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Review

Ras nanoclusters: Versatile lipid-based signaling platforms ☆

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ABSTRACT

Ras proteins assemble into transient nanoclusters on the plasma membrane. Nanoclusters are the sites of Ras effector recruitment and activation and are therefore essential for signal transmission. The dynamics of nanocluster formation and disassembly result in interesting emergent properties including high-fidelity signal transmission. More recently the lipid structure of Ras nanoclusters has been reported and shown to contribute to isoform-specific Ras signaling. In addition specific lipids play critical roles in mediating the formation, stability and dynamics of Ras nanoclusters. In consequence the spatiotemporal organization of these lipids has emerged as important and novel regulators of Ras function. This article is part of a Special Issue entitled: Nanoscale membrane organisation and signalling.

1. Introduction

Ras proteins are small GTPases that oscillate between an active GTP-bound and inactive GDP-bound state to function as plasma membrane localized molecular switches in growth factor regulated signaling pathways [1,2]. The level of Ras.GTP is normally tightly regulated by restricted, controlled access to exchange factors that catalyze GTP binding and GTPase activating proteins (GAPs) that enhance GTP hydrolysis to return activated Ras back to the inactive ground state. This control is subverted in ~15% of all human tumors which express Ras proteins with oncogenic mutations that prevent GAP action and lock Ras in the GTP-bound state [3,4]. Three major Ras isoforms, H-, K- and N-Ras are ubiquitously expressed in mammalian cells. K-Ras has two alternative splice variants: K-Ras4A and K-Ras4B. Since K-Ras4B is the ubiquitously expressed splice variant, we will focus mainly on K-Ras4B in this review, and all references to K-Ras hereafter imply K-Ras4B unless otherwise stated. All these Ras proteins share a common set of exchange factors and effectors, but the efficacy with which each isoform activates a specific effector varies significantly [1,3]. Isoform signaling specificity is encoded not by the highly conserved Ras G-domains (amino acids 1–165) that directly interact with effector proteins, but by the highly divergent C-terminal hypervariable regions (HVR, amino acids 166-188/89) [5,6]. The HVR contains a linker region and a C-terminal membrane-anchoring domain, which undergoes posttranslational modification to attach different lipid anchors to each Ras isoform (Fig. 1). Complex interactions between plasma membrane constituents and the different Ras lipid anchors, which are further modified by the activation state of the G-domain, determine the spatial distribution of Ras proteins on the plasma membrane. In this review we will consider how plasma membrane lipids and Ras interact to generate multiple types of signaling nanocluster on the plasma membrane, each with distinct lipid compositions. These newly defined Ras-lipid interactions can account for the different efficacies with which each isoform recruits and activates effectors to explain isoform-specific signaling. These same Ras-lipid interactions also lead to intriguing emergent control networks for Ras signaling platforms.

2. Ras proteins have a complex spatiotemporal distribution on the plasma membrane

Quantitative imaging techniques, such as electron microscopy (EM)-spatial mapping (Fig. 2) [7–10], fluorescence lifetime imaging microscopy-fluorescence resonance energy transfer (FLIM-FRET) [10–12], single particle tracking [13] and fluorescence recovery after photobleaching (FRAP) [10,14], reveal a highly dynamic spatiotemporal organization of Ras proteins on the plasma membrane. Approximately 40% of Ras proteins exist in immobile nanodomains, termed nanoclusters, with the remaining proteins freely diffusing as mobile monomers (Fig. 3 and Table 1) [8,9]. Ras nanoclusters are ~9 nm in radius and contain ~6-7 Ras proteins per nanocluster. Ras nanoclusters turnover rapidly with lifetimes in the order of 0.1-1 s [9,13], thus nanoclusters are constantly forming and disassembling.

Recent work suggests that Ras dimer formation is a critical prerequisite for the assembly of the larger nanoclusters [15–19]. Mathematical

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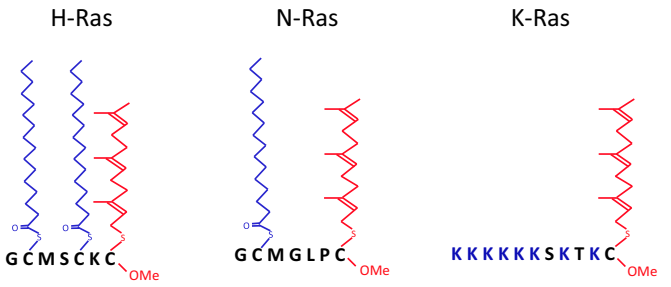

Fig. 1. Membrane anchors of three Ras proteins. After posttranslational modification all Ras isoforms are farnesylated and methyl esterified on a C-terminal cysteine. H-Ras is then dual-palmitoylated on Cys 181 and Cys 184 while N-Ras is mono-palmitoylated on Cys 181. K-Ras is not further lipid-modified but has a polybasic domain consisting of 6 contiguous lysines, plus two other lysines.

modeling shows that cooperativity between Ras monomers and dimers is a required parameter to replicate K-Ras nanocluster formation *in silico* [15]. MD simulations also reveal that H-Ras lipid anchors spontaneously form excess dimers as well as nanoclusters on a lipid bilayer, suggesting that dimers possess intrinsic stability [16]. This is further supported by computational modeling and experiments using purified N-Ras [17] or H-Ras [18] on model bilayers, which again show that Ras proteins form spontaneous dimers with a conformational orientation that is optimal for effector interactions. Furthermore, CRAF, a downstream effector of Ras, dimerizes upon binding to K-Ras.GTP on the plasma membrane [19], indirectly supporting the existence of K-Ras dimers. Preliminary results also suggest that the maintenance of a monomer pool may require nanocluster assembly to uncouple dimers followed by nanocluster disassembly to regenerate monomers (Fig. 4).

