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# Does Ras Activate Raf and PI3K Allosterically?

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The mechanism through which oncogenic Ras activates its effectors is vastly important to resolve. If allostery is at play, then targeting allosteric pathways could help in quelling activation of MAPK (Raf/MEK/ERK) and PI3K (PI3K/Akt/mTOR) cell proliferation pathways. On the face of it, allosteric activation is reasonable: Ras binding perturbs the conformational ensembles of its effectors. Here, however, we suggest that at least for Raf, PI3K, and NORE1A (RASSF5), that is unlikely. Raf's long disordered linker dampens effective allosteric activation. Instead, we suggest that the high-affinity Ras–Raf binding relieves Raf's autoinhibition, shifting Raf's ensemble from the inactive to the nanocluster-mediated dimerized active state, as Ras also does for NORE1A. PI3K is recruited and allosterically activated by RTK (e.g., EGFR) at the membrane. Ras restrains PI3K's distribution and active site orientation. It stabilizes and facilitates $PIP_2$ binding at the active site and increases the PI3K residence time at the membrane. Thus, RTKs allosterically activate PI3Kα; however, merging their action with Ras accomplishes full activation. Here we review their activation mechanisms in this light and draw attention to implications for their pharmacology.

**Keywords: allosteric, allostery, B-Raf, KRas, K-Ras, NORE1A, BRAF**

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## INTRODUCTION

*Is allostery driving Ras activation of its effectors*? The presumption that this is the case is easy to understand. Active Ras binds its effectors, and direct binding always perturbs the structures, initiating and promoting dynamic and at least some conformational changes (1–4). The relevant question is though—does Ras binding promote signals that propagate, through some allosteric pathways, *and lead to a functional change*? That is, do these signals prompt conformational and dynamic changes that affect the active site and are the dominant mechanism of effector activation? Even though not directly observed, the premise in the community has been that this is likely to be the case.

This premise has recently been revisited. Experimental and computational data indicated that at least for phosphatidylinositide-3-kinase α (PI3Kα) this is *not* the case (5, 6). Indeed, PI3Kα is known to be recruited and activated by epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK), at the membrane (7, 8). For Raf the premise still prevails. Here we overview PI3Kα and Raf activation, as well as activation of Ras association domain family 5 (RASSF5, a.k.a. NORE1A) tumor suppressor (**Figure 1**). We suggest that these Ras effectors are not activated via allosteric activation through Ras interaction. Further, even though to date there are no data relating to other Ras effectors, we suspect that this holds. In the case of Raf, a long disordered linker joins the kinase domain with the regulatory domain containing the Ras binding domain (RBD) and

