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Oligomerization and nanocluster organization render specificity

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Abstract

Nanoclusters are anchored to membranes, either within them or in the cytoplasm latched onto the cytoskeleton, whose reorganization can regulate their activity. Nanoclusters have been viewed in terms of cooperativity and activation; here we perceive nanocluster organization from a conformational standpoint. This leads us to suggest that while single molecules encode activity, nanoclusters induce specificity, and that this is their main evolutionary aim. Distinct, isoform-specific nanocluster organization can drive the preferred effector (and ligand) interactions and thereby designate signalling pathways. The absence of detailed structural information across the nanocluster, due to size and dynamics, hinders an in-depth grasp of its mechanistic features; however, available data already capture some of the principles and their functional ‘raison d’être’. Collectively, clustering lends stability and reduces the likelihood of proteolytic cleavage; it also increases the effective local concentration and enables efficient cooperative activation. However, clustering does not determine the ability of the single molecule to function. Drugs targeting nanoclusters can attenuate activity by hampering cooperativity; however, this may not perturb activation and signalling, which originate from the molecules themselves, and as such, are likely to endure. What then is the major role of nanoclustering? Assuming that single molecules evolved first, with a subsequent increase in cellular complexity and emergence of highly similar isoform variants, evolution faced the threat of signalling promiscuity. We reason that this potential risk was thwarted by oligomerization and clustering; clustering confers higher specificity, and a concomitant extra layer of cellular control. In our Ras example, signalling will be more accurate as a dimer than as a monomer, where its isomer specificity could be compromised.

Keywords

nanoclusters; signalling; oligomerization; structure; Ras; microclusters; cytoskeleton; allostery; conformational states; allosteric; scaffolding; co-localization

I. INTRODUCTION

Some of the most important principles emerging over the last few decades from cell signalling research relate to the physical location of signalling proteins and their

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organization (Bray, 1998). We now know that many of the protein kinases, scaffolding proteins, transmembrane receptors, signalling proteins and their related enzymes and regulators that process and transmit messages in a living cell are associated with clusters of molecules within, or attached to, cell membranes or the cytoskeleton. We also know that each receives one or more inputs and produces specific outputs, and that they operate as transient units. Key questions include what are the mechanisms through which they form and operate; how do they robustly regulate cellular functions and what happens in disease; and can they be usefully harnessed in drug discovery and signaling circuit design? A conformational view of nanoclusters based on the free energy landscape (Frauenfelder, Sligar & Wolynes, 1991; Nussinov, 2013), which offers a mechanistic insight into nanocluster activation and signalling at the basic level, can address such questions. It also underscores the body of evidence that single molecules are activated by their inherent allosteric mechanisms, with subsequent binding of the activated conformation to its effector to initiate signalling. Clustering of the molecules (Fig. 1) will cooperatively enhance these activities; here however our thesis is that the main reason for their existence is that they drive specificity. Clustering is likely to have emerged at a later stage for more robust signalling among the evolving isoforms.

II. A MECHANISTIC OVERVIEW OF CELL SIGNALLING AND NANOCLOUDS

Recent advances paint cell signalling as an interplay between two organizational levels. The top level is the global cellular network, pathway cross-talks and feedback loops; the bottom level is the players, molecules and their complexes. The two levels are orchestrated: molecules integrate input signals transmitted from the pathways, including post-translational modification (PTM) events. Each signal changes the distribution of their conformational ensembles. The integration mechanism is functionally important: it transforms the order of input events acting on the molecule to output which controls pathway branching and signal strength (Nussinov *et al.*, 2013b; Nussinov & Tsai, 2014). It does this by translating temporal input events into distinct binding sites shapes and dynamics, which in turn recruit surface-complementary molecular partners. This spatial and temporal description of cell signalling illuminates a key problem which has plagued cell biology: how is specificity achieved at each step along the pathway? Such an account contends that beyond the effective local concentration, because each event alters the conformational ensemble in a distinct way, the type, combination and order of events decides the recruited partner. Recent discoveries append an additional coordinated spatial and signal transduction level: nano-(or micro-)clustering organization. Clusters are transient nanoscale arrangements of plasma membrane proteins localized in, or anchored to fluidic membrane domains, with lipid-lipid, protein-lipid, and protein-protein interactions. The clusters may also be connected to the cytoskeleton (Fig. 1), which is also an active component of the membrane clusters. Clusters may consist of tens to hundreds of molecules spanning both outer and inner membrane leaflets. The dynamics and time frame of the assembly and disassembly of these spatially constrained signalling systems, within or adjoining the membrane, emerges as a crucial factor in the control of the signal output. Recently, there have been excellent reviews of nanoclusters (Bethani *et al.*, 2010; Blanco & Alarcon, 2012; Ferrao *et al.*, 2012; Filipp,