Within this framework H-, K- and N-Ras isoforms each form distinct, spatially non-overlapping nanoclusters, thus the Ras isoforms laterally segregate with high fidelity on the plasma membrane [8,11,12,16,20–22]. In addition Ras proteins exhibit guanine nucleotide-dependent segregation such that each isoform assembles into spatially distinct, non-overlapping GTP and GDP nanoclusters (Table 1) [8,11,12,16,20–22]. Ras nanocluster formation is biologically important because nanoclusters are the sole sites for effector recruitment and activation, and is therefore essential for signal propagation [1,9,23]. Extensive signaling experiments as well as computational modeling show that the fraction of Ras proteins in nanoclusters is fixed over a multi-log range of expression levels [9,23]. One result of this non-equilibrium behavior is that increasing the number of K-Ras.GTP molecules on the plasma membrane leads to a linear increase in the number of K-Ras.GTP nanoclusters. This coupled with switch-like activation of the RAF-MEK-ERK cascade in nanoclusters allows the Ras nanocluster system to operate as an analog-digital-analog (ADA) converter for high-fidelity signal transmission (Fig. 5) [23–26].

3. Structural basis of Ras plasma membrane localization and spatial segregation

Ras membrane anchors: the minimal membrane anchors of each Ras isoform are required and sufficient to target and anchor their cognate isoform to the plasma membrane. These anchors comprise a common fanesyl-cysteine-methyl ester attached posttranslationally at the extreme C-terminus of the Ras proteins and one of three different "second" signals to complete the anchor [27,28]. These alternate second signals comprise palmitoylation on Cys 181 and Cys 184, for H-Ras, and palmitoylation on Cys 181 alone for N-Ras [28] (Fig. 1). In contrast, K-Ras is not further lipidated but possesses a polybasic domain (PBD) consisting of six contiguous lysines (aa175-180) [27,29] (Fig. 1). Each of these membrane anchors visualized by GFP tag undergoes trafficking to the plasma membrane and organizes into non-overlapping nanoclusters [8]. The minimal membrane anchors of H-

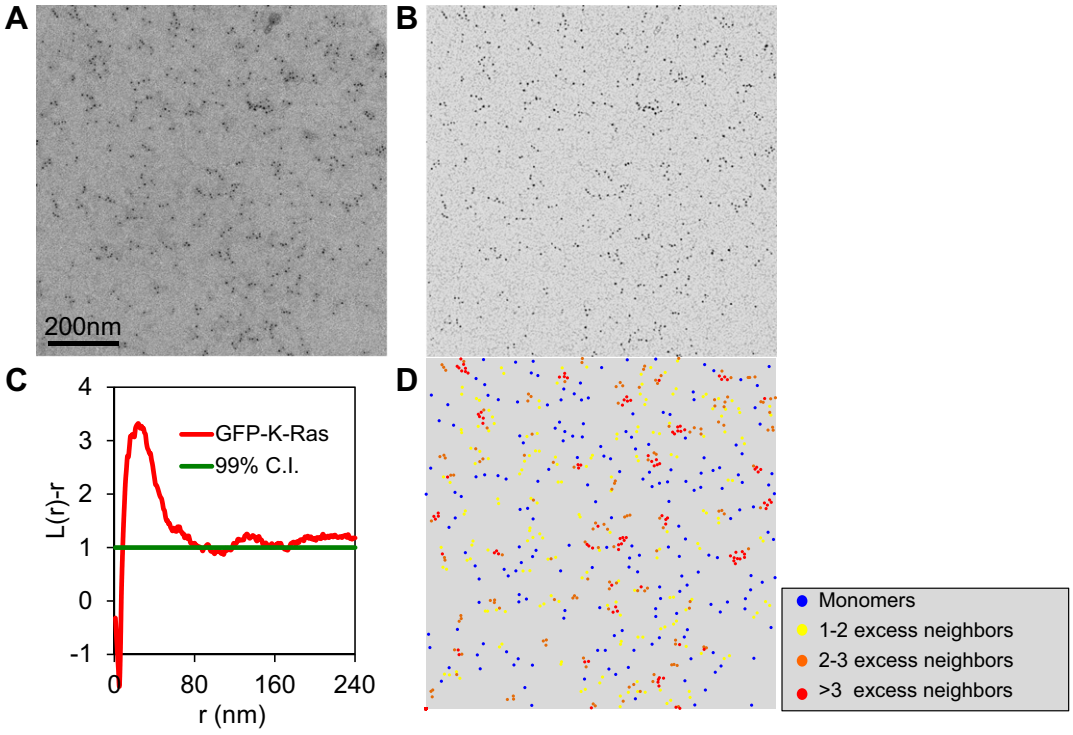

Fig. 2. Electron microscpy (EM) combined with spatial mapping. An intact plasma membrane sheet of BHK cells expressing GFP-K-Ras was labeled with 4.5 nm gold nanoparticles coupled to anti-GFP antibody. EM image was acquired using a transmission EM (**A**) and further processed using ImageJ (**B**). The spatial distribution of the gold particles was analyzed using Ripley's K-function ($L(r)$-r), showing that the pattern is highly clustered (**C**). A local L(r) was then calculated for each point in the image in B and used to constuct a heat map, which codes each particle according to the excess number of neighbors (over that expected for a random pattern of the same density) detected within a radius of 15 nm (**D**). This allows a visualization of clusters.

