009) (Fig. 1) and photoactivated localization microscopy (PALM) (Sengupta et al., 2011). Single-particle tracking (SPT) and fluorescence correlation spectroscopy (FCS) studies on nanoscale volumes (Eggeling et al., 2009) have provided further evidence for the existence of small GPI-AP nanoclusters in living cell membranes – although, as recently pointed out, an entirely consistent picture has not yet emerged owing to the high number of different techniques employed (Klotzsch and Schütz, 2013).

Other lipid-tethered proteins at the inner leaflet of the membrane, e.g. the Ras family of GTPases, also form nanoclusters that are composed of small numbers of proteins, as observed by immunogold electron microscopy (EM) (Parton and Hancock, 2004). Ras isoforms are distinguished by differences in the structure of their lipid anchors; e.g. K-Ras is tethered to the membrane by a farnesyl group and a polybasic region, whereas H-Ras is anchored by two palmitoyl chains and a farnesyl group. EM imaging of the membrane-targeting regions of these two Ras isoforms has shown that fewer than ten copies of both K-Ras and H-Ras are localized in largely separated nanoclusters of less than 30 nm within the inner leaflet of the plasma membrane (Plowman et al., 2005; Prior et al., 2003). Ras nanoclusters had lifetimes of less than 1 second, were immobile and constituted about 40% of the total Ras population, with the remaining population being monomeric and laterally mobile (Murakoshi et al., 2004). The properties of Ras nanoclusters are complicated by their dependence on the structure of the membrane anchor and the hyper-variable region of the protein moiety, the presence of cholesterol, extrinsic clustering factors and whether the isoform is loaded with GTP or GDP (Harding and Hancock, 2008). For example, when loaded with GDP but not GTP, H-Ras nanoclusters were localized in lipid rafts based on the affinity of their lipid chains for raft domains and by the fact that the localization required cholesterol (Harding and Hancock, 2008). By contrast, the K-Ras targeting domain also localized in nanodomains but this localization did not depend on cholesterol. In general, the field appears to be moving towards a consensus that GPI-AP and Ras nanoclusters constitute nucleation sites for signaling at the cell membrane that can grow and be stabilized by the recruitment of additional components to create active functional platforms for specific cellular functions (Cebecauer et al., 2010; Mayor and Rao, 2004; van Zanten et al., 2009).

Of considerable interest is the nature of the factors that regulate and stabilize nanoclusters of lipid-anchored proteins. In general, lipid-linked protein nanoclusters might be stabilized by *cis* associations of their protein ectodomains, cytosolic domains, lipid anchors or a combination of these. For example, Akihiro Kusumi's group recently showed that ectodomain interactions are the primary factor in transiently stabilizing homodimers of the GPI-AP CD59, with cholesterol and other lipids having secondary stabilizing roles (Suzuki et al., 2012). Furthermore, in supported lipid bilayers, Ras GTPases dimerize using a specific motif in their cytosolic domains (Lin et al., 2014). These studies suggest that protein–protein interactions among identical lipid-linked proteins play a significant role with respect to the stability of lipid-linked protein nanoclusters.

Accessory molecules that are located outside of the membrane might also be key to the formation, size and stability

Table 1. Super-resolution microscopy approaches to image nanoclusters in membranes

Method	Description	Benefits	Limitations
Single-molecule localization microscopy (SMLM)	Localization of individual molecules that results in high-precision maps of molecular positions. This group of methods is based on stochastic activation of dispersed fluorophores and subsequent analysis to obtain the centers of the fluorescent spots within the images. Main variants: –PALM and fPALM, based on photoactivatable fluorescent probes –STORM, dSTORM and the BLINK technique, based on photo-switchable fluorescent probes	High lateral precision (10–50 nm) spatial mapping of molecular positions. SPT-PALM of slowly diffusing components is achievable in live cells. Multi-color imaging is possible.	Slow (data acquisition minutes to hours). Requires additional equipment to achieve axial super resolution (e.g. interferometry PALM). Highest resolution requires fixed samples. Blinking and/or switching of organic dyes requires deoxygenated buffers.
Stimulated emission depletion (STED) microscopy	Enhanced resolution results from narrowing of the point-spread function. It is based on a doughnut-shaped excitation beam superimposed on the primary Gaussian profile excitation beam resulting in depletion of emission from the outside ring of the excitation spot. A super-resolution image is generated by scanning the overlapping excitation beams over the sample.	High lateral resolution (30–100 nm). FCS-STED is possible in living cells. 3D-imaging of sub-cellular structures. Applicable to living specimens.	Involves expensive, specialized lasers and optics. High intensity of the depletion beam. Limited combination of fluorophores can be employed. Dual-color imaging with equal spatial resolution is challenging.
Near-field scanning optical microscopy (NSOM)	Excitation light is guided through a sub-wavelength aperture probe that is scanned over the sample. It relies on near-field excitation and requires close proximity of the NSOM probe (tapered optical fiber) and the sample.	Scanning tip delivers a simultaneous topography map. Multi-color imaging with equal resolution. Limited evanescent field eliminates cytosolic background. Perfectly suited to image apical cell membrane. FCS-NSOM possible in living cells.	Spatial resolution is dictated by probe diameter (70–90 nm). Mostly used for fixed samples. Technically challenging. Probe fragility.

dSTORM, direct STORM; FCS, fluorescence correlation spectroscopy; fPALM, fluorescence PALM; PALM, photoactivated localization microscopy; SPT, single-particle tracking; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy.

of lipid-linked protein nanoclusters. These accessory molecules include proteins associated with the actin cortex, the extracellular matrix and other molecules – including galectins – that have the capacity to bind galactose or galactose-derivative containing polysaccharides (Vasta, 2009). For example, intracellular galectins were shown to be important in stabilizing and enlarging GTP-loaded Ras nanoclusters (Belanis et al., 2008). In response to growth factor receptor activation, galectin-1 – which has the capacity to bind intracellular prenyl groups – translocates from the cytosol to the membrane and becomes important in recruiting GTP-loaded H-Ras to nanoclusters that do not require cholesterol and function as signal transduction

platforms (Belanis et al., 2008; Prior et al., 2003). In this case, galectin-1 appears to replace cholesterol as the factor that stabilizes H-Ras-GTP nanoclusters. Moreover, when H-Ras is depalmitoylated, galectin-1 acts as a chaperone to transfer H-Ras to the Golgi.