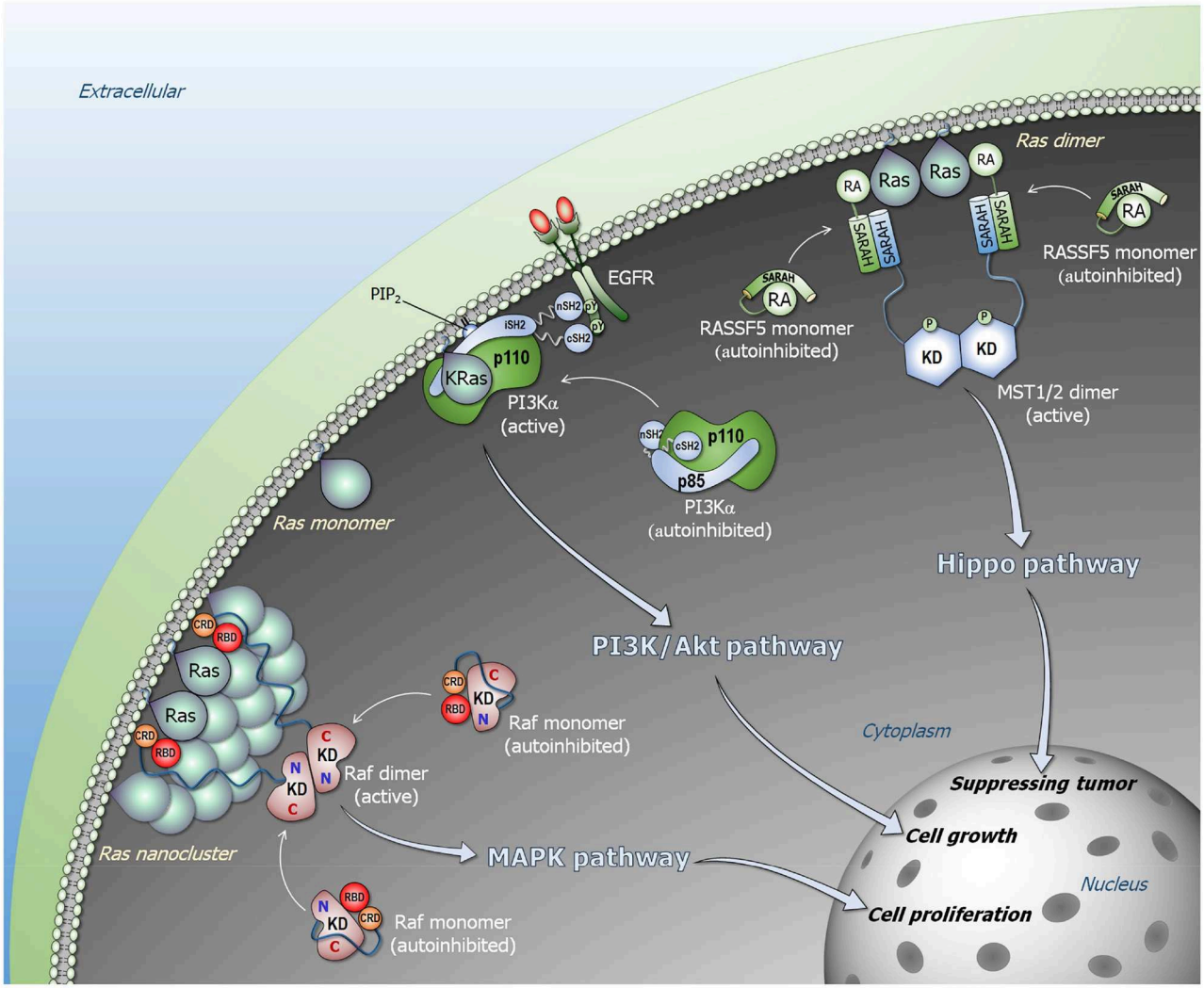


**FIGURE 1 |** Ras signaling pathways. Ras forms nanoclusters and promotes Raf dimerization in the Raf/MEK/ERK (MAPK) pathway (lower left). Monomeric Raf is autoinhibited in cytosol, and the high-affinity Ras–RBD interaction releases the autoinhibition, activating Raf through side-by-side dimerization. PI3Kα is allosterically activated by EGFR (middle). The C-terminal phosphorylated tyrosine motif of EGFR liberates the SH2 domains of p85α regulatory subunit from the p110α catalytic subunit, releasing the autoinhibition of PI3Kα. Ras binding is not link to the allosteric activation of PI3Kα, but its binding contributes to further increase in the residence time of active PI3Kα at the membrane. NORE1A (RASSF5) is an adaptor protein and autoinhibited by its RA domain interacting with its SARAH domain (upper right). In the presence of proximal Ras molecules, the Ras–RA interaction liberates NORE1A SARAH to recruit MST1/2 SARAH, promoting MST1/2 dimerization through their kinase domains that activates MST1/2 via cross phosphorylation. In the presence of Hippo signal, the active MST1/2 kinase promotes phosphorylation cascade signal, leading to YAP1 phosphorylation and degradation that result in tumor suppressing.

the cysteine-rich domain (CRD), which attaches Raf to the membrane (9–11). Protein disorder inherently implies no preferred interactions, no matter the sequence length. In the absence of specific interactions between the linker and RBD and the kinase domain, no allosteric propagation can take place. If no allosteric propagation, it is like there is no linkage between the two domains. The high-affinity Ras–RBD interaction (12, 13)—vs. the low affinity autoinhibition—argues in favor of activation via a shift in Raf's population toward the Ras-bound active state. In the case of PI3Kα, it is allosterically activated by the binding of the phosphorylated EGFR C-terminal motif to PI3Kα's Src homology 2 (SH2) domains (7, 14, 15); not by Ras. These binding events promote a conformational change which relieves PI3Kα autoinhibition and recruit PI3Kα to the membrane. Notably, EGFR activates PI3Kα even in the absence of Ras (16), albeit to a lesser extent. Activation of NORE1A tumor suppressor resembles the activation of the Raf proteins (17, 18). Taken together, these lead us to suggest some guidelines as to when allostery may not be involved in activation in binding events. This is important, since the mechanisms of activation are considered in drug discovery (19–26). If allostery is at play, disrupting propagation pathways is often deliberated.