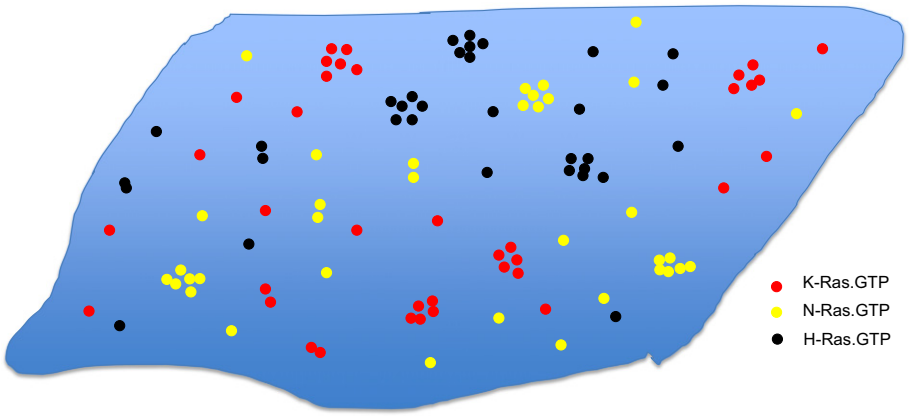

Fig. 3. Ras proteins form isoform- and guanine nucleotide-dependent nanoclusters. Ras proteins distribute heterogeneously on the plasma membrane into two predominant populations: mobile monomers and dimers (~56%) and immobile nanoclusters (~44%). The fraction in clusters (= the clustered fraction) is independent of expression level. A typical Ras nanocluster has a radius of <10 nm and contains ~6 Ras proteins. The average lifetime of a Ras cluster is between 0.1 – 1 s. H-, N- and K-Ras isoforms segregate into non-overlapping isoform-specific nanoclusters. Each isofrom further segregates into non-overlapping GTP-bound clusters and GDP- bound cluster, so called GTP-dependent lateral segegregation (not shown on the diagram).

Ras (tH) and K-Ras (tK) assemble into cholesterol-dependent and cholesterol-independent nanoclusters respectively. This clustering behavior has been replicated in MD simulations indicating that nanocluster formation is an intrinsic property of lipid anchor / lipid bilayer interaction [16,30]. Theoretical modeling simulating tH clustering in a ternary lipid mixture shows that approximately 34% of tH molecules pack into nanoclusters [16], consistent with the clustered fraction of ~40% Ras measured in EM spatial mapping experiments. tH preferentially localizes at the domain boundary between the cholesterol-enriched liquid-ordered (L_o) and the cholesterol-poor liquid-disordered (L_d) domains in phase separating bilayers [16]. Interestingly, the L_d-favoring farnesyl chain and the L_o-favoring palmitoyl chains on tH combine to determine the final localization of tH at the domain boundary [16]. This is also consistent with the cholesterol-dependence of GFP-tH nanoclustering in EM spatial mapping experiments. Domains in biological membranes equivalent to L_o domains in synthetic membranes are frequently called lipid rafts. However, stable L_o domain formation on the length and time scales observed in synthetic membranes does not occur in the plasma membrane of intact cells. This topic has been discussed in numerous reviews (for example [31,32]); our perspective here is that cholesterol-dependent nanoclusters likely reflect lipid raft formation on very short time and length scales and thus have similar biophysical properties to classical L_o domains, albeit transiently.

MD simulations of tK reveal that the PBD forms a pseudo-helix lying parallel to the inner leaflet of a negatively charged (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine / 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; POPC/POPG) lipid bilayer [30]. Strikingly, not all lysines participate in the membrane-association in the same manner: only 5 out of the 8 lysines (excluding Lys 175, Lys 176 and Lys 179) directly interact with the acidic lipids [30]. The positively charged lysines coral acidic lipids to induce phospholipid lateral segregation in the bilayer [30] providing interesting evidence of the impact of PBDs on membrane heterogeneity. The nature of K-Ras4A nanoclustering is not known. In addition to a PBD and a farnesyl chain, the C-terminus of K-Ras4A is also palmitoylated. We speculate that this unique membrane anchor will cause K-Ras4A to laterally segregate from other Ras isoforms and form nanoclusters with a distinct proteolipid composition.

Integration between the HVR and the G-domain: although the C-terminal lipid anchors play the primary role in membrane binding the Ras G-domains must contribute to or modify these interactions to account for the GTP-dependent lateral segregation observed in intact cells. The mechanism, whereby this occurs, involves changes in the G-domain conformational orientation [33–35]. GTP-loading triggers structural rearrangements in switch I and II that are transmitted through a network of salt bridges involving D47 and E49 in the β2–β3 loop, R161 and R164 in helix-α5 that ultimately release R169 and K170 membrane binding to allow, in the case of H-Ras, an ~100° rotation of the G-domain (Fig. 6A). After rotation, R128 and R135 in helix-α4 now interact with membrane lipids and stabilize the new orientation (Fig. 6A). The coupling mechanism that transmits G-domain conformational changes has been referred to as switch III, and the charged residues in helix-α4 or the proximal HVR that engage in mutually exclusive interactions with the lipid bilayer, switched elements [11,12,33]. In addition the correct orientation of the H-Ras G-domain is critical for effector and scaffold interactions [11,12,36]. A similar switch III operates in N-Ras and K-Ras, but each isoform has a different G-domain orientation that is optimal for binding to CRAF [11,12]. In consequence,

Table 1
Ras nanoclusters have complex composition. Ras nanoclusters contain distinct lipid/actin composition.

	CHOL-dependence	Cluster lifetime	Actin	Gal-1	Gal-3
H-Ras.GDP	Yes	<0.1 s	Yes	No	No
H-Ras.GTP	No	~1 s	No	Yes	No
K-Ras.GDP	No	<0.1 s	Yes	No	No
K-Ras.GTP	No	~1 s	Yes	No	Yes
N-Ras.GDP	No	N/A	No	No	No
N-Ras.GTP	Yes	N/A	No	No	No

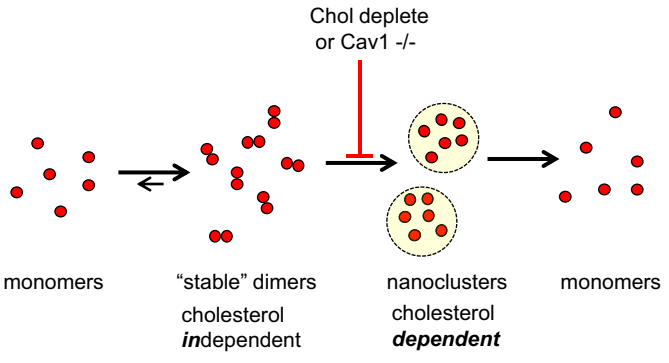

Fig. 4. Ras dimers act as intermediate between monomers and nanoclusters on the plasma membrane. Theoretical modeling and experiments using purified Ras proteins suggest that Ras isofomrs form dimers as fundamental units for more extensive oligomerization into nanoclusters. Changes in plasma membrane lipid composition, such as depleting choelsterol or disruption of caveolae, potentially interfere with the oligomerization process and retains Ras proteins in dimers. Within nanoclusters dimers are disassembled so monomers are released when nanoclusters in turn disassemble.