In the case of GPI-APs, the cortical actin cytoskeleton must be considered because it can regulate the extent of nanoclustering (Goswami et al., 2008). This regulation can be either passive or active. In the first case, and as discussed in the Introduction, the membrane skeleton can be coupled to unspecified transmembrane proteins that act as 'picket fences' to create corrals in the plane of the membrane with dimensions that vary between 30 and 250 nm
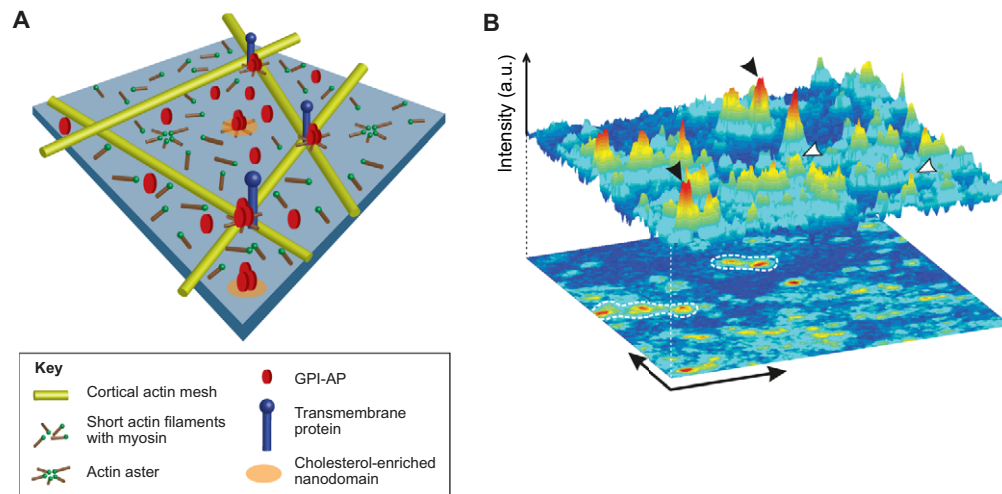
Fig. 1. Nanoscale organization of GPI-APs on the cell membrane. (A) The organization of GPI-APs on plasma membranes as co-existence between monomers and small nanoclusters containing a few molecules. Nanocluster formation appears to be driven by cortical actin (hypothetical actin asters) and maintained by weak interactions with cholesterol (Gowrishankar et al., 2012). These small nanoclusters can be further stabilized by cortical actin through asters and/or proximal transmembrane proteins that act as 'posts' attached to the cortical actin. The physical separation between GPI-AP nanoclusters has been observed to be within 200–250 nm (van Zanten et al., 2009). (B) (Top) 3D intensity projection of a super-resolution NSOM image showing the co-existence of nanoclusters (black arrowheads) and monomers (white arrowheads) of GPI-APs. (Bottom) Areas encircled by a dashed line on the 2D image illustrate that nanoclusters prefer to concentrate at specific sites as hotspots that are typically separated by less than 250 nm. This characteristic separation might reflect the physical dimensions of the actin meshwork and/or spatial distribution of actin asters (van Zanten et al., 2009; Gowrishankar et al., 2012). a.u., arbitrary units. Image reproduced with permission (van Zanten et al., 2009).

depending on the cell type (Kusumi et al., 2011) (Fig. 1A). These fences could determine the upper limit on the size of nanoclusters by interposing a collection of transmembrane proteins acting as fences between adjacent nanoclusters.

It has been also proposed that dynamic, short actin filaments form aster-like aggregates under the plasma membrane that actively drive lipid-anchored proteins into nanoclusters (Gowrishankar et al., 2012) (Fig. 1A). The observation that GPI-AP nanoclusters are found to be spatially separated by a characteristic distance of less than 250 nm (van Zanten et al., 2009), further indicates that the assembly of actin asters close to the inner leaflet of the plasma membrane occurs at this spatial scale (Fig. 1B). However, a main unresolved issue is what gives rise to the coupling of GPI-AP nanoclusters in the outer leaflet with the sub-membranous actin cortex. One possible mechanism is that GPI-APs are coupled to complementary nanodomains (consisting of lipids, proteins or both) that reside in the inner membrane leaflet. These putative inner-leaflet nanodomains could associate with small, dynamic actin filaments in the cortex in a yet unknown fashion and actively draw GPI-APs into nanoclusters through myosin motors. Alternatively, GPI-AP nanoclusters could associate with transmembrane proteins that are coupled to the dynamic actin filaments.

Indeed, transmembrane proteins that are anchored to the cytoskeleton, either permanently or in a transient and regulated manner, might also serve as 'posts' that stabilize GPI-AP nanoclusters through *cis* interactions they undergo with nanocluster components. This mechanism was, in fact, originally proposed to explain how membrane proteins are linked to the underlying membrane-associated cytoskeleton, as observed during patching and capping (Singer, 1977). This is a phenomenon in which membrane proteins that have been crosslinked by antibodies passively form patches that are subsequently actively cleared to a distal part of the cell surface through coupling to a retrograde cortical actin flow. However, a

key issue is the identity of the transmembrane proteins involved and how the linkage to the cytoskeleton might be regulated. In this regard, GPI-AP nanoclusters that have been induced by antibody-coated, multivalent gold particles undergo transient anchorage (Chen et al., 2006), whereby their lateral diffusion is arrested for several seconds before they become mobile again. Moreover, this transient anchorage is dependent on cholesterol and one of the seven transmembrane adaptor proteins (TRAPs), namely CBP (officially known as PAG1) (Hořejší et al., 2004). CBP is involved in anchoring GPI-AP nanoclusters in a manner that depends on phosphorylation and the cytoskeletal adaptor ERM-binding protein 50 (EBP50; officially known as SLC9A3R1), which binds to members of the ezrin–radixin–moesin (ERM) family (Chen et al., 2009). Presumably, the CBP–EBP50–ERM complex could link a GPI-AP nanocluster to the underlying actin cytoskeleton. A similar transient anchorage, probably also mediated by components of the membrane-associated cytoskeleton, termed stimulated temporary arrest of lateral diffusion (STALL), has been observed for ligand-induced nanoclusters of the GPI-AP CD59. Moreover, these STALL locations were associated with downstream intracellular signaling processes (Suzuki et al., 2007).