Ballek & Manning, 2012; Harding & Hancock, 2008; Henis, Hancock & Prior, 2009; Kholodenko, Hancock & Kolch, 2010; Klammt & Lillemeier, 2012; Klein, 2012; Manz & Groves, 2010; Mattila *et al.*, 2013; Omerovic & Prior, 2009; Rossy *et al.*, 2013; Tian *et al.*, 2007). Here our purpose is to provide an overview of cluster activation from the conformational standpoint, and to relate it to the spatial organization of cell signalling. This is important, as it may shed light on their mode-of-action, explain observations, make testable predictions, and present a coherent unifying framework. Our premise is that the nano-(micro-)scale cluster organization can be described by dynamic, loosely pre-organized multimolecular complexes that function as allosteric signalling network modules. The transient interactions can be *via* direct oligomerization (Bethani *et al.*, 2010; Earl, Bi & Baum, 2011; Guldenhaupt *et al.*, 2012; Nikolov, Xu & Himanen, 2013; Smith & Milligan, 2010) or *via* cross-linked oligomers, mediated by scaffolding molecules (Harding & Hancock, 2008). Multiple signals can be perceived and transmitted by the same complex because of its diverse temporal content of molecular types and interactions. Conformational and dynamic changes of the stimulated molecules increase allosterically the sensitivity (affinity) of neighbouring molecules for their ligands resulting in efficient signal transduction and amplification of weak signals. In our broadened definition, scaffolding molecules have multiple partners and transmit allosteric signals (Nussinov, Ma & Tsai, 2013a). They can be proteins or lipids. Such conformational description unifies the spatial organization of plasma membrane proteins, including homotypic and heterotypic receptor associations, molecules located in membranes of adjacent cells, and membrane-anchored proteins. It also underscores the fundamental role of allostery in specific cell activation *via* oligomer and cluster assembly.

Below, we interpret clusters within such a conformational framework. We first describe the concept. We illustrate how it bears upon membrane microdomains (rafts) as signalling platforms. Their association with cytoskeleton proteins links transient patterns of cell membrane receptors and membrane-anchored proteins to micrometer-scale inter- and intra-cellular signalling (Nussinov, 2013). We synthesize existing information on lipid rafts, the dynamic cytoskeleton, clusters, and signalling, and cast it into a conformational framework. Finally, we suggest that the advent of nanoclusters in evolution was in response to increasing cellular complexity and appearance of isoforms, rendering more robust signalling. Because the principles governing conformational ensembles are based on the free energy landscape (Frauenfelder *et al.*, 1991), such a point of view is powerful in relating observations to fundamental molecular behaviour.

III. A CONFORMATIONAL VIEW OF NANOCLUSTERS

Early on it was observed that when chemotactic receptors cluster on the bacterium cell surface, the activity of one receptor influenced that of its neighbours (Bray, Levin & Morton-Firth, 1998). Around the same time, T lymphocyte (T-cell) signalling was found to depend on the assembly of multiple T-cell receptor/peptide-major histocompatibility complex (TCR/peptide-MHC) complexes, with the assembly extent determining the strength and nature of the signal (Reich *et al.*, 1997). This explained how T cells maintain sensitivity to antigens present in only low abundance on the antigen-presenting cell. Both of these early observations, as well as others (Bethani *et al.*, 2010), implied cluster cooperativity.

Cooperativity acts through all interactions, direct, and protein or lipid mediated. From the conformational standpoint, cooperativity is allostery, and allostery is the major vehicle responsible for signalling within and between cells (Nussinov, Tsai & Ma, 2013c). Allostery can take place through any perturbation of the molecular structure, such as that incurred by non-covalent binding of cholesterol (Sengupta, 2012), ions (Dawe *et al.*, 2013; Liu *et al.*, 2012), inhibitors (Lavoie *et al.*, 2013), and proteins, and through covalent PTMs (Nussinov *et al.*, 2012). Allostery is highly specific, works through a shift of the conformational ensemble, and propagates across molecules, interfaces and pathways. Clusters can consist of homo- or hetero-oligomers, or they can be mediated by scaffolding proteins or lipids that transiently link proteins together (Nussinov *et al.*, 2013a). Like Lego bricks (Bray, 1998), scaffolding proteins have evolved for the purpose of making protein clusters with varied partners (Fig. 1), except that they are flexible bricks, presenting dynamic ensembles. Since the distribution of the conformational ensembles changes with time and depends on prior allosteric events acting on the scaffolding protein, it controls partner selection. Scaffolding proteins, such as growth factor receptor-bound protein 2 (Grb2), which provides a link [*via* SHC-transforming protein 1 (SHC) and son of sevenless (SOS)] between the activated epidermal growth factor receptor (EGFR) and the Ras proteins, have no enzymatic activity. However, *via* allosteric population shift, their multiple SH2 and SH3 domains serve as connectors that can actively control ligand recruitment. Lipids can similarly act as scaffolding molecules *via* allosteric population shifts, as shown by phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P₃; PIP₃] which mediates Akt (also known as protein kinase B, PKB) and phosphoinositide-dependent protein kinase-1 (PDK1) (Tsai & Nussinov, 2013). Mediated clusters extend the signalling capacity of direct oligomerization, with the scaffolding proteins cross-linking pathways, essentially transforming the cluster into a signalling network. The Ras binding domain of Raf (RBD) can also be viewed as a scaffolding component (Fig. 2). RBD mediates not only the interaction of Ras with the Raf catalytic kinase domain, but also *via* Raf catalytic domain dimerization the interaction with another Ras molecule, creating mediated Ras oligomers, resulting in cooperative activation. Since many scaffolding proteins, such as IQ motif containing GTPase activating protein 1 (IQGAP1), bind not only a kinase (e.g. B-Raf) but also actin (Le Clainche *et al.*, 2007), itself also a scaffolding protein and a component of the membrane microdomains and the cytoskeleton, the effects of the network are micrometer scale, across the global spatial structure of cell signalling. Scaffolding proteins like IQGAP1 (Fig. 1) can have tens of partners; and the decision of which partner to select at any given time can be governed by allostery.

Here, we envision cell signalling as transient, allostery-driven cluster activation and signal integration cellular events (Nussinov, 2013). Because allostery involves specific interactions, and lipid microdomain composition and fluidity vary across tissues and environments, even subtle changes in protein sequences and structures will influence the preferred localization, protein partner choice and signalling consequences, and these are linked to nanocluster formation and pathway activation (Harding & Hancock, 2008). As we suggest below, it also results in preferred conformational orientation with respect to the membrane and the protein environment (Abankwa *et al.*, 2008; Gorfe *et al.*, 2007; Kapoor *et al.*, 2012; Mazhab-Jafari *et al.*, 2013), and this dynamic gel-like preorganization of loosely

networked molecules - proteins and lipids - is conducive to robust signalling and fast timescales.