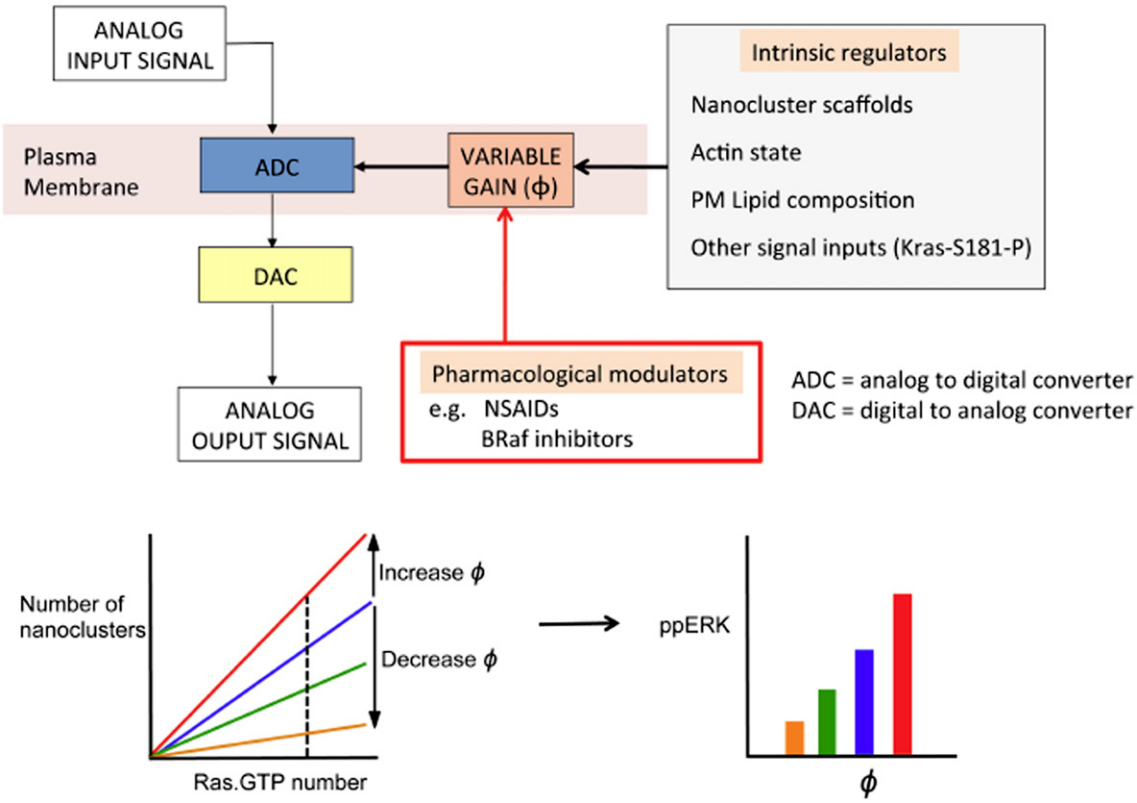

Fig. 5. Ras nanoclusters are analog-digital convertors that digitize signal input. A fixed clustered fraction is set for Ras on the plasma membrane, which yields a linear correlation between the number of nanoclusters and the Ras.GTP level (left graph). Thus, a change in clustered fraction corresponds to a direct change in ERKpp sigal output (right graph). Ras clustered fraction is highly dynamic and is a function of intrinsic cell plasma membrane properties, which can be manipulated pharmacologically.

mutations in helix-α4 that enhance CRAF and PI3K binding by H-Ras.GTP by stabilizing helix-α4 interactions with the membrane have precisely the opposite effect on CRAF and PI3K binding by K-Ras.GTP [11]. G-domain orientation is therefore a novel codec for regulating effector interactions. It is worth emphasizing that the key residues in the switched elements are basic, thus electrostatic interactions with anionic lipids are as important for H- and N-Ras PM interactions as for K-Ras. Work with N-Ras and K-Ras in model membrane systems further confirm the role of the membrane in constraining Ras G-domain conformations [37,38]. An important consequence of the H-Ras GTP orientation change is a partial extraction of the C-terminal anchor peptide from the lipid bilayer with a change in the structure of the two palmitates (Fig. 6A). The palmitates are highly ordered in the GDP-bound state but disordered in the GTP-bound state [22,33–35] (Fig. 6A). These differences likely account for the assembly of cholesterol rich, liquid-ordered lipid raft like domains by H-Ras.GDP and disordered, cholesterol-independent domains by H-Ras.GTP [11,34–36].

The difference in K-Ras G-domain orientation between GDP- and GTP-bound states is much smaller than that of H-Ras. MD simulations show that switch 1, switch 2, loop 3, helix α3 and loop 7 in K-Ras.GTP all adopt multiple conformations and display much higher degrees of fluctuation than H-Ras.GTP [11,39] (Fig. 6B). This is consistent with atomic force microscopy (AFM) experiments showing that GDP-bound K-Ras has almost all of its helices aligned with the bilayer plane while the GTP-bound K-Ras adopts an almost random orientation [37]. As a result the PBD attached to the plasma membrane in both GDP- and GTP-bound states displays slight differences between the active and the inactive states. While the entire PBD is in contact with the membrane in GDP-bound K-Ras, the C-terminal section of the PBD is displaced from the plasma membrane when bound to GTP, possibly caused by the high level of fluctuation of the G-domain [11] (Fig. 6B).