Below, we first provide a brief background of allosteric activation. Next, we discuss activation of three Ras effectors, Raf, PI3Kα and NORE1A, and why allostery is unlikely to be involved. Finally, we lay out guidelines relating to when allostery is unlikely.

## ALLOSTERIC ACTIVATION: DEFINITION AND BACKGROUND

Classically, allosteric activation is defined as inducing a conformational change in the active site of the enzyme by binding at a location other than the active site. We suggested that if a conformational change is not observed, then it is likely due to limitations in the experimental approach used to detect a conformational change (27). Thus, with this definition, if Ras only has a role in recruiting the enzyme to the membrane, it would not be allostery since it does not elicit a conformation that alters the active site. Similarly, if Ras were to only restrict the orientation of the active site relative to the membrane to make productive catalysis more likely, by definition, this would also not be allostery because it would not involve a conformational change.

Allostery is linked to structural perturbation events (27–37). The events can be covalent changes, such as mutations, allosteric post-translational modifications (PTMs) or covalent allosteric drugs (38–42), or non-covalent, such as binding of small molecules (drugs, membrane signaling lipids, cofactors, water molecules, ions) or macromolecules, such as proteins (43–45). Allosteric events can take place near or away from the functional (active, protein-protein interaction, etc.) site; both can elicit efficient communication and productive allosteric events (29, 46, 47). Whether covalent or non-covalent, the perturbation breaks and forms new atomic interactions. In turn, the local changes promote additional adjustments in the interactions in their environments. These remodeling perturbations propagate along multiple pathways, with favored paths extending to the functional site, shifting the ensemble, thereby accomplishing distinct conformational and dynamic changes that switch the protein from the inactive to the active state (*vice versa* for repressors) (**Figure 2**). Thus, conformational dynamics is implicitly at play since allosteric events take place by a shift of the ensemble from energetically less favored states to more favored ones. Notably, the active conformation already exists in the ensemble; however, the shifts in the ensemble that allostery promotes increase its population. This conformation is primed to bind the substrate.

Allostery involves propagation which argues that the location of the allosteric event with respect to the active site is an important factor in determining its efficiency. Even though compact structures can act as efficient vehicles in allosteric transmission, dynamic segments, such as loops, linkers and hinges, respond and can efficiently mediate function (48, 49). Ras effectors are multidomain proteins, and to date no statistics have been published of the distributions of cancer driver mutations in multidomain proteins with respect to the functional (active) site. We expect that driver mutations tend to occur in the domain whose function is targeted. Mutations occurring in the catalytic domain make the active site conformation substrate-favored; those in a regulatory domain that acts in autoinhibition through its interaction with the catalytic domain, would relieve the autoinhibition. We are unaware of driver mutations occurring in non-catalytic domains whose actions propagate via disordered linkers to alter the active site conformations, as would be the case if Ras binding to the Raf's RBD were to allosterically activate it. To our knowledge, to date no driver mutations have been identified in Raf's RBD to substitute for its interaction with Ras.

To explain how Ras activates Raf, we consider two fundamental physical tenets. First, every biomacromolecule exists in an ensemble of conformations. For rigid molecules the ensemble is more restricted; for flexible (especially disordered) it is broad. Second, the most stable state is the most populated state. The ensemble of Raf monomers can be classified into three states: an active Ras-bound "open" state; a free "open" conformational state, and an autoinhibited "closed" state, where the kinase domain is blocked by another segment of Raf which prohibits it from dimerization (**Figure 1**). In the absence of Ras, Raf largely populates the microensemble of the autoinhibited state; however, a certain fraction of the population will be in the free state. The autoinhibited state is unlikely to be stable, since if it were, it should be possible to experimentally determine it (by crystallization, NMR). This is not the case for the very stable Ras–RBD complex. In the presence of Ras, Raf is most highly populated in the Ras-bound state due to a shift of the free state fraction. The equilibrium between the autoinhibited state and the free state will then be restored by a certain shift of the autoinhibited state to the free state. Kinase domain dimerization can take place even in the absence of Ras; however, GTP-bound active Ras raises the otherwise low population of the active species, with the exposed kinase domain prepped for dimerization. Ras' action in NORE1A's activation resembles its action in Raf's activation (**Figure 1**).