Recent two-color super-resolution studies also suggest that the cortex has an important role because enhanced cortical actin was found to be associated with nanodomains of the influenza envelope transmembrane protein hemagglutinin (HA) (Gudheti et al., 2013). This association could limit the lateral mobility of HA in actin-enriched regions. Moreover, the authors were able to alter domain size and HA density within the domain by using reagents that perturb F-actin. In addition, the actin-binding and actin-disassembling factor cofilin is predominantly excluded from actin-enriched regions that underlie the HA nanodomains, suggesting that HA and/or proteins within the HA cluster mediate this exclusion (Gudheti et al., 2013). All of the studies described above not only demonstrate that the actin cortex is an

important determinant of nanocluster organization but also that nanoclusters have the potential to regulate the distribution of actin-binding proteins in the underlying cortex.

Transmembrane proteins involved in the immune system

In addition to lipid-linked proteins, many transmembrane proteins also form nanoclusters. Below, we will discuss examples of transmembrane protein nanoclusters with relevance for immunological processes.

LFA-1

The main integrin receptor involved in leukocyte adhesion is the lymphocyte function-associated antigen-1 (LFA-1, officially known as ITGAL), which forms nanoclusters of ~70 nm on monocytes prior to ligand binding (Cambi et al., 2006). The integrity of these nanoclusters is independent of cholesterol or the actin cytoskeleton and the clusters contain on average six LFA-1 molecules (Cambi et al., 2006). By using NSOM, a first level of hierarchy for LFA-1 organization was defined because ligand-independent LFA-1 nanoclusters resided within 50–100 nm of GPI-AP nanodomains (van Zanten et al., 2009) (Fig. 2). Upon ligand-induced LFA-1 activation, a higher level of hierarchy that is associated with function was observed (van Zanten et al., 2009). Indeed, coalescence of the two nanodomain types and further recruitment of GPI-APs to these sites resulted in the formation of stable cell adhesion nanoplatforms (van Zanten et al., 2009) (Fig. 2). Although there is no clear explanation yet with regard to what keeps LFA-1 in nanoclusters, it is tempting to

speculate that LFA-1 and GPI-AP nanoclusters are somehow interconnected by the actin cytoskeleton to the sub-membranous actin asters described above (Gowrishankar et al., 2012). More recently, using a combination of NSOM and SPT, the importance of the actin cytoskeleton was demonstrated for inside–out activation of LFA-1, its stable ligand binding and firm cell adhesion (Bakker et al., 2012). Although LFA-1 nanoclustering in resting monocytes was observed prior to ligand activation, it is worth mentioning that other integrins, such as β_1 and β_3, form nanoclusters upon ligand activation (Rossier et al., 2012; Yu et al., 2012). These nascent nanoclusters are thought to play a key role in the initiation of cell adhesion (Yu et al., 2012) and in the formation of focal adhesion complexes (Rossier et al., 2012; van Zanten et al., 2009; Yu et al., 2012).

DC-SIGN

The pathogen recognition receptor dendritic cell-specific ICAM-3-grabbing non-integrin 1 (DC-SIGN; officially known as CD209) binds an astonishingly large variety of human infectious agents ranging from HIV to dengue viruses to yeasts. It is found on the surface of immature dendritic cells for the purpose of subsequent antigen presentation. Biochemical studies have shown that DC-SIGN predominantly forms tetramers (Feinberg et al., 2005; Serrano-Gómez et al., 2008; Yu et al., 2009). Recently, work from our laboratories using EM and different super-resolution approaches demonstrated that DC-SIGN forms nanoclusters on immature dendritic cells (or following ectopic expression in rodent cells) prior to pathogen
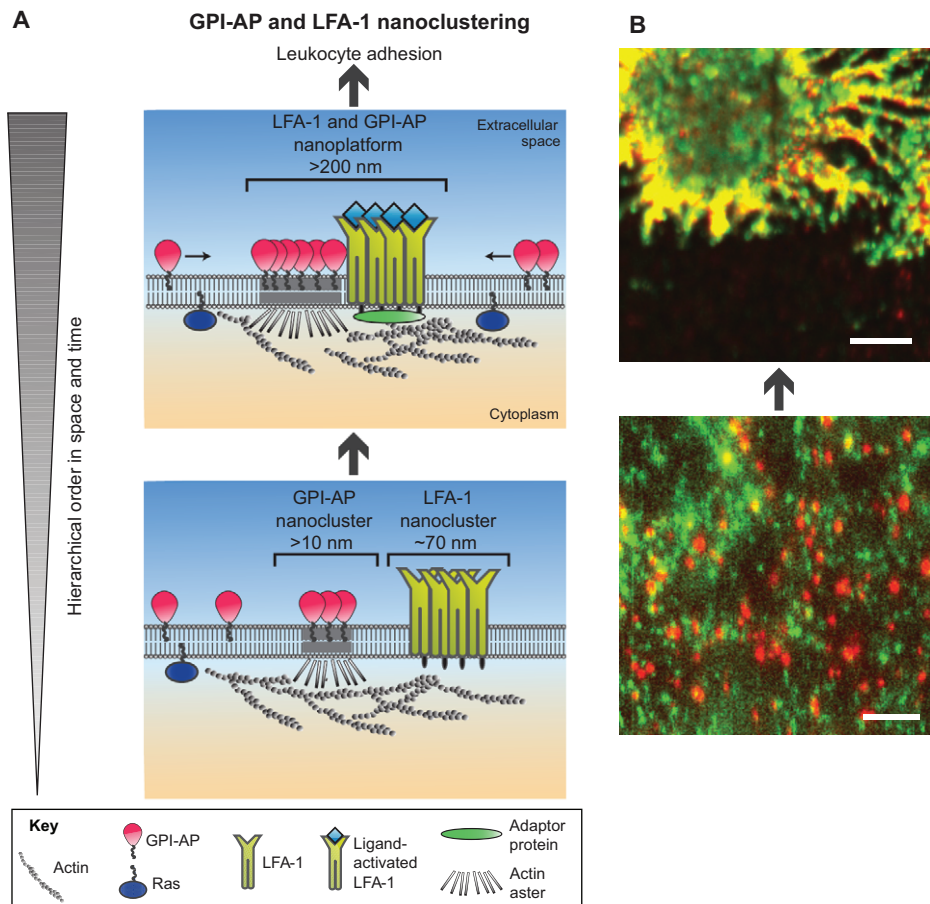