As a result of the different lipid anchor structures and conformations and the different G-domain residues in contact with the plasma membrane, each Ras isoform in the GDP- and GTP bound state engages in a distinct set of molecular interactions with the lipid bilayer. In consequence it is reasonable to expect that each type of nanocluster comprising a group of like Ras proteins will collect or sort a corresponding distinct set of phospholipids. This prediction has recently been validated, at least for a cohort of plasma membrane lipids, sufficient to support the idea that each Ras nanoclusters has a distinct lipid structure.

4. Lipid composition within Ras nanoclusters

4.1. Cholesterol

The first evidence for different lipid compositions of Ras nanoclusters came from studies of Ras nanocluster stability under conditions of cholesterol depletion. These experiments showed that H-Ras.GDP forms cholesterol-dependent nanoclusters while H-Ras.GTP segregates into cholesterol-independent clusters [8]. Conversely, N-Ras.GDP nanoclusters are cholesterol-independent, and N-Ras.GTP nanoclusters are cholesterol-dependent [8]; thus H-Ras.GDP and N-Ras.GTP assemble into lipid raft-like nanodomains. Similarly the minimal H-Ras anchor, −tH, assembles into cholesterol dependent nanoclusters, and extensively co-localizes with H-Ras.GDP, but not H-Ras.GTP. GDP-bound and GTP-bound K-Ras both form cholesterol-independent nanoclusters that are spatially segregated from each other [8,9]. These data clearly demonstrate that cholesterol distributes differently in, and / or is structurally important in only certain types of Ras nanocluster. Incorporation of cholesterol into Ras nanoclusters is relevant to effector binding and signaling transduction. Whereas RAF, a major downstream effector of Ras, robustly activates the MAPK cascade when targeted to cholesterol-

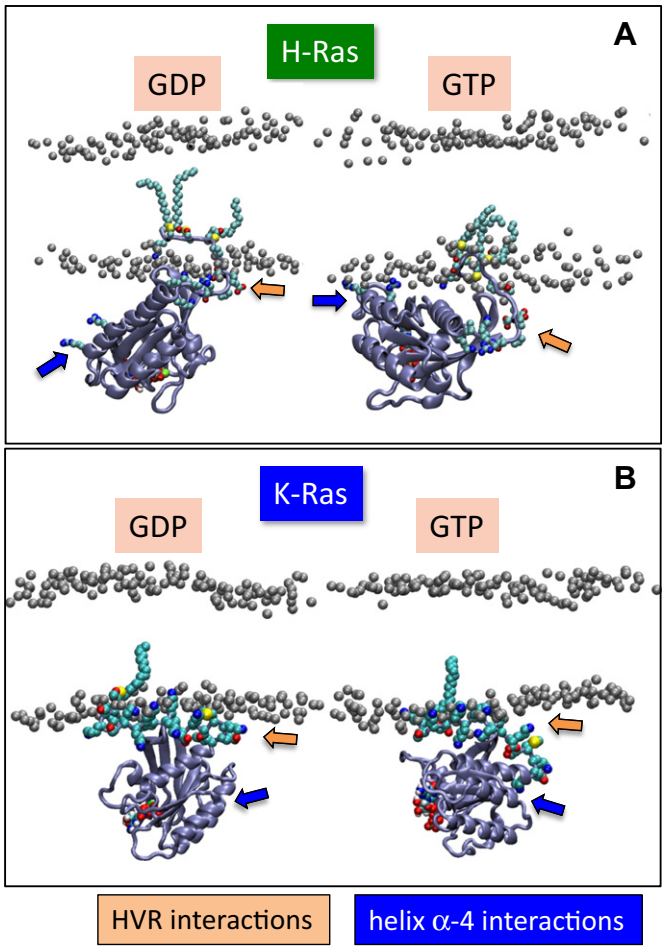

Fig. 6. Ras proteins have different G-domain orientations with respect to the plasma membrane. (**A**) In addition to the minimal membrane anchor H-Ras makes additional contacts with the plasma membrane either through the HVR (when GDP-bound) or helix α4 in the G-domain (when GTP-bound) . In consequence upon GDP to GTP exchange, the G-domain swings ~100° to change its conformational orientation. There are attended changes in the ordering of the palmitates in the anchor. (**B**) K-Ras interactions with the plasma membrane are limited to the HVR. The PBD on K-Ras is fully associated with the membrane when to GDP-bound but partially detached when GTP-bound.

independent nanodomains of the plasma membrane (using a tK anchor) [40–42], RAF is completely inactive when targeted to cholesterol-dependent nanodomains using a -tH anchor [40].

More broadly cholesterol can be viewed as facilitating the lateral segregation or de-mixing between different types of Ras nanoclusters in the plasma membrane. In model membranes, cholesterol non-linearly drives phase separation between lipids favoring tightly packed L_o and lipids favoring highly fluid L_d domains [43–48]. Only cholesterol levels between ~25-35% in three component model bilayers drives phase separation between the L_o and the L_d domains [43,44]. At lower or higher cholesterol levels saturated and unsaturated lipids mix and there is no phase separation [43,44]. In this context, in cell plasma membranes, cholesterol drives lateral segregation between cholesterol-independent H-Ras.GTP and cholesterol-dependent H-Ras.GDP [49]. If the cholesterol content in the plasma membrane is depleted by β-methyl-cyclodextrin, lateral spatial segregation between GDP- and GTP-bound H-Ras fails leading to the formation of heterotypic nanoclusters composed of both H-Ras.GDP and H-Ras.GTP [49]. Efficient Ras lateral segregation is required for effective effector activation and signal transmission [8,40,50], thus as expected the formation of mixed clusters of H-Ras.GTP and H-Ras.GDP significantly compromises H-Ras dependent MAPK signal transduction [49,51].