IV. NANOCCLUSERS ARE OFTEN ASSOCIATED WITH LIPID MICRODOMAINS AND THE CYTOSKELETON

Clusters have been observed in a broad range of systems, particularly within, or adjacent and anchored to membranes. Clear advantages include lower entropic cost and restricted diffusion; however, a mere reduction of the dimensionality of the diffusion from three dimensions (in the cytoplasm) to two dimensions (in the membrane) cannot provide sufficiently robust signalling, nor can the restriction to confined regions. By contrast, a dynamic preorganization of the proteins and their neighbouring molecules, which presets the stage for allostery can be powerful in favouring both. Networked molecules in a cluster are essentially transformed into infinite functional entities. They bestow preferred localization and conformational orientation, which accomplishes this prerequisite. In the membrane, clusters are often associated with sphingomyelin and cholesterol-rich lipid raft microdomains (although not always, K-Ras clusters in cholesterol-insensitive, non-raft domains), effectors, scaffolding and cytoskeleton proteins (Allen, Halverson-Tamboli & Rasenick, 2007). All components are dynamically, although specifically, associated, resulting in more effective orchestrated temporal activation (del Sol *et al.*, 2009; Kar *et al.*, 2010; Ma *et al.*, 2011; Tsai, Del Sol & Nussinov, 2009). The cytoskeleton can also act as scaffolding proteins (Nussinov *et al.*, 2013a), allosterically influencing transient cluster formation/dissociation and localization, and through these, the potency of receptors, transporters and membrane-anchored signalling proteins. Monomers, dimers and microfilaments of microtubules and actin interact specifically with proteins clustered in lipid rafts. These interactions perturb the structures of both partners, and set in motion bidirectional information transfer from, and to, the cytoplasm. As a result, both cytoskeleton proteins and their membrane-associated partners undergo specific redistributions of their ensembles, which from the conformational standpoint is how signalling takes place. Clusters can locate in planar (non-caveolar, glycolipid) and caveolae lipid rafts, with diameters ranging between 25 and 100 nm (Allen *et al.*, 2007; Simons & Toomre, 2000), and with their component flotilins and caveolins, respectively, acting as cluster scaffolding proteins. In addition to these, and to actin and tubulin, other scaffolding proteins mediate the interactions of cluster members, including IQGAP1 (Malarkannan *et al.*, 2012) (Fig. 1) acting in the mitogen-activated protein kinase (MAPK) and β -catenin pathways and interacting with Raf, mammalian target of rapamycin (mTOR)-Akt (Tekletsadik, Sonn & Osman, 2012), calmodulin and the cytoskeleton; galectin-1, a major regulator of H-Ras nanoclusters (Belanis *et al.*, 2008); linker for activation of T cells (LAT), which is anchored *via* palmitoylation and interacts with signalling molecules related to T-cell differentiation (Balagopalan *et al.*, 2010); ezrin, which dynamically tethers the plasma membrane to actin filaments and regulates B lymphocyte activation *via* B-cell receptor (BCR) diffusion and microclustering (Parameswaran *et al.*, 2013); Vav1, which facilitates formation of signalling microclusters (Knyazhitsky *et al.*, 2012); Grb2 (Balagopalan *et al.*, 2010), p18 and MP1, a scaffolding protein in the Raf/MAPK kinase/extracellular signal-regulated kinase (Raf/MEK/ERK) pathway which together with p14 forms a heterodimer localizing MEK

(Magee & Cygler, 2011), ERK and more (Fig. 1). All coordinate multiple signalling pathways *via* localization and redistributions of the conformational ensembles. By crosslinking cluster members, scaffolding proteins can influence the signal. Further, because scaffolding proteins are themselves also regulated, largely *via* PTMs such as phosphorylation, palmitoylation, farnesylation, and ubiquitination, which are also allosteric effectors, they impart an additional layer of control. In addition, lipid modifications affect the clustering and its organization (Li & Gorfe, 2013). Adding to this picture of nanoclusters are lipids such as cholesterol (Katritch, Cherezov & Stevens, 2013; Liu *et al.*, 2012; Oates *et al.*, 2012) which interact specifically with membrane receptors and are also established allosteric effectors (Bethani *et al.*, 2010). Collectively, through these, other proteins, lipids, ions and ordered water molecules (Liu *et al.*, 2012), clusters and membrane domains may robustly mediate cooperative signalling.

V. CYTOSKELETON SIGNALLING AND RAFTS DYNAMICS

How does cluster signalling modify the cytoskeleton and how do cytoskeleton signals modify the clusters (Allen *et al.*, 2007)? Studies of TCRs and BCRs provide some clues (Hering, Lin & Sheng, 2003). TCR activates regulators of actin polymerization, such as cell division control protein 42 homolog (Cdc42), Wiskott-Aldrich syndrome protein family member 2 (WASF2) and Wiskott-Aldrich syndrome protein (WASP), which remodel the cytoskeleton. Cluster formation, within minutes of TCR-pMHC (major histocompatibility complexes displaying the antigenic peptide) recognition, is driven and shaped by the actin cytoskeleton which acts as a template: when TCR clusters encounter an actin barrier at an angle with respect to the driving actin flow, they continue their motion tangential to the barrier. Of particular interest from the conformational standpoint, the actin and tetraspanin networks also organize BCR-mediated nanocluster signalling (Mattila *et al.*, 2013) independently of ligand engagement (Monroe, 2006). Ligand-independent (tonic) BCR signalling is triggered and regulated by the dynamic reorganization of the actin cytoskeleton (Kusumi *et al.*, 2005; Treanor & Batista, 2010), although the detailed mechanism is still unclear. Current data suggest that in resting B cells BCR and coreceptor CD19 nanoclusters exist in distinct, actin-bordered compartments. Upon cytoskeleton disruption, the BCR nanoclusters are released to interact with co-receptor CD19 to trigger signalling (Mattila *et al.*, 2013), with the CD19 cytoplasmic domain acting as an adaptor to recruit phosphatidylinositol 3-kinase (PI3K) [Vav, phosphoinositide phospholipase C- γ -2