Allostery is unlikely to be at play in Ras' contribution to PI3Kα activation either. RTK binds PI3Kα (**Figure 1**). Binding promotes relief of PI3Kα's autoinhibition and exposure of the active site to the lipid substrate at the membrane through conformational change (6). However, no conformational change in PI3Kα is stimulated by Ras. Consequently, it is reasonable to conclude that the mechanism of Ras' activation of PI3Kα is not allosteric. Thus, even though the mechanisms of Ras activation of its effectors differ, in none of those explored here allostery is incurred by Ras action. Below we provide the mechanistic details.

## ACTIVATION OF RAS EFFECTORS RAF, PI3K AND NORE1A

### If Not Allostery, What Is Ras Role in PI3Kα Activation?

PI3Kα is a lipid kinase that phosphorylates phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$). Binding of Akt protein kinase to $PIP_3$ at the membrane is a key step in the AkT/mTOR signaling pathway leading to cell growth and proliferation. Inactive PI3Kα is a

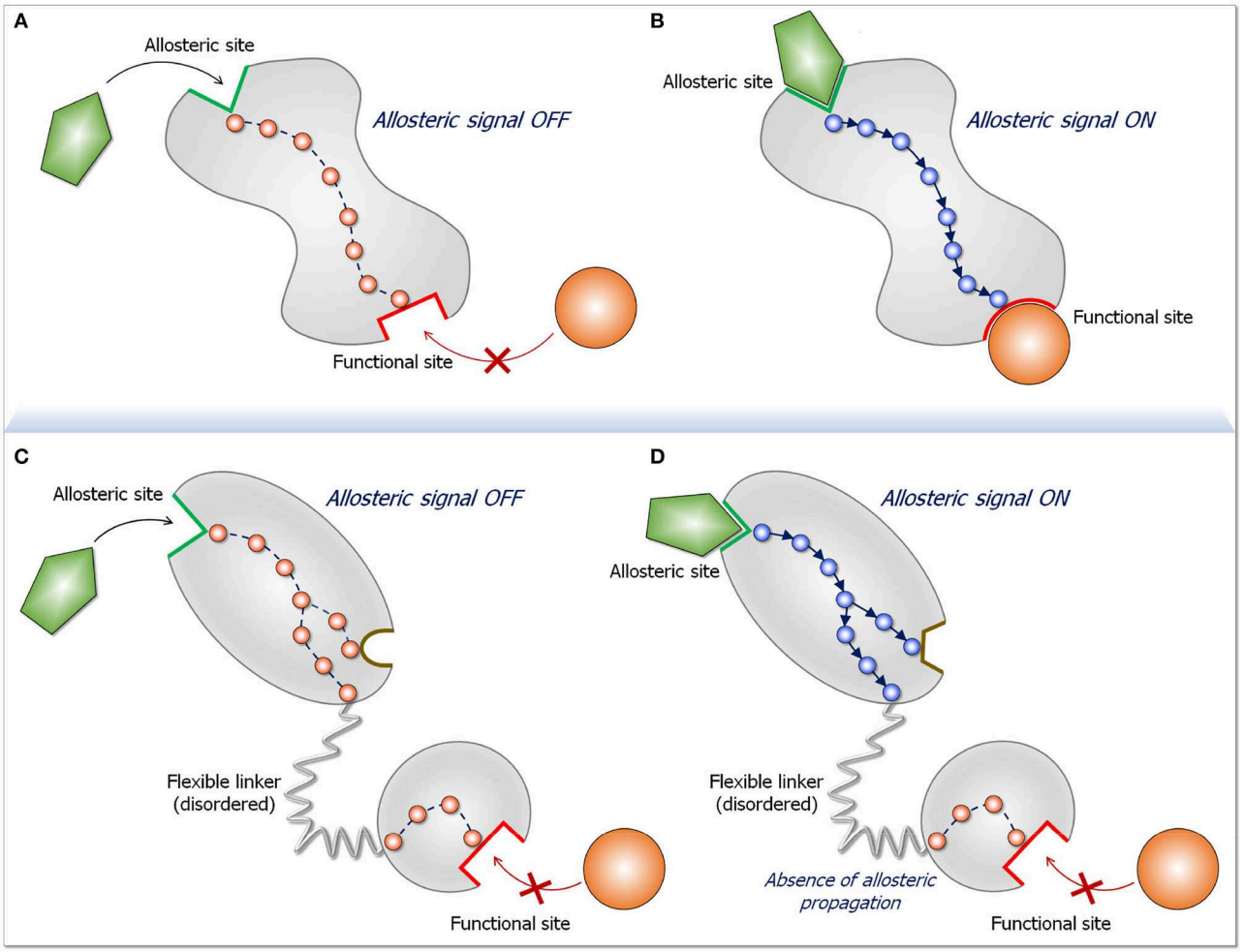


**FIGURE 2 |** Schematic diagram for an allosteric propagation pathway and its absence in long disordered linkers. The top two panels display a two-state dynamic allosteric switch. Both states pre-exist in the population. In the absence of the ligand **(A)** the protein populates a conformation in the ligand-free state. Upon ligand binding at the allosteric site **(B)**, a functional switch that is in favor of a ligand-bound state initiates at the binding site and propagates down to the functional site. The two bottom panels **(C,D)** depict what happens when two domains are joined by a long, disordered linker. The two-state switch takes place only in the domain to which the allosteric ligand binds, but do not propagate down the linker. The reason for the absence of allosteric propagation through the long linker is that the disordered state is distributed in multiple conformations. Since in the disordered state there are no specific stabilized interactions, there is no preferred propagation pathway. Preferred propagation pathways are required for population shift. In practice, identification of an allosteric propagation pathway in the structure can be achieved through superposition of the two (active and inactive) structures and locating changes in interactions of residues along pathways extending from the allosteric