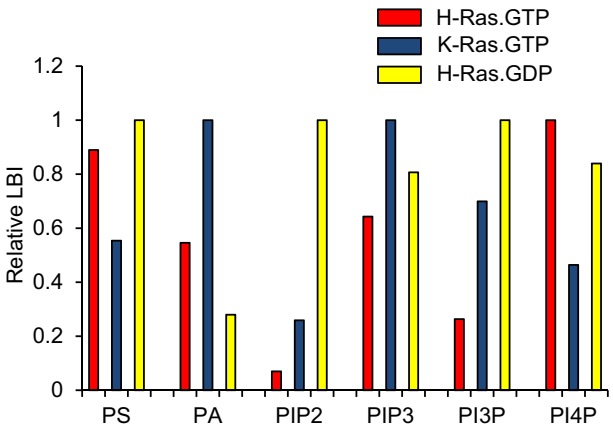

Fig. 7. Ras nanoclusters have different lipid compositions. Relative lipid content of three Ras nanoclusters asayed in intact plasma membrane sheets using bivariate EM analysis to comap the distibution of specific probes for each lipid onto the distribution of each Ras protein.

4.2. Anionic lipids

The prevalence of other lipids in Ras nanoclusters was revealed in a recent study [10] (Fig. 7). Phosphatidylserine (PS) is distributed relatively evenly among K-Ras.GTP, H-Ras.GTP and H-Ras.GDP nanoclusters [10]. Phosphatidic acid (PA) is more enriched in K-Ras.GTP than H-Ras.GTP nanodomains while phosphatidylinositol 4,5-biphosphate (PIP_2) preferentially co-localizes with H-Ras.GDP [10]. Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is also found in all three Ras nanoclusters currently investigated. On the other hand, phosphatidylinositol 3-phosphate (PI_3P) preferentially associates with H-Ras.GDP while phosphatidylinositol 4-phosphate (PI_4P) is found mostly in H-Ras.GDP and H-Ras.GTP nanoclusters but not K-Ras nanoclusters [10]. These results are interesting because they clearly illustrate that each Ras nanocluster may sort and assemble a distinct cohort of plasma membrane phospholipids (Fig. 7). Ras effectors have distinct lipid co-activators or lipid substrates, in this context the lipid composition of H-Ras.GTP and K-Ras.GTP nanoclusters can be correlated with their known effector activation profiles. Enrichment of PIs in H-Ras nanoclusters is consistent with the observation that H-Ras is a more efficient activator of PI3Ks, which bind specifically to PIP_2 via the P110 subunit [52–54]. On the other hand, extensive co-localization of K-Ras with PA is consistent with its preferential activation of RAF, which has a specific PA-binding domain [55]. Thus the unique lipid environment within each Ras nanodomain directly determines the ability of Ras to recruit specific effectors, which could be the underlying mechanism for Ras isoform-specific activities.

As with cholesterol, anionic lipids play different roles within Ras nanoclusters. For example, PS depletion experiments show that plasma membrane PS is required for the structural stability of K-Ras.GTP nanoclusters, but not H-Ras.GDP or H-Ras.GTP nanoclusters [10]. This may reflect the different interactions between PS and K-Ras versus PS and H-Ras. K-Ras interacts with PS mainly via electrostatic interactions with the PBD [21,56] while H-Ras most likely interacts with PS acyl chains via hydrophobic palmitate lipid anchors. As hydrophobic interactions are less specific, H-Ras clustering would be less sensitive to changes in PS levels and lateral distribution in the plasma membrane. Consistent with this interpretation PS depletion directly mislocalizes K-Ras from the plasma membrane and disrupts the nanoclustering of K-Ras molecules that remain localized to the plasma membrane, leading to a significant abrogation of K-Ras signaling [10,57,58]. Since PS is not required for H-Ras nanocluster formation, changing PS levels in the plasma membrane has no effect on H-Ras signaling [10,57,58]. In an intriguing analogy with cholesterol, PS non-linearly regulates lateral

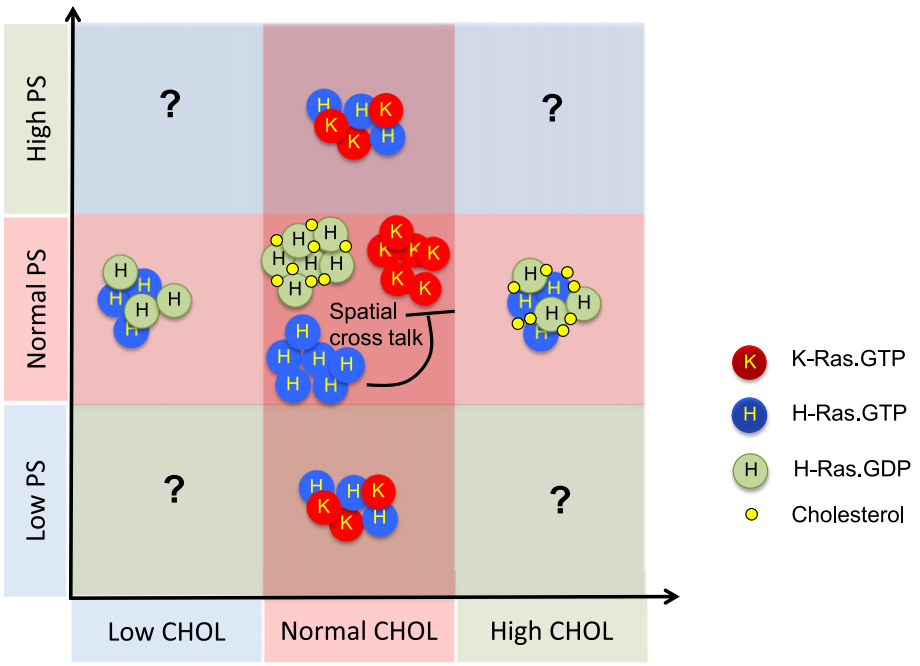

Fig. 8. PS and cholesterol (CHOL) regulate Ras spatial segregation on the plasma membrane. Under normal levels of cholesterol and PS, Ras proteins undergo efficient isoform- and guanine nucleotide-dependent lateral segregation. As both H-Ras and K-Ras compete for PS and PS is only structurally required for K-Ras clusters, H-Ras remotely regulates K-Ras nanoclustering and signaling via spatial cross talk. When PS and/or CHOL level in the plasma membrane are changed, spatial segregation fails and mixed clusters composed of multiple Ras components form. As spatial segregation is key to efficient effector activation, cluster coalescence markedly attenuates signaling. Lipid combination changes that have not been investigated are shown as "?".

segregation between H-Ras.GTP and K-Ras.GTP [10] (Fig. 8). Thus efficient spatial segregation between H- and K-Ras nanoclusters only occurs over a narrow range of PS levels [10]. Either a lower or higher PS content in the plasma membrane leads to failed segregation and the formation of heterotypic nanoclusters composed of both H- and K-Ras, which a concomitant attenuation of MAPK signaling [10] (Fig. 8).