(PLC γ 2), and additional kinases] to the BCR (Fearon & Carroll, 2000; Srinivasan *et al.*, 2009). Thus, such a mechanism connects cytoskeleton dynamics and compartmentalization of receptors, where actin reorganization, triggered by some protein other than the ligand, appears allosterically to facilitate receptor cross-talk resulting in tonic signalling (Mattila *et al.*, 2013). CD19 resembles LAT in T cells, and TCR and LAT also form distinct membrane domains in resting T cells, raising the possibility of a general mechanism. Other receptor–ligand interactions, such as B-cell activating factor (BAFF) and BAFF-R (Fearon & Carroll, 2000), could similarly trigger local cytoskeleton reorganization. Actin, an allosteric protein, further mediates long-range interactions by physically transporting surface molecules, such as TCRs and the integrin IFA1, and by directly interacting with components of signalling cascades. As such, it extends pre-organized dynamic signalling in the cell.

Below, we provide a detailed example illustrating the importance of a conformational view in understanding the mechanism of activation. The example focuses on the Ras protein due to the considerable data available and its importance in cancer. 20–30% of cancers are associated with Ras protein mutations.

VI. A CONFORMATIONAL VIEW ILLUSTRATED BY THE RAS EXAMPLE

(1) Background: Ras clusters, signalling and isoforms

Ras-family proteins are small GTPases (Cebecauer *et al.*, 2010). Ras is a key component of signalling in the cell. Almost half of Ras plasma-membrane-bound proteins are associated with nanoclusters. Clustered, but not individually distributed, Ras proteins recruit and activate their downstream effectors. Interference with the clustering of Ras proteins abolishes signalling. Among the body of papers addressing Ras nanocluster signalling, the series by J.F. Hancock and his colleagues spanning decades and lately expanded and complemented in collaboration with A.A. Gorfe, whose molecular dynamic simulations provide the conformational preferences, as well as recent work by R. Winter (Weise *et al.*, 2012) narrate a compelling, and still unfolding story.

Upstream of Ras, is SOS, which through downstream of receptor kinase (Drk)/Grb mediates receptor tyrosine kinase (RTK) activation of Ras (Fig. 1). SOS is a cytoplasmic protein, but is recruited to the membrane when receptors are activated, by the action of the SH2/SH3-containing proteins. SOS1, the Ras guanine nucleotide exchange factor (GEF) in the MAPK/ERK pathway exchanges GDP→GTP in Ras. The high effective concentration of Ras-GTP in nanoclusters may allow SOS to remain bound at the plasma membrane. SOS has an allosteric binding site for Ras-GTP, creating a positive feedback loop between Ras and SOS. Downstream Ras signalling takes place through effectors, many of which are also membrane-associated, such as Raf, the scaffolding protein galectin-1 and phosphoinositide-3-kinase α (PI3K α). Some of their downstream effectors are also membrane-bound, such as scaffolding protein IQGAP1 (Fig. 1), a Raf partner, which also interacts with cytoskeleton proteins (actin and microtubules) and mTOR. A change in the conformation and orientation of membrane-bound Ras will impact its binding to effectors and consequently perturb cell signalling (Abankwa *et al.*, 2010; Kapoor *et al.*, 2012). There are three major Ras isoforms: K-Ras, H-Ras and N-Ras. Their sequences and structures are almost identical, except for their C-terminal membrane-associated hypervariable region (HVR), which is associated with trafficking, subcellular localization, and lateral nanoclusters segregation. All three are anchored in the membrane *via* lipidated PTMs (farnesyl, palmitoyl) at their C-terminals. In addition, the K-Ras HVR is highly positively charged (through multiple lysine residues), unlike the H- and N-Ras. Recently, Weise *et al.* (2012) observed how farnesylated K-Ras may be delivered to the membrane. The delta subunit of cGMP phosphodiesterase (PDE δ) has been suggested to serve as a cytosolic carrier for farnesylated Ras. Since the K-Ras4B/PDE δ complex which formed in bulk solution is unstable in the presence of heterogeneous membranes, delivery of PDE δ -solubilized K-Ras4B to the plasma membrane appears to be followed by the dissociation of the complex upon plasma membrane contact, and membrane binding of the released farnesylated K-Ras4B.

Isoform expression is cell specific and relates to distinct cancers: 95% of pancreatic, 45% of colorectal and 35% of lung cancers relate to K-Ras (K-Ras4B) mutations; the incidence of other cancers *via* the N- and H-isoforms is much lower. These outcomes are expected to stem from altered patterns of isoform signalling. Key questions are what the differences are; how to understand them on the basic, conformational level; and what possible therapeutic strategies they may suggest. The selective retention of C-Raf in K-Ras, but not in H-Ras, nanoclusters (Plowman *et al.*, 2008; Prior *et al.*, 2003; Tian *et al.*, 2007) may provide clues to these challenging questions. Raf's long residence times can result in elevated levels of Ras signalling through the Raf-MEK-ERK pathway.