site to the functional site.

stable heterodimer. It consists of the p85α regulatory subunit and p110α catalytic subunit (6, 50) whose active site is blocked by p85α (15). Conformational changes, elicited primarily by the nSH2 domain of p85α, are a key step in PI3Kα activation (51, 52). These are the outcome of allosteric perturbation by EGFR (or another RTK). The phosphorylated tyrosine motif (pYxxM) in the C-terminal of RTK, interacts with high affinity with the nSH2 domain (7, 14). This interaction breaks the nSH2–p110α helical interface eliciting a conformational change that releases the nSH2 from p110α, as well as the p85α iSH2 domain from the p110α C2 domain, and the movement of the p110α's adaptor binding domain (ABD). iSH2 forms strong hydrophobic interactions and salt bridges with p110α's ABD, C2 and the kinase domains. Its rotation breaks its interaction with p110α's ABD consistent with hydrogen deuterium exchange mass spectrometry (HDX-MS) data (53). These conformational changes expose the PI3Kα membrane binding surface (5, 53–55). The mechanism of PI3Kα activation that we determined underscores the action of the RTK motif via its interaction with the nSH2 and the associated large conformational change. The release of nSH2 permits the C-lobe of the kinase domain to get away from the C2 domain, priming PI3Kα for phosphorylation of the $PIP_2$ lipid substrate to $PIP_3$ (15, 56). In oncogenic Ras, in the absence of RTK, calmodulin (CaM)'s phosphorylated tyrosine can similarly target the nSH2 (and cSH2 domains), recruiting and activating PI3Kα (57–59). Alternatively, EGFR overexpression can take place.

What is then Ras' role in PI3Kα activation? The RTK motif already accomplishes recruitment to the membrane with the coupled conformational change that relieves the autoinhibition and switches it from the inactive to the active state. The conformational change created by Ras binding is insignificant, and unlikely to play a role in activation. However, the PI3Kα population which is favorably positioned and oriented, primed for substrate binding and catalysis, is limited. We conclude that Ras binding serves to further increase the PI3K residence time at the membrane, stabilizing and facilitating $PIP_2$ binding at the active site. Thus, RTKs allosterically activate PI3Kα; however, merging their action with Ras accomplishes full activation (5).