Fig. 2. Hierarchical organization of GPI-APs and the cell adhesion integrin receptor LFA-1. The inverted triangle at the left indicates the increase in hierarchical order in space and time from bottom to top of each panel. (A) Bottom panel: GPI-AP nanoclusters (containing two to four molecules in total) and pre-formed LFA-1 nanoclusters (six to ten molecules in total) have been observed in close proximity from each other (50–150 nm) before LFA-1 is activated through ligand binding. Top panel: Activation of LFA-1 through ligand binding, correlates with an increase in the number of GPI-AP molecules in each nanocluster that is likely to be mediated by local rearrangements of the cytoskeleton through adaptor proteins (green). Furthermore, incorporation (arrows) of mobile monomeric GPI-APs and diffusible LFA-1 nanoclusters (Bakker et al., 2012) can further strengthen the nanoclusters and lead to the assembly of LFA-1- and GPI-AP-containing nanoplatforms that are adhesion competent. (B) Bottom panel: Representative super-resolution NSOM images of GPI-AP (green) and LFA-1 nanoclusters (red) in resting monocytes show that LFA-1 and GPI-AP are not associated but in close proximity to each other. Scale bar: 1 mm. Top panel: Ligand activation of LFA-1 leads to aggregation of GPI-APs and LFA-1 (visualized by the substantial increase in yellow areas, which indicates spatial colocalization at the nanoscale) into adhesion-competent nanoplatforms. Scale bar: 5 μm. Images have been taken with permission from van Zanten et al., 2009.
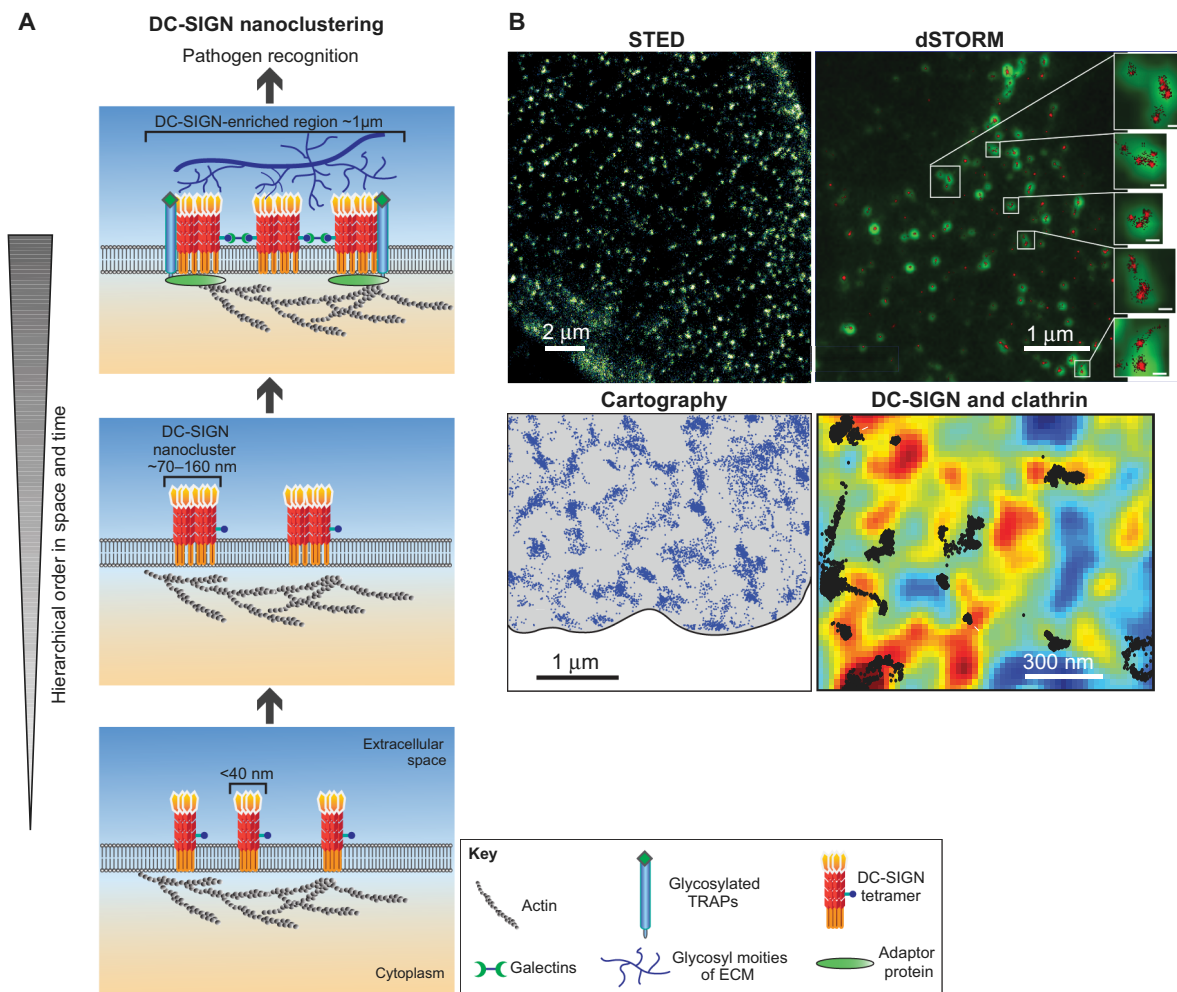
Fig. 3. Hierarchical organization of the pathogen recognition receptor DC-SIGN. The inverted triangle to the left indicates the increase in hierarchical order in space and time from bottom to top of each panel. (A) DC-SIGN is thought to be expressed predominantly in the form of tetramers (bottom panel) on the cell surface. These then aggregate further and form DC-SIGN nanoclusters (middle panel). Nanoclusters, in turn, are recruited to specialized regions of the cell membrane (top panel) and maintained through a number of additional interactions with, e.g. TRAPs (see text). (B) Representative images from different super-resolution and SPT methods illustrate the highly hierarchical organization of DC-SIGN. The stimulated emission–depletion (STED) microscopy image (top left), with a resolution of ~90 nm, clearly shows DC-SIGN nanoclusters. As shown in the enlarged regions of the direct stochastic optical reconstruction microscopy (dSTORM) image (top right), nanoclusters appear to be in close proximity to each other. The cartography map (bottom left) represents the reconstructed molecular positions (blue dots) obtained from single-particle tracking (SPT) movies of several DC-SIGN nanoclusters as they explore the cell membrane (Torreno-Pina et al., 2014). This map demonstrates that DC-SIGN nanoclusters explore restricted areas of ~1 μm, which is consistent with static dSTORM images. Superimposition of a DC-SIGN map with images of clathrin illustrate that DC-SIGN compartments are enriched with clathrin (bottom right). Black areas indicate the position of DC-SIGN, the colored background represents the intensity of the clathrin signal from low (blue) to high (red) intensity. Adapted with permission from Torreno-Pina et al., 2014.