(2) Ras conformations and oncogenic potential

The different membrane preferences of Ras isoforms appear partly to underlie the isoform-specific differences in network signalling. Inactive H-Ras associates with lipid rafts and caveolae; active H-Ras shifts to the disordered plasma membrane. By contrast, K-Ras is predominantly (>85%) localized to the negatively charged, disordered plasma membrane irrespective of its activation state, indicating a preference for distinct plasma membrane properties. The membrane affects Ras orientation, and thus exposure of its effector binding surface (Abankwa *et al.*, 2008; Gorfe *et al.*, 2007; Mazhab-Jafari *et al.*, 2013). Although the interactions between the Raf's RBD and H-Ras and K-Ras are equivalent, signalling takes place over longer timescales in K-Ras nanoclusters. In K-Ras nanoclusters, following activation, the C-Raf RBD dissociates from Ras; however, it remains bound to the membrane through its cysteine-rich domain (CRD)-lipid interactions (Abankwa *et al.*, 2010). Raf lingers in the highly acidic phosphatidylserine and phosphatidic acid membrane environment of K-Ras nanoclusters, which provide avid binding sites for Raf's CRD. This results in Raf's dynamic preorganization, primed for its interaction with Ras whose orientation is also coupled to its lateral segregation in the membrane (Ghosh *et al.*, 1996; Ghosh *et al.*, 1994).

H-Ras was observed to adopt two major orientations with respect to the membrane: in the GTP-bound state, H-Ras helix $\alpha 4$ interacts with the membrane, and stabilizes it in an orientation that facilitates Raf binding and the MEK-ERK (MAPK) pathway activation. In the GDP-bound state, Ras reorients, stabilized by HVR-membrane contacts, with the $\beta 2$ - $\beta 3$ -loop and helix $\alpha 5$ controlling the switching between the two states. The different HVR properties in K-Ras and H-Ras and different membrane environments argue for different HVR conformations with respect to the membrane, and suggest distinct mechanistic details to achieve the same aim. This is borne out by molecular dynamic simulations which illustrate that while H-Ras is stabilized by helix $\alpha 4$, K-Ras is stabilized by its positively charged HVR, with the active G-domain orientation of K-Ras inverted with respect to that of H-Ras.

Raf initiates the MAPK signalling from activated Ras (Yan *et al.*, 1998). MAPK is the major signalling pathway responsible for oncogenic Ras in cancer. Thus the Ras orientation, coupled with high residence time of Raf in the highly acidic K-Ras membrane nanocluster, may provide clues to its high oncogenic potential. Raf dissociates from the membrane in H-Ras nanoclusters, but not in K-Ras nanoclusters where it is dynamically poised in a binding-

competent state to reinitiate the MAPK signalling pathway. Mutations in the Ras switch elements result in different outcomes, likely due to the isoform-specific orientations and their preferred membrane environments. Interactions with other effectors, such as PI3K α , and galectin-1 appear to be similarly orientation sensitive (Abankwa *et al.*, 2010), reflected in the exposed binding interface.

Above we have considered Ras as a monomer. Solution studies and structural data pointed to the monomer being the functional state. However, dimerization of membrane-anchored Ras could play a role in Ras activation (Guldenhaupt *et al.*, 2012; Inouye *et al.*, 2000). This is also in line with receptors and other membrane-associated proteins (Smith & Milligan, 2010). Further support for a role for Ras dimer in function comes from residues in the modelled dimer interface shown to influence Ras signalling. However, how dimers relate to Ras activation is unclear. While the affinity may be enhanced in membrane-anchored states, Ras shape and stability as a monomer suggest that the major functional state is a monomer. Apart from direct Ras dimerization, Ras dimers can also be indirect, mediated by Raf, whose RBD acts as a scaffolding protein, and the Raf catalytic domain dimers (Fig. 2).

Raf-mediated indirect Ras dimerization can further cooperatively enhance the nanocluster network. Formation of Ras nanoclusters requires scaffold proteins of the galectin family and possibly also soc-2 suppressor of clear homolog/suppressor of Ras-8 (Shoc2/SUR-8) (Udell *et al.*, 2011). However, in the absence of high- (and low-) resolution structural data on a larger scale it is challenging to model clustering and activation.

(3) Ras clusters feature binding-competent states

From the conformational standpoint, to be networked in a cluster, the molecules should be favourably spatially juxtaposed and oriented with respect to each other already prior to their interaction. Ras adopts distinct isoform-specific orientations with respect to the membrane in the GTP- and GDP-bound states and to Ras partners, which appear governed by the chemical properties of the HVR and helix $\alpha 4$. Interactions of H-Ras with PI3K α , and with scaffolding protein galectin-1 were observed to be orientation sensitive, and the N-Ras and K-Ras interactions with the C-Raf-binding domain are differentially affected by mutations in the switch elements which relate to Ras orientation (Abankwa *et al.*, 2010; Kapoor *et al.*, 2012). The inverted active G-domain orientation between K-Ras and H-Ras indicates selective access of C-Raf to different Ras isoforms. Raf monomers also have to be correctly oriented with respect to Ras and to each other; interaction with Ras shifts the conformation of the Raf monomer to a dimer-favoured state, and dimerization is required for activation (Lavoie & Therrien, 2011; Poulikakos *et al.*, 2011). That Raf orientation is important for downstream signalling is further supported by Raf's interaction with scaffolding proteins kinase suppressor of Ras(KSR) (Udell *et al.*, 2011) and IQGAP1. In turn, IQGAP1's orientation is also important, as it interacts with scaffolding proteins actin and tubulin, in addition to p21 activated kinase (Pak)/Raf/MEK1/2-mediated ERK1/2 activation (Malarkannan *et al.*, 2012). Ras is also suspected of dimerizing when attached to the membrane, and Fourier transform infrared spectroscopy, molecular simulations, and Förster resonance energy transfer experiments of N-Ras have suggested a dimerization interface between α -helices 4 and 5 and the loop between $\beta 2$ and $\beta 3$ (Guldenhaupt *et al.*, 2012). This