## If Not Allostery, How Does Ras Activate Raf?

Raf is a multidomain protein. It has a variable length N-terminal tail that was proposed to mediate calcium-dependent B-Raf homo- and hetero-dimerization (60), interact with the C-terminal (61), and be responsible for A-Raf low basal activity. It also includes the RBD and CRD domain that latches Raf to the membrane, a variable-length linker containing the Ser/Thr-rich segment (10, 11), and the kinase domain. In the inactive state, monomeric Raf is autoinhibited. It's likely autoinhibited organization has recently been reviewed (9) along with the supporting experimental data and theoretical considerations (11, 61–87).

The high affinity (nanomolar range) active Ras–Raf's RBD binding recruits Raf to the plasma membrane (61, 88). CRD's anchorage to the membrane (89–91) is stabilized by its 'membrane insertion' loop residues (89, 92) in an organization that is similar to the one it adopts when alone, not in the Ras–RBD context (89). The Raf-1 linker connecting RBD and CRD consists of only 6 residues that further constrain and stabilize the Ras–RDB-CRD organization at the membrane. No interactions are observed between KRas4B, including the farnesyl, and CRD. This is not the case for the HRas farnesyl group. However, different than KRas, HRas has also two palmitoyls, and the two membrane-anchored palmitoyls lend stability to the system (93). Additional interaction details of the different Ras–Raf systems have also been uncovered (59, 89, 94–96). In a favored orientation, KRas4B attaches to the membrane through its farnesylated hypervariable region (HVR) in a way such that the effector binding site faces away from the membrane and is largely exposed. This permits the RBD to interact at the effector binding site while the CRD is anchored at the membrane through its loop. The nanomolar affinity of the Ras–RBD interaction has been measured in solution. However, under physiological conditions at the membrane, fluctuations that take place and molecular dynamics (MD) simulations indicate that these can be significant. The tethered Ras–RBD-CRD organization reduces the Ras–RBD fluctuations, thus increases the residence times of the productive organization. The enhanced affinity promotes a population shift of the Raf ensemble toward this Ras-bound state, relieving the autoinhibition.

High affinity is not the sole factor controlling the relief of Raf's autoinhibition and population shift toward the open state. Whereas, the disordered linker (∼180 residues in B-Raf; ∼170 residues in Raf-1) between CRD and the kinase domain deters allosteric transmission, it also encodes residues whose phosphorylation enhances or abrogates the autoinhibition. Ser446 phosphorylation of B-Raf weakens the autoinhibition; phosphorylated Ser259 of Raf-1 is recognized by 14-3-3 proteins (86, 87, 97, 98), promoting the autoinhibition. Dephosphorylation by protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) releases it, shifting the equilibrium toward open state (11, 80, 99–101). 14-3-3 also binds phosphorylated Ser621 of Raf-1 (86, 97, 98). The interaction of the N-terminal with the kinase domain is likely to be weak (9). Simultaneous binding at both sites can promote the autoinhibited state by stabilizing the interaction of the N-terminal segment and the kinase domain (11, 73, 87, 102–104). However, these distinct sites that assist in regulating the switch controlling the On/Off open/closed states, may not need such long linkers.

Taken together, this raises the question of *why long linkers*? We believe that the long linkers permit distancing the kinase domains from Ras–RBD-CRD at the membrane. The membrane is crowded. The linker efficiently connects the protein assemblies at the cytoplasm with signals communicated through receptor proteins, such as RTKs. In the cytoplasm, dimers of Raf kinase domains gather in large complexes, including mitogen-activated protein kinase (MEK) and extracellular signal-regulated kinase (ERK) dimers. Large scaffolding and adaptor proteins are also involved, e.g., kinase suppressor of Ras (KSR) (105, 106), IQ motif-containing GTPase activating protein (IQGAP) (107), heat shock protein (HSP90) (108), and galectin (109). All are large multidomain proteins that interact with additional proteins, such as IQGAP1 with Arp2/3 which stimulates branching of actin assemblies (110). The long linker provides an effective and pragmatic solution, enabling formation of clusters in the cytoplasm thus signaling efficiency. The large clusters are further favored by the water layer at the membrane surface which "pushes" or drives the proteins away from the membrane surface unless there are lipid-favoring residues at the protein surface, as in the case of CRD. The long linkers also vacate the requirement for Ras dimerization for Raf's activation. They allow Ras nanoclusters-mediated Raf's dimerization and activation (**Figure 1**).