binding (Fig. 3). The nanoclusters vary in size from 70–160 nm and contain around ten molecules per nanocluster (Cambi et al., 2004; de Bakker et al., 2007; Itano et al., 2012; Liu et al., 2014; Manzo et al., 2012).

Interestingly, the 'neck' region of DC-SIGN is presumably not only responsible for tetramer formation (Serrano-Gómez et al., 2008) but also, in part, for its nanoclustering, indicating that its organization on the cell membrane is also hierarchical (Manzo et al., 2012). Furthermore, mutation studies suggested that ectodomain interactions that involve the carbohydrate recognition domain (CRD) of DC-SIGN serve to stabilize nanoclusters (Liu et al., 2012). DC-SIGN organization in nanoclusters has been shown to be crucial for enhancing its binding capability to small pathogens such as viruses (Cambi et al., 2004; Manzo et al., 2012), indicating that hierarchy in

organization correlates with function. There are several possibilities for how DC-SIGN nanoclusters can become stabilized (Liu et al., 2012). The DC-SIGN CRD could bind to glycosaminoglycans or glycosyl moieties of ECM proteins, or to transmembrane proteoglycans that link to the ECM or to TRAPs, which might be directly or indirectly linked to the membrane-apposed cytoskeleton. More recently, we found that DC-SIGN nanoclusters further organize into larger compartments of ~1 μm in size that are maintained by interactions between the receptor glycosylation motif and surface galectins (Torreno-Pina et al., 2014) (Fig. 3). These regions appeared enriched in the endocytic vesicle coat-component clathrin, and its interactions with DC-SIGN are highly affected by the glycan network. As such, cell surface glycan-mediated interactions might represent another, previously unidentified, layer of cell membrane organization at

the microscale that could have functional relevance in regulating clathrin-mediated endocytosis of the receptor and its bound ligands. Given that many membrane receptors are glycosylated, it is conceivable that glycan interactions have a role in the spatiotemporal organization of other receptors, in a manner that is similar to what has been observed for DC-SIGN.

T-cell receptors, linkers for activation of T cells and B-cell receptors

Several other receptors involved in the immune system have also been shown to form nanoclusters (Schamel and Alarcón, 2013). For instance, there is accumulating evidence that T-cell receptors (TCRs) form nanoclusters on the surface of unstimulated T cells. Initial biochemical and EM experiments carried out in Alarcon's group showed that monomers and TCR nanoclusters co-exist on resting T cells (Schamel et al., 2005). Another study confirmed this by using the two-color coincidence-detection technique but found a smaller population of nanoclusters compared to monomers (James et al., 2007). More recently, TCR

nanoclustering has been demonstrated by EM and PALM on fixed cells (Lillemeier et al., 2010). Although the molecular mechanisms for the formation of TCR nanoclusters are still highly debated, nanocluster stability appears to depend on cholesterol and sphingomyelin (Molnár et al., 2012; Schamel et al., 2005). Because T cells can regulate the extent of TCR nanoclustering depending on their activity, i.e. naïve versus memory T cells, it has been suggested that nanoclustering facilitates a quick memory response upon T-cell activation (Kumar et al., 2011).