loop has also been identified as controlling the switch between Ras-membrane active/inactive orientation states (Abankwa *et al.*, 2010; Kapoor *et al.*, 2012). Galectin-1 monomers also dimerize, which may lead to formation of higher-order multimers with increased valency, linking Ras proteins (Earl *et al.*, 2011). Collectively, these point out a complex transient network organization, with the components adopting orientation-productive binding-competent states, able to form signalling links on short time scales. These data further underscore the importance of dimerization.

(4) The role of lipid modifications

Cell-biological and biophysical data indicate that H-, N- and K-Ras isoforms differ in their C-terminal sequences and in the number and type of their lipid modifications (Karnoub & Weinberg, 2008), suggesting that lipid modifications play an important role in the clustering, nanocluster organization and distribution in the plasma membrane (Abankwa, Gorfe & Hancock, 2007; Hancock, 2003). K-Ras contains a single farnesyl modification; N-Ras is modified by a palmitoyl and a farnesyl. K-Ras prefers liquid-disordered lipid domains enriched with unsaturated lipids (Weise *et al.*, 2011); N-Ras opts for the liquid-ordered/disordered domain boundary (Nicolini *et al.*, 2006); the double palmitoylated and farnesylated H-Ras favours liquid-ordered domains (Plowman *et al.*, 2005). Studies of model membranes with raft-like liquid-ordered and non-raft liquid-disordered lipid domains present similar behaviour (Nicolini *et al.*, 2006; Weise *et al.*, 2011). Computations show that asymmetric insertion of the differentially lipidated peptides results in a vertical relative displacement of the raft domains and deforms the bilayer, with the domain boundary serving as the centre of deformation (Li & Gorfe, 2013). The extent of deformation varies, and depends on the type and number of lipid modifications. We reason that the differential preference of palmitoylated and farnesylated peptides for ordered or disordered membranes or their domain boundaries is a yet another regulation layer to avoid redundancy, ensuring robust signalling specificity among the evolved isoforms. Taken together, the different – intracellular *versus* cytoplasmic – membrane preferences by the H-, N- and K-Ras isoforms, through their lipid modifications, argue for distinct isoform nanocluster localization and segregation, however, not higher order assembly and specificity, which may relate to the different, preferred orientation with respect to the membrane.

VII. A CONFORMATIONAL VIEW OF EphA CLUSTERS

A conformational view of ephrinA receptor (EphA) isoform clusters exquisitely illustrates specificity *via* oligomerization and nanocluster organization. Cell proliferation and differentiation, migration and adhesion are key processes in cell life, morphing cells into anatomical structures during development. The precise positioning of cells as they approach each other, as in the case of vascular patterning or in controlling axon growth in the assembly of topographic neural maps, is highly complex. Two membrane-anchored protein families, the Eph receptor tyrosine kinases and their ephrin ligands are tasked with orchestrating signal transduction: Ten EphA and six EphB receptors which interact with six ephrinA and three ephrinB ligands, respectively. The interactions between the Eph receptors and ephrins of the same subclass are promiscuous; however, cross-subclass binding is observed only for two receptors. EphA3 can bind ephrin-B2, and EphA4 interacts with all

nine ephrin ligands, which have different functions. Previously, nine EphA4 ligand-binding domain (LDB) conformations in complex with the ligands were observed in nine crystal structures providing the fundamental principles of the interactions (Bowden *et al.*, 2009). Two additional EphA4 crystal structures allowed further insight into the conformational dynamics of EphA4 and its signalling pathways. The heterogeneous ensemble and loop dynamics observed in their analysis suggested how the EphA4 receptor is able to bind multiple A- and B-ephrin ligands and small molecules *via* conformational selection, fine-tuning cellular signal response in receptor and ligand cells (Nussinov & Ma, 2012).

An important step of Eph signalling involves the assembly of higher-order signalling clusters, setting off bidirectional signalling in both interacting cells. The crystal structure of the complexed EphB2 and ephrinB2 binding domains revealed a tetrameric arrangement with two contact surfaces that are involved in the assembly of Eph/ephrin tetramers (Himanen *et al.*, 2001; Himanen *et al.*, 2010): an extensive high-affinity ephrin-binding channel, which could be responsible for the initial interaction, and a smaller lower affinity Eph-ephrin interface (Himanen, Saha & Nikolov, 2007) which could be important for the generation and function of Eph/ephrin signalling at the cell–cell contact points, possibly involving individual assembling Eph/ephrin complexes into hetero-oligomeric clusters. High-resolution structures of the complete EphA2 ectodomain and complexes with ephrinA1 and ephrinA5 revealed extended arrays with Eph/Eph interactions within and outside of the Eph ligand-binding domain (Himanen *et al.*, 2010). These array-like EphA2 ectodomains arrangements in the crystal lattice are compatible with the EphA2 clustering in *cis* on the cell membrane. More recently (Seiradake *et al.*, 2013), crystal structures of the EphA4 ectodomain alone and in complexes with ephrinB3 and ephrinA5 were obtained. Remarkably, in contrast to the elongated EphA2 ectodomain architecture, EphA4 presented closed clusters with a dimeric or circular arrangement in the crystal lattice (Fig. 3). The EphA4 ectodomains, compatible with *cis* interaction in the cell surface, consisted of two, three or six copies of EphA4 in a dimeric or circular arrangement. Localization microscopy further showed that ligand-stimulated EphA4 induces smaller clusters than does EphA2. Mutating EphA4 converted its organization from circular to an extended array. Further, concomitant changes were observed in its specificity and function, suggesting a conformational mechanism by which distinctive ectodomain surfaces determine clustering, which in turn govern specificity, i.e. the signalling pathway. Taken together, structural data suggest that the mode of clustering of one isoform cannot be extrapolated to others. The sequence, as well as other environmental factors, such as lipid composition (as in the Ras case above), concentration and other binding events (e.g. by cofactors), all are likely to play a role in oligomerization and cluster organization. Changes in the sequence or the physical environment are allosteric effectors, and work *via* a population shift inducing different preferred conformational states and thus oligomerization and higher order structures. In turn, the altered arrangement of the different cluster architectures may induce restricted specificities; that is, a different preferred partner and thus cellular pathway.