Thus, rather than allostery, current data argues for a shift of the ensemble through release of the autoinhibited, closed state. In the absence of active Ras molecules, Raf mostly populates a closed autoinhibited state, with access to the kinase domain hindered by other segments. In the presence of Ras, the high affinity Ras–RBD interaction at the membrane shifts the ensemble. This mechanism is also supported by the dual 14-3-3 interaction, phosphorylation (dephosphorylation) experiments and mutational data [e.g., alanine and acidic substitutions at phosphorylation sites in the activation loop (73–75)]. It can explain why Raf evolved tight interaction with Ras and why Ras nanoclusters can function effectively in Raf's activation (111). It can also clarify how the large Raf assemblies with MAPK kinases and scaffolding proteins can form, act efficiently (112), and allow signaling dynamics (113) despite the crowded membrane surface.

## If Not Allostery, How Does Ras Activate NORE1A?

Different from Raf and PI3Kα, NORE1A (RASSF5) Ras effector is not a kinase, but essentially an adaptor protein, mediating the interactions of Ras and mammalian sterile 20-like kinase 1/2 (MST1/2). Ras-bound NORE1A activates the MST1/2 kinase (17, 114–118), which via the Hippo pathway phosphorylation cascade, leads to Yes-associated protein 1 (YAP1) phosphorylation and degradation. Overexpression of YAP1 induces cell proliferation (119). In the absence of active Ras, it is in a closed conformation, with its Ras association (RA) domain interacting weakly with the Sav-RASSF-Hippo (SARAH) domain. The linker between the two domains is short (5 residues) and contains a flexible hinge. In the presence of active Ras, the equilibrium shifts in favor of the tight Ras–RA interaction. The dissociated SARAH domain heterodimerizes with the MST1/2 SARAH domain. The tightly bound SARAH domain heterodimer releases the MST from its autoinhibited state, where the kinase domain interacts weakly with the MST SARAH domain. This shift in the MST ensemble from the inactive closed state to the open state permits kinase domain homodimerization and activation via trans-autophosphorylation. The affinity of the MST1/2 SARAH homodimer is lower than that of the hetero-SARAH dimer (120, 121), putting it under Ras control. NORE1A bridges Ras and MST (17), with Ras interaction acting to bring MST1/2 kinase domains into spatial proximity (18, 122), just like it activates Raf. Thus, rather than allostery activating NORE1A to promote its activation of MST kinase, the high (micromolar) affinity of the SARAH heterodimer drives the equilibrium toward NORE1A open active state, driving MST1/2 kinase activation via population shift.

## CONCLUDING REMARKS

Conformational ensembles and their shifts underlie biological processes (1–4, 30, 32, 123–131). Population shifts between two states due to differences in the stabilities follow the thermodynamic rule that systems are always driven to their free energy minima. In the case of the two Ras effectors described here, Raf and NORE1A, the higher stability of the interaction with Ras vs. that of the autoinhibited state drives the changes in the equilibrium. In the third, PI3K, Ras increases the population time at the membrane, facilitating $PIP_2$ insertion. Understanding how Ras effectors are regulated is of paramount importance since it can help in pharmacological discovery. Ras has additional effectors, including Tiam1, RalGDS, AF6, RIN, and more. Scenarios involving high affinity to Ras and long disordered interdomain linkers are likely to discourage allosteric transmission. A tell-tale is the presence (or absence) of observable conformational changes (27, 132). If binding promotes a conformational change, allostery is likely at play (**Figure 2**). This is the case for RTK's phosphorylated motif promoting conformational change in the interactions of the nSH2 domain of the p85α, which expose p110α active site. On the other hand, in our MD simulations of PI3Kα RBD complexed with KRas4B, we observed only insignificant conformational changes in RBD making an allosteric mechanism unlikely, in line with experimental data discussed here.

Finally, Ras does not have an allosteric role for the three effectors discussed above. However, this is not necessarily always the case for Ras or Ras-like GTPases. One example is the Ras family GTPase RHEB that appears to have a primary role as an allosteric activator of the mTORC1 complex (133).

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.