The adaptor proteins linker for activation of T-cells 1 and 2 (LAT1 and LAT2, hereafter referred to as LAT) are members of the TRAP family (Hořejší et al., 2004) and also form nanoclusters on resting T cells, although the size of these nanoclusters and their participation in T-cell signaling are still debated. Lillemeier et al. showed that TCR and LAT exist as separate and discrete nanoclusters (also termed 'protein islands' by the authors) 70–140 nm in diameter that concatenate (without mixing) during
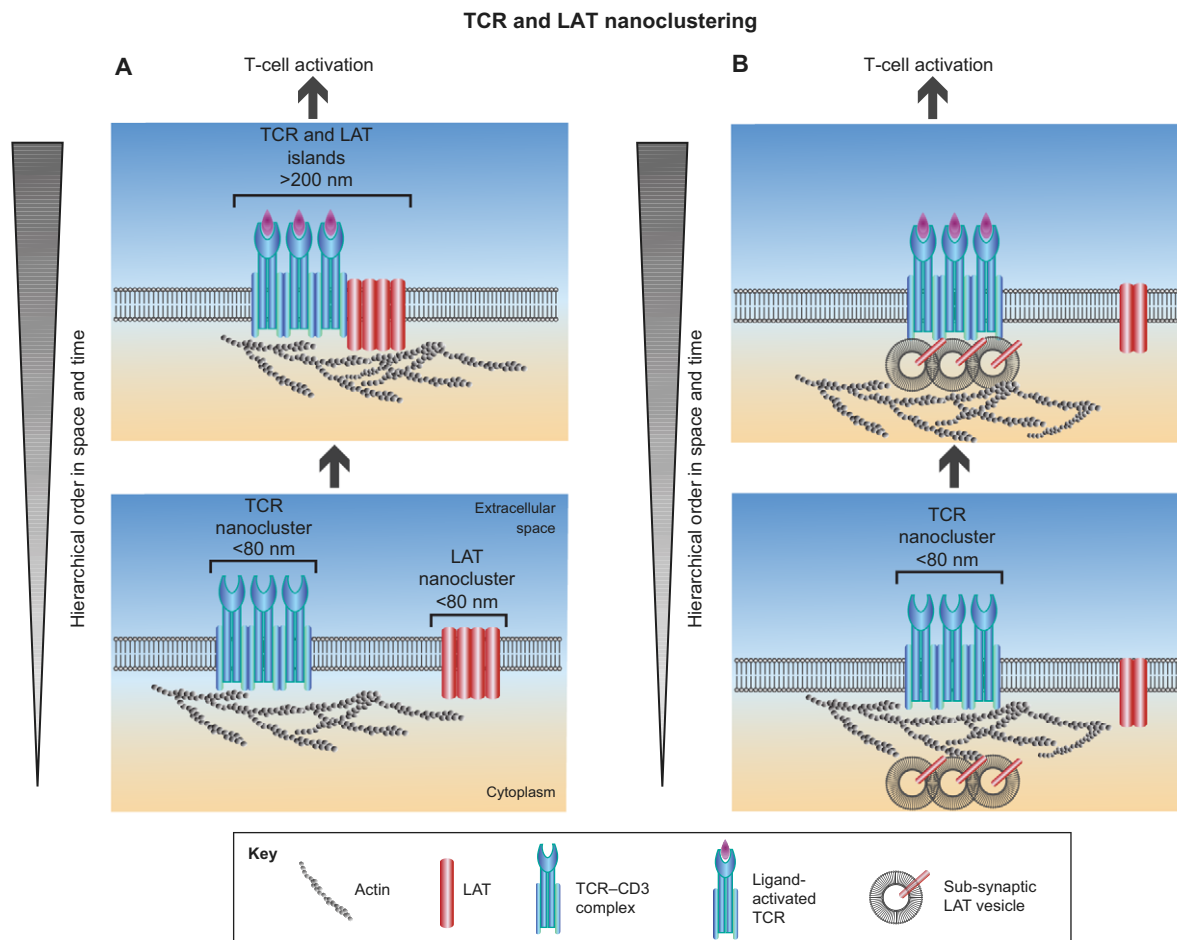
TCR and LAT nanoclustering

Fig. 4. Two possible pathways for the hierarchical organization of TCR and LAT molecules at T cells. The inverted triangle to the left indicates the increase in hierarchical order in space and time from bottom to top of each panel. (A) One possible pathway considered in the field, termed the 'protein island' model. TCR–CD3 [comprising TCR and the T-cell co-receptor cluster of differentiation 3 (CD3), also known as Cde3] complexes and LAT molecules are already present as small preassembled nanoclusters on the surface of resting T cells (bottom panel). After antigen recognition and TCR activation, TCR-CD3 and LAT nanoclusters concatenate, but do not mix (top panel). The actin cytoskeleton is thought to play a main role in this process (Lillemeier et al., 2010). Evidence for the protein island model comes from EM and PALM images before and after TCR activation. (B) An alternative assembly pathway of TCR and LAT nanoclusters, involving sub-synaptic LAT vesicles. TCR-CD3 complexes, as well as some LAT molecules, exist as small nanoclusters on the surface of resting T cells (bottom panel). LAT can also be found in sub-synaptic vesicles (top panel). After TCR activation, only LAT molecules within the sub-synaptic vesicles in close proximity to the cell membrane participate in signal transduction (Williamson et al., 2011). Evidence for the involvement of LAT sub-synaptic vesicles comes from PALM images in living cells, which show LAT recruitment in close proximity to the cell membrane. These LAT nanoclusters appear and disappear quickly over time, suggesting that vesicles dock and undock at the membrane without undergoing any appreciable lateral movement (Williamson et al., 2011).

activation (Lillemeier et al., 2010) (Fig. 4A). By contrast – using similar super-resolution approaches – another study showed that TCR and LAT nanoclusters are highly mixed, and that LAT nanoclusters are composed of only a few molecules (Sherman et al., 2011). These nanoclusters might not participate in early T-cell signaling events because sub-synaptic vesicles of LAT were seen to dock to the plasma membrane to TCR-activation sites (Williamson et al., 2011) (Fig. 4B), a process that is dependent on the vesicle (v)-SNARE protein VAMP7 (Larghi et al., 2013). The reasons for these contrasting results are not yet clear but they might be related to differences in the TCR activation times used by the different groups.