VIII. CONCLUSIONS

1. Clustering stabilizes the molecules, reducing the likelihood of proteolytic cleavage; it also increases the effective local concentration and is required for activation *via*

cooperativity among cluster members. However, clustering does not determine the ability of the single molecule to function.

2. Coming back to the Ras example above, Ras functional efficacy is determined not by its clustering, but by its allosteric switching mechanism. Ras switching from GDP- to GTP-bound states through GEF allosterically activates Ras and initiates signalling; the reverse action through GTP→GDP hydrolysis helped by GAP, terminates signalling. The importance of these steps in oncogenesis can be seen from Ras mutations: rather than hyperactivation of Ras signalling, oncogenic mutations compromise Ras GTPase activity which under normal physiological conditions is controlled through a negative feedback mechanism, ensuring that signalling is transient. Ras downstream effectors bind at the allosteric lobe, interaction that is facilitated by Ras orientation with respect to the membrane, which positions it in a binding-competent state.
3. What is then the major role of nanoclustering? Assuming that single molecules evolved first, followed by increased cellular complexity and emergence of highly similar isoform variants, evolution faced the danger of signalling promiscuity. This potential risk was foiled by oligomerization. Oligomerization and clustering bestows higher specificity. If Ras indeed exists as dimers, which is what we suspect, its signalling will be more accurate than had it acted as a monomer, where its isomer specificity could have been compromised.
4. The compelling recent crystal structures of EphA4 ectodomain alone and in complexes with ephrinB3 and ephrinA5 appear to support this proposition (Seiradake *et al.*, 2013). The remarkable crystal lattices of the EphA4 ectodomain indicated closed clusters with a dimeric or circular arrangement in the crystal lattice, contrasting with extended arrays previously observed for the EphA2 ectodomain isoform (Himanen *et al.*, 2010). Further, engineered EphA4 mutations converted its circular organization to extended arrays with concomitant changes in specificity and function, thus linking organization to signalling specificity. For the small Ras protein and its large multidomain effectors, specificity may be imparted *via* additional interactions.
5. Taken together, clusters may be driven by intracellular (as in Ras), extracellular (as in Eph), or intramembrane interactions. Their gel-like properties, conferred by the preorganized networks that they create lend stability and specificity, thereby equipping the cell with an additional layer of regulation. Are nanoclusters ‘good’ drug targets? Targeting nanoclusters will diminish activity by hampering cooperativity, however, it may not affect activation and signalling, which emanate from the molecules themselves, and as such, are likely to endure.
6. Finally, clustering takes place through binding events, which redistribute the ensemble. Each step perturbs the conformation, with the allosteric effects propagating across the molecule, and through interfaces, across their neighbours. Sites are coupled; changes in one can be reflected in another. This is the basis of the cooperativity noticed for T-cell receptors in the 1990s (Bray *et al.*, 1998; Reich *et al.*, 1997).

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Fig. 1.

An example of a cluster illustrating Ras activation. Ras signalling affects many cellular functions, including cell proliferation, apoptosis, migration, fate specification, and differentiation. Ras is a membrane-anchored GTPase that is normally activated in response to the binding of extracellular ligands, such as epidermal growth factor (EGF), to a receptor tyrosine kinase (RTK), e.g. epidermal growth factor receptor (EGFR). Under normal physiological conditions, the EGFR kinase domain is intrinsically autoinhibited. EGF binding to the extracellular domain transmits a signal which propagates through the transmembrane domain resulting in EGFR dimerization and activation. EGFR activation