Taken together these studies show that Ras nanoclustering is highly sensitive to both the PS and cholesterol content of the plasma membrane such that only a narrow window of overlap exists whereby the concentration of these two major PM lipids allow high fidelity lateral segregation of the various Ras isoforms [10] (Fig. 8). Because the basis for Ras lateral segregation is a set of interactions between plasma membrane constituents and lipid anchors and PBDs, it is reasonable to hypothesize that other membrane-associating proteins with lipid anchors and / or PBDs will potentially behave in a similar manner (Fig. 8). For example, other small GTPases, including Rac and Rho, certain G-protein-coupled receptors and large G-proteins all contain lipid anchors, PBDs, or both. To the extent that these proteins occupy spatially distinct, lipid-dependent domains on the plasma membrane aberrant mixing and coalescence may result if lipid composition is changed in the plasma membrane with attendant signaling consequences.

5. More complex biological implications of PS dependent lateral segregation

5.1. Spatial cross talk

Ectopic-expression of H-RasG12V to increase the density of H-Ras.GTP molecules on the plasma membrane significantly alters the spatial distribution of PS and inhibits K-Ras nanoclustering [10] (Fig. 8). This remote communication between spatially segregated H- and K-Ras is termed spatial cross talk. This is a lipid-mediated effect because a similar phenomenon is observed when over-expressing only the C-terminal HVR (CTH) of H-Ras without the G-domain, thus the mechanism does not require any signal output from H-Ras. Over-expression of either full-length H-Ras.GTP (as H-RasG12V) or CTH also disrupts clustering of the minimal membrane-anchoring domain of K-Ras (tK) [10], further illustrating that this is a membrane-mediated effect. Lentiviral expression of full-length H-RasG12V (H-Ras.GTP) or GFP-CTH in a cohort of endometrial and pancreatic cancer cells blocks proliferation of cell lines addicted to oncogenic K-Ras, but has no effect on those cell lines expressing wild-type K-Ras [10]. This observation is remarkable because expressing a second oncogenic Ras mutant (H-Ras.G12V) in K-Ras transformed cancer cells does not enhance cell proliferation, but rather compromises proliferation and viability. This result is consistent with the concept that ectopic expression of activated H-Ras, or GFP-CTH disrupts PS-mediated K-Ras clustering and thereby compromises K-Ras signaling.

The proposed mechanism for spatial cross talk is competition for PS between H-Ras and K-Ras nanoclusters. However, PS is a highly abundant plasma membrane phospholipid, so how might this competition arise? One hypothesis is that not all plasma membrane PS is equally available for Ras nanocluster interaction. This leads to the concept of different operational pools of PS, only some of which are relevant for domain formation (Fig. 9). If the key pool for nanoclustering is of low abundance then spatial cross talk mediated by competition for this limited resource becomes a realistic mechanism. Mathematical models based on the premise of different pools of PS can realize many of the PS dependent and spatial cross talk phenomena observed between K-Ras and H-Ras [10]. In favor of the concept of multiple pools of PS in the plasma membrane, FRAP and FCS experiments show that only ~40% of PS molecules are mobile on time and length scales relevant to Ras nanocluster formation [10,13,59], Even the PS molecules in the mobile pools cannot all participate in Ras clustering because a variety of membrane proteins associate and sequester PS in the plasma membrane (Fig. 9). Thus, only a small "reactive" pool of mobile PS likely participates in Ras nanoclustering at any given time point even though the overall PS level in the inner leaflet of the plasma membrane is high [10] (Fig. 9). Spatial cross talk occurs because various Ras nanoclusters are competing for this limited reactive pool of PS (Fig. 8). This means that Ras isoforms can communicate by remotely inducing changes in PS distribution without physically contacting each other.

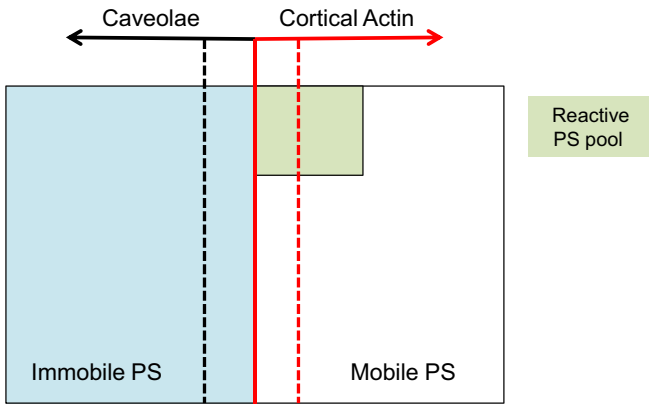

Fig. 9. PS exists in multiple pools in the plasma membrane. A large fraction of PS (~60%) is immobilized by cortical actin. Caveolae are enriched in PS and also inhibit cortical actin organization. Only a small fraction of the mobile PS pool is available for lipid platform assembly (the reactive pool). If cortical actin increases (red dotted line) the mobile pool and reactive pool decrease. The converse occurs as caveolae increase (black dotted line). Since the reactive pool is small K-Ras nanoclustering is highly sensitive to PS changes.