The multi-chain high-affinity IgE ε receptor subunit β (FcεRI; also known as MS4A2) and BCR are other examples of receptors that pre-cluster prior to antigen binding (Veatch et al., 2012; Wilson et al., 2001; Yang and Reth, 2010). For instance, by using a combination of direct stochastic optical reconstruction microscopy (dSTORM) and SPT, BCR and its co-receptor CD19 were found to exist in spatially distinct preformed nanoclusters in resting B cells (Mattila et al., 2013). Although the actin cytoskeleton regulates BCR, CD19 mobility appears restricted by the tetraspanin CD81, indicating that both CD19 and CD81 can form scaffolds to support BCR signaling (Mattila et al., 2013).

Outstanding investigations – biogenesis, formation and stability of nanoclusters

The above examples illustrate that nanoclustering is a common organizing principle for many membrane receptors, although the clusters might be subject to different regulatory mechanisms, depending on the particular receptor involved. Nevertheless, the prevalence of nanoclustering has raised a number of important questions that have not yet been fully answered, such as any that relate to biogenisis, formation and stability of nanoclusters as well as the potential role of the non-clustered molecules.

Nanocluster biogenesis, formation and stability

The full intracellular molecular complement of membrane nanoclusters (e.g. individual protein monomers, preformed protein homo-oligomers, preformed protein hetero-oligomers and/or particular lipid components) is typically unknown. Of interest here is whether a given nanocluster arrives at the plasma membrane as an almost fully assembled entity – as appears to be the case for LAT (Williamson et al., 2011) – with only a few factors to be subsequently recruited and/or eliminated or, by contrast, almost completely unassembled. It is unlikely that most membrane nanoclusters are composed solely of close-packed, identical proteins, although the idea is conceptually attractive. Instead, the current evidence strongly suggests that a specific complement of lipids and (most probably) other proteins will be present in nanoclusters. Indeed, at least in the case of DC-SIGN (Itano et al., 2011; Itano et al., 2012), LFA-1 (van Zanten et al., 2009) and BCR (Mattila et al., 2013), nanoclusters are not fully packed with their respective receptors.

The mechanism by which nanocluster precursors undergo initial reorganization after their delivery to the plasma membrane is a key issue. One might expect at least passive reorganization to occur in response to the differences in chemical composition between the intracellular and plasma membrane environments. These arise from at least three specific characteristics of plasma membranes: (1) molecular components have specific orientations and cannot be treated as soluble proteins with the freedom to reorient in three dimensions; (2) the environment contains

additional structural features, i.e. the outer and inner membrane environments are considerably different; and (3) the membrane is very crowded. Involvement of an active rather than passive process in the formation of nanoclusters and their stabilization is also possible (Gowrishankar et al., 2012).

If the mechanisms of nanocluster formation involve at least the partial self-association at the plasma membrane, then how are these associations limited to the nanoscale? The limiting factors could be of biochemical nature (i.e. they might constitute specific molecular interactions), reflect cell biological aspects (e.g. transmembrane pickets attached to the cytoskeleton) (Kusumi et al., 2011) or result from physical effects (e.g. kinetics trapping or the entropic costs associated with nanocluster existence). Importantly, at least in several cases, there is no particular size limit; instead nanoclusters of varying sizes simultaneously coexist (de Bakker et al., 2007; Gudheti et al., 2013; Itano et al., 2012; Williamson et al., 2011).

Elucidating the factors that contribute to nanocluster stability is crucially important in understanding nanocluster function and regulation. Generally speaking, nanoclusters could merge, partially disassemble, change their biochemical composition or completely dissolve. A dynamic equilibrium may also exist between nanoclusters and their components in the nearby membrane environment; for instance, although DC-SIGN molecules in nanoclusters do not appreciably exchange with those in the surrounding membrane, HA molecules in nanoclusters do (Itano et al., 2011). Another characteristic that is intrinsically related to nanoclusters is their lateral mobility within the membrane plane. Examples, of functions that require nanocluster mobility, are the transport of complexes between viruses and DC-SIGN nanoclusters to sites of cellular internalization (van der Schaar et al., 2007), rearrangement of the relative spatial distributions of TCR and LAT nanoclusters after T cell activation (Lillemeier et al., 2010), and rearrangement and further recruitment of GPI-APs to activated LFA-1 nanoclusters (van Zanten et al., 2009). Finally, the role of the membrane-associated actomyosin cell cortex has only just began to be investigated in detail, both structurally and functionally (Gowrishankar et al., 2012; Gudheti et al., 2013; Kusumi et al., 2011; Luo et al., 2013; Xu et al., 2013). Determination of its actual organization and structural association with membrane components should be an area of intense investigation in the future.