results from the formation of an asymmetric dimer in which the C-terminal lobe of one kinase domain plays a role analogous to that of cyclin in activated cyclin-dependent kinase (CDK)/cyclin complexes (Zhang *et al.*, 2006). Signalling proceeds through SHC-transforming protein 1 (SHC), Growth factor receptor-bound protein 2 (Grb2) and Son of sevenless (SOS). SOS1, the Ras guanine nucleotide exchange factor (GEF) in the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway, is recruited by the adaptor protein Grb2 in response to EGFR activation. Under physiological conditions, Ras is in its inactive GDP-bound state. GEF exchanges GDP for GTP and by so doing activates Ras. Active, GTP-loaded Ras binds monomeric Raf, thereby leading to Raf dimerization, which is the active form of Raf (see Fig. 2). Ras activates a number of signalling pathways, including the Raf/MAPK kinase/extracellular signal-regulated kinase (Raf/MEK/ERK) pathway, the MEK kinase/SAPK-ERK kinase/Jun N-terminal kinase (MEKK/SEK/JNK) pathway, a phosphatidylinositol 3-kinase (PI3K) Akt for proliferation, and the nuclear factor-kappa B (NF-B) pathway which is mostly related to inflammation. There are three major Ras forms, K-Ras, H-Ras and N-Ras, which largely differ in their hypervariable region (HVR), and in their lipophilic post-translational modification (PTM) group, farnesyl, palmitoyl, prenyl, etc. Calmodulin inhibits K-Ras signalling, but not H-Ras or N-Ras. Ras cofactors, and proteins in their pathways, are partners of the Ras GTPase-activating-like protein (IQGAP1) scaffolding protein. IQGAP1 is a large multi-domain protein with tens of partners already identified. Cytoskeleton proteins actin and myosin, as well as other proteins, are also partners of IQGAP1. Some of these proteins are scaffolding proteins too, including actin which itself also has tens of partners. Further, like Ras, some of these proteins are also anchored in the membrane, including Raf (see Fig. 2) and actin. Ras acts as a binary signal switch cycling between ON and OFF states. Ras returns to its OFF state *via* GTP→GDP hydrolysis with the help of the GTPase-activating protein (GAP). GDP-bound Ras is unable to bind its effectors. IQGAP1 binds to and regulates the function of numerous proteins. Over 90 proteins have been reported to associate with IQGAP1, directly or as part of a larger complex (White, Erdemir & Sacks, 2012). Most of the binding is to the IQ or C-terminal regions. Some proteins interact with expected binding motifs, such as actin to the CHD, and calmodulin and S100 to the IQ motifs. Unfortunately, no structure is available for complexes of any of IQGAP1 protein partners with the respective IQGAP1 domain. As the figure illustrates, IQGAP1 is also a scaffold in the MEK/ERK cascade. It binds to both MEK1/2 and ERK1/2 and modulates their activation to proximal Raf. There are three Raf kinases, A-Raf, B-Raf and C-Raf. IQGAP1 binds B-Raf, except in the heart where it binds C-Raf. Together with binding Ca²⁺/calmodulin, it couples Ca²⁺ to the Ras-Raf-MEK-ERK cascade. IQGAP1 also binds directly to actin and promotes cross-linking of actin filaments. The dynamic cross-linking through IQGAP1, acting to both increase the effective local concentration and allosterically to coordinate function hints at how higher level cellular regulation takes place; in our case here, it also provides insight into how actin dynamically controls and is controlled by cluster molecules, such as Ras. A cluster consists of multiple copies of each of the proteins, and is highly dynamic persisting only sufficiently long for a burst of signalling activity to go through. Abbreviations used in the figure are: APC, adenomatous polyposis coli; Cdc45, cell division control protein 42 homolog; Clip-170, cytoplasmic linker protein-170; Rac1, Ras-related C3 botulinum toxin substrate 1; S100B, S100 calcium binding protein B.

Fig. 2.

Raf activation by Ras. Raf consists of a catalytic domain which has two lobes, a cysteine-rich domain (CRD) and Ras-binding domain (RBD), with the CRD connecting to the catalytic domain *via* a hinge region. In resting cells (top of figure), Raf monomers are autoinhibited by their CRD domain which interacts with the catalytic domain. Interaction with membrane-anchored GTP-bound Ras relieves the autoinhibition. RBD at the N-terminal of Raf interacts with Ras, swinging the CRD away, and exposing the Raf dimerization surface. Raf dimerizes by a side-to-side interaction, creating an asymmetric

active dimer. Raf's CRD domain interacts with the membrane, stabilizing the complex. This architecture effectively creates a Raf-linked Ras dimer. However, in addition, it is currently believed that Ras also forms direct dimers. While dimerization and clustering provide co-localization and leads to cooperativity, here our major tenet is that they drive specificity and that this is the major reason that they evolved. Clustering can help thwart pathway redundancy of Ras isoforms. We note that here the structure of the (inactive) symmetric dimer with inhibitors bound to both monomers is shown. The protein databank (PDB) codes for the structures shown here are: 1UWH (Raf dimer), 2L05 (RBD), and 3GFT (Ras).

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Fig. 3.

Examples of clusters representing oligomeric units of EphA4 ectodomain (EphA4_{ecto}) in the crystal lattices. (A) The monomeric unit of ligand-free (LF) EphA4_{ecto} (left panel) and the dimeric cluster formation (middle and right panels). The protein databank (PDB) code for the structure shown for LF-EphA4_{ecto} is 4BK4. The extracellular EphA4_{ecto} contains ligand-binding domain (LBD), sushi, epidermal growth factor (EGF) like, and two fibronectin repeats (FN1 and FN2) (Seiradake *et al.*, 2013). (B) The monomeric unit of EphA4_{ecto} interacting with methylated ephrinA5 receptor-binding domain (RBD) (left panel) and the

dimeric cluster formation (middle and right panels). The PDB code for the structure shown for the complex of EphA4_{ecto}-ephrinA5 RBD (methylated) is 4BK5. (C) The monomeric unit of EphA4_{ecto} interacting with native ephrinA5 RBD (left panel) and the trimeric cluster formation (middle and right panels). The PDB code for the structure shown for the complex of EphA4_{ecto}-ephrinA5 RBD (native) is 4BKA. (D) The dimeric unit of EphA4_{ecto} interacting with ephrinB3 RBD (left panel) and the hexameric cluster formation (middle and right panels). In the dimeric unit, protein structures are represented with the surface and the ribbon representations for each unit. The PDB code for the structure shown for the complex of EphA4_{ecto}-ephrinB3 RBD is 4BKF. Cartoons shown here were regenerated from the original artwork (Seiradake *et al.*, 2013).