The phenomenon of spatial cross talk potentially has deep biological implications. If minor lipids, such as PS and PIP_2, exist in multiple pools, which effectively limit the amount of lipid molecules available for binding, it is not unreasonable to hypothesize that other key signaling lipids, such as PA, PIP_3, PI_3P, etc., may also have multiple reactive pools. In this scenario, those membrane-associating proteins with specific lipid-binding domains, highly charged juxtamembrane domains and PBDs will have to compete for binding with the lipids in the small reactive pools, as in the case of H-Ras and K-Ras (Fig. 8). This may potentially lead to spatial cross talk among various seemingly unrelated membrane proteins and surface signaling cascades. In this manner, the plasma membrane may interconnect multiple signaling cascades into an integrated lipid based remote communication network with powerful signal integration capacity.

5.2. Lipid mediated regulation and cellular control of Ras nanoclustering

Given the above discussion an important expectation is that controlling the availability or operational pool size of cholesterol or PS, and possibly other lipids, will modulate the operation of Ras nanoclusters and modify Ras signal transmission. There are several well documented examples of this phenomenon:

5.2.1. Actin cytoskeleton changes

Perturbing the actin cytoskeleton has multiple effects on Ras nanoclustering. Early experiments showed that depleting cortical actin with latrunculin inhibits K-Ras and –tH nanoclustering but has no effect on H-Ras.GTP clustering [8,9]. Cortical actin is also an important regulator of plasma membrane PS. Disruption of actin by latrunculin significantly increases the mobile fraction of PS [10,59] effectively increasing the PS level in the plasma membrane that is available for interacting with Ras. This leads to a failure of H- and K-Ras lateral segregation and the formation of mixed heterotypic nanoclusters composed of both H- and K-Ras [10]. As such actin depletion results in a partial phenocopy of increasing the total amount of PS in the plasma membrane [10].

5.2.2. Caveolin and Cavin expression

A cellular organelle that regulates lipid distribution in the membrane is the caveola [49]. Depletion of caveolae by knocking down expression of the caveolar structural components caveolin-1 (Cav-1) or cavin leads to significant changes in cell lipidomics [49]. Relevant to Ras nanoclustering and signaling caveloae regulate plasma membrane PS distribution. The molecular mechanisms are unclear, but PS is enriched in caveolae [60–62] and all mammalian cavin isoforms directly bind PS [60,63–67]. Caveolae also negatively regulate cortical actin by inhibiting activation of Rac1 [68], and since actin immobilizes PS loss of caveolae decreases the plasma membrane PS mobile fraction [68]. Together these changes in plasma membrane PS as a result of cavin or Cav-1 knock down (Cav-1 KD), or functionally depleting caveolae by hypotonic stress, enhance K-Ras clustering [10,49]. Conversely in cells co-expressing activated H-ras and K-Ras limited availability of mobile PS in Cav-1 KD cells results in failure of H- and K-Ras lateral segregation and the formation of mixed heterotypic nanoclusters composed of both H- and K-Ras [10]; a functional phenocopy of depleting overall cellular PS levels [10]. Cav-1 KD also compromises lateral segregation between GDP- and GTP-bound H-Ras, likely a consequence of the role of caveolae in regulating the lateral availability of cholesterol in the plasma membrane [49,51,69]. In migrating cells Cav-1 expression is polarized and mostly found at the leading edge [68]. It is interesting to speculate that high fidelity lateral Ras lateral segregation and hence efficient Ras signal transduction might therefore be restricted to the leading edge as a result of differing PS spatiotemporal dynamics induced by different caveolar distributions. Such behavior is observed in *Dictyostelium* where rapid activation of Ras signaling at the leading edge and quenching of Ras signaling at the trailing edge is critical for rapid migratory responses to chemotactic gradients [70–72].

5.2.3. Pharmacological agents

5.2.3.1. Fendiline and Staurosporines (STS). Since plasma membrane lipids play such important roles in the formation of signaling nanoclusters, changing lipid content should have biological and physiological implications. Fendiline specifically disrupts K-Ras but not H-Ras nanoclustering by mislocalizing PS from the plasma membrane, the molecular mechanism is presently unknown but is unrelated to the known pharmacology of fendiline as a an L-type calcium channel blocker [58]. Fendiline-induced disruption of K-Ras nanoclustering blocks K-Ras signal transmission and inhibits of the growth of K-Ras transformed cancer cell lines [58]. Staurosporines also redistribute PS from the plasma membrane to endomembrane leading to disruption of K-Ras nanoclustering and attenuated K-Ras signaling [57]. This effect is unrelated to the ability of the staurosporines to inhibit PKC as the concentrations that inhibit K-Ras clustering and signaling are well below the drug concentrations needed to inhibit PKC [57].

NSAIDS Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, naproxen and indomethacin, specifically stabilize cholesterol-enriched domains in synthetic model bilayers [73,74] and intact cell plasma membranes [50,73]. The NSAID-induced stabilization of cholesterol-enriched domains effectively increases the available cholesterol pool and induces mixing of Ras clusters between H-Ras.GDP and H-Ras.GTP and between K-Ras.GTP and H-Ras.GDP. In both cases, activation of CRAF by either oncogenic mutant H-RasG12V or oncogenic mutant K-RasG12V is compromised by NSAID treatment [50].

6. Conclusions

Ras proteins form spatially segregated, isoform-specific nanoclusters on the plasma membrane that are further regulated by guanine nucleotide binding state. As sole sites for effector binding, nanocluster formation is essential for Ras signal transmission. As a result of the different conformational orientations adopted by each Ras isoform in their GDP and GTP-bound states, each associates with different lipids in the plasma membrane, giving rise to distinct lipid compositions within each Ras nanocluster. These lipids, which include cholesterol, PS, PA, PIP_2, PIP_3, PI_3P and PI_4P, play different roles in mediating the structural integrity of homotypic Ras nanoclusters. Interesting emergent properties also arise as a result of competition for operationally limited pools of lipids including remote spatial cross talk between different types of nanocluster, as well as heterotypic nanoclustering between normally

segregated clusters. Plasma membrane lipid content and lipid spatio-temporal dynamics offer new levels of control for plasma membrane based signaling complexes that are arrayed in nanoclusters. Such regulation may be effected both through endogenous cellular mechanisms such as vesicular trafficking and endosomal lipid sorting as well as exogenous mechanisms using pharmacological agents.

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