Role of non-clustered components

Although the forces that drive and limit nanocluster assembly are still under discussion, it is important to note that clustering is rarely complete and that, in many cases, nanoclusters co-exist with randomly distributed non-clustered components, as shown for TCR, LAT, GPI-APs, CD71 (van Zanten et al., 2009) and CD36 (Jaqaman et al. 2011), suggesting that such co-existence has a functional or regulatory role (Fig. 5). For example, nanoclusters could act as pre-assembled units that have the potential to become activated upon ligand binding because they spatially concentrate receptors and commonly exhibit restricted diffusion or are even spatially trapped. Yet, they might exist below a functional threshold because of their small size and, as such, are not able to stably bind their ligands. Alternatively, binding of ligands to such small nanoclusters might not be sufficient to elicit a downstream response. Non-clustered components, typically characterized by faster and unconstrained diffusion, might modulate nanocluster function by being
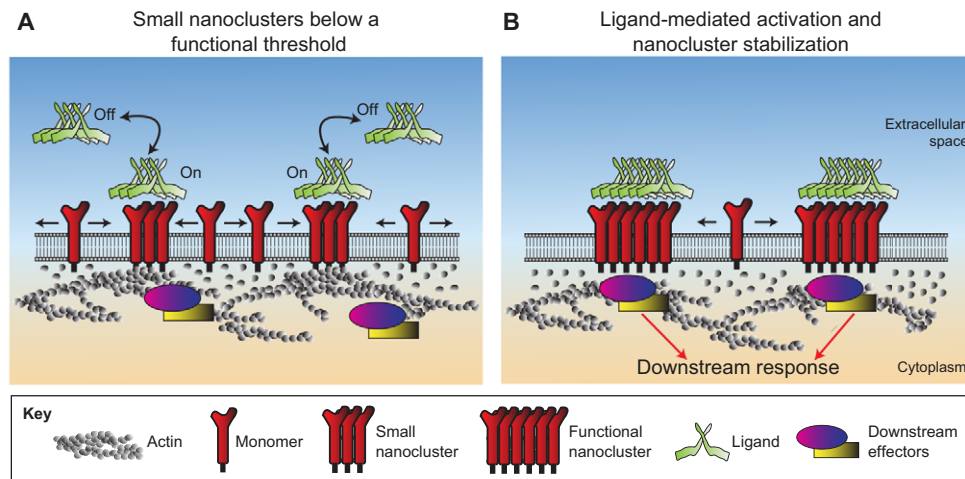
Fig. 5. Co-existence of transmembrane protein monomers and nanoclusters at the cell membrane. (A) Monomers and small inactive nanoclusters co-exist within the cell membrane. Small nanoclusters generally diffuse only slowly or, as shown here, might be anchored to the actin cytoskeleton. Monomeric species, by contrast, have greater mobility. At resting condition, i.e. in their inactivated non-ligand-bound state, it is possible that the size of the nanoclusters is below a functional threshold, at which they cannot stably bind their ligands (the on–off arrows indicate an equilibrium between extracellular and bound ligands). Alternatively, binding of ligands to small nanoclusters might not be sufficient to elicit a response to downstream effectors. (B) Upon ligand binding, a pre-existing small nanocluster can incorporate further monomers. Nanocluster activation through ligand binding and further recruitment of protein monomers stabilize the – now – larger cluster and render it functional, resulting in a downstream response. How this process is mediated in unknown but it might require the assistance of the local actin cytoskeleton and/or other signaling molecules, or involve stable conformational changes of the bound receptors that are transmitted to the monomer species.

incorporated into pre-existing nanoclusters, thereby acting as switches that provide the necessary threshold of nanocluster assembly in order to initiate their function. According to this hypothesis, dynamic pre-formed nanoclusters may not be functional when their size is below a specific threshold. Non-clustered receptors could provide a positive feedback to create larger domains as a way to trigger nanocluster assembly and hierarchical organization in a functional context. Such a positive feedback would, in principle, come into play upon nanocluster activation, and be mediated by lateral diffusion and encounter with nanoclusters, as recently suggested for TCRs (Schamel and Alarcón, 2013).

Why nanoclusters?

The ubiquitous presence of protein nanoclusters in mammalian plasma membranes prompts the crucial question of why nature has chosen this feature. Possible answers might come from elucidating why many soluble proteins are found in oligomeric states. This fundamental question has been addressed in several reviews that discuss plausible hypotheses, many of which may also be applicable to membrane protein nanoclusters (Ali and Imperiali, 2005; Hashimoto et al., 2011; Lynch, 2013). One important feature of soluble, oligomeric proteins is that they present the possibility of allosteric regulation; thus membrane protein nanoclusters might also be subject to this type of regulation. Another potential function of oligomerization of soluble proteins is to provide multiple, identical, ligand-binding sites in close proximity. For multivalent ligands, nanoclustering might increase the effective ligand-binding affinity (Cebecauer et al., 2010). For example, DC-SIGN nanoclusters recognize and bind to a large variety of glycosylated pathogens, and this ability might not only depend on the monosaccharide-binding ability of its CRD but also on the multivalency of the receptor within nanoclusters. Furthermore – compared with non-clustered receptors – in the case of monovalent ligands, oligomerization

of their receptors can result in a higher dwell time of the ligand because ligand rebinding is facilitated (Lagerholm and Thompson, 1998). Moreover, the time of ligand engagement to the nanocluster might be regulated by altering the density of clustered ligand-binding sites because ligand-rebinding probability increases with the square of the binding-site density.

With respect to signal transduction, Hancock and co-workers (Harding and Hancock, 2008; Tian et al., 2007) have proposed that nanoclustering in the plasma membrane offers a general way to 'digitalize' analog input signals according to their strength by increasing the number of nanoclusters, because each of these provides a discrete switch-like output because of the short lifetime of nanoclusters (Murakoshi et al., 2004). For example, in response to EGF stimulation, ligation of the EGF receptor activates K-Ras, thereby generating nanoclusters that provide digital inputs to the Ras–Raf–MEK–ERK kinase signaling cascade. This allows the pathway to be sensitive to small amounts of the activating ligand. The 'quantal' outputs from each signaling nanocluster are 'counted', i.e. integrated, in the cytoplasm by the kinase cascade to generate the bulk, analog cellular response.

Conclusions and perspectives

A number of recent studies have demonstrated that membrane protein nanoclusters and their functional remodeling is an emerging theme in eukaryotic plasma membrane organization. It is reasonable to expect that, as the field progresses – particularly in light of the increasing availability of super-resolution microscopy – even more examples will be discovered. The development of technologies that permit simultaneous, multiplexed observation of different nanocluster types in living cells might, in the near future, give rise to a new field of systems nanobiology to specifically address the structural and functional properties of membrane protein nanoclusters. Although each imaging technique has potential drawbacks, the convergence of

observations obtained with different approaches strongly suggests that nanoclustering will be increasingly recognized as a predominant feature. One of the challenges that lies ahead is to determine with certainty why nanoclustering is so prevalent.

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