The Mystery of Rap1 Suppression of Oncogenic Ras

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Abstract

Decades ago, Rap1, a small GTPase very similar to Ras, was observed to suppress oncogenic Ras phenotype, reverting its transformation. The proposed reason, persisting since, has been competition between Ras and Rap1 for a common target. Yet, none was found. There was also Rap1's puzzling suppression of Raf-1 versus activation of BRAF. Reemerging interest in Rap1 envisages capturing its Ras suppression action by inhibitors. Here, we review the literature and resolve the enigma. *In vivo* oncogenic Ras exists in isoform-distinct nanoclusters. The presence of Rap1 within the nanoclusters reduces the number of the clustered oncogenic Ras molecules, thus suppressing Raf-1 activation and mitogen-activated protein kinase (MAPK) signaling. Nanoclustering suggests that Rap1 suppression is Ras isoform dependent. Altogether, a potent Rap1-like inhibitor appears unlikely.

Introduction

Decades ago, a paper published what was to become a pioneering and baffling finding: the discovery of the Rap1 protein (identical to Krev-1), with a high sequence similarity (53% identical) to Ras [1]. Rap1 has two isoforms, Rap1A and Rap1B, that differ in only a few amino acids. The work was intriguing, since the authors observed Rap1 to suppress oncogenic Ras phenotype, or as they put it, revert Ras transformation. They puzzled over the mechanism and suggested that Rap1 may antagonize Ras proteins, either by competing for a common target or by functioning as a G protein in transducing an inhibitory growth signal, opposing the Ras growth-promoting signal in fibroblasts. The question of why, or how, Rap1 suppresses Ras oncogenic signaling lingered in the decades that followed. A few years later, Rap1^{G12V} expressed at physiological levels was observed to interfere with signal transduction from Ras to MAPKs *in vivo* [2]. Activated Rap1 inhibited extracellular signal-regulated kinase (ERK)s' – but not Ras – activation, ruling out the involvement of nucleotide exchange factors. Nonetheless, in all experiments using conditional expression of Rap1^{G12V},

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MAPK inhibition was incomplete. As in the first publication, the authors suggested two possible explanations; in the first, Rap1[G12V]–GTP antagonizes Ras by competing for a Ras effector. In the second, Rap1[G12V] sends a downstream signal, antagonizing the MAPK pathway. The authors favored the first model since Ras and Rap1 proteins share a cluster of identical amino acids (residues 32–44 in HRas) in the effector-binding region. In further support of this rational, they cited Rap1–GTP potent inhibition of the interaction between Ras and p120 GTPase-activating protein (GAP) and that overexpressed Rap1 was unable to activate Raf-1 [2]. Altogether, this and subsequent publications (e.g., [3–7]; see also the references that follow) led to the still persistent view that Rap1 is a Ras antagonist, acting by competing with Ras and assembling into unproductive complexes with Ras effectors. However, more recently, biochemical, cellular, and developmental observations revealed that Rap1 has Ras-independent functions; primary among these is the control of cell adhesion-related events [4,7–29]. Such critical cellular functions are further ratified by Rap1 conservation across species, raising the question: is Rap1's raison d'être indeed Ras suppression, and if a different mechanism is involved, as this appears to suggest, the question reemerges: what is it?

The quest for the underlying mechanism becomes particularly important under the premise that the apparent Rap1 suppressor action can be exploited in Ras drug discovery. Multiple experiments testify to Rap1 interfering with Ras downstream MAPK signaling, underscoring its significance. To date, the paradigm lingers: Rap1 attenuates, or abates Ras transforming activity through competitive binding to a common, though still unidentified, target. Later, we suggest another explanation: the presence of Rap1 in a Ras nanocluster reduces the effective local concentration of oncogenic Ras molecules in the nanocluster, thus Raf activation and MAPK signaling (Figure 1, Key Figure). As we noted recently, such a simple and straightforward explanation also holds for several other oncogenic Ras-related apparent puzzles, such as how wild-type Ras suppresses the oncogenicity of its oncogenic mutant variant [30,31]. It is also in line with earlier Rap1 observations [32,33]. Those findings suggest that the strength of the interaction of the Raf cysteine-rich domain (CRD) is a critical determinant in Raf's activation. Thus, our rational simply stipulates that the presence of Rap1 'dilutes' the effective concentration of the oncogenic Ras in the nanocluster. If Rap1 is active, it will activate BRAF – but not Raf-1; if inactive, which is the expected state *in vivo*, neither will, even though Raf-1 CRD whose Rap1 binding is nucleotide independent, will still bind. Altogether, oncogenic Ras signaling will shrink, albeit not entirely, in line with the experimental data [2]. Ras signaling through BRAF will go; via Raf-1 it will not.

Excellent reviews on Rap1 functional roles have been published over the years (these include [5–7,16–20,34–36]). Thus, here we refer the reader to these rather than review them again. Instead, we prefer to focus on the apparent puzzling Rap1 observations and how they can be understood, and also the implications of our simple explanation.

Key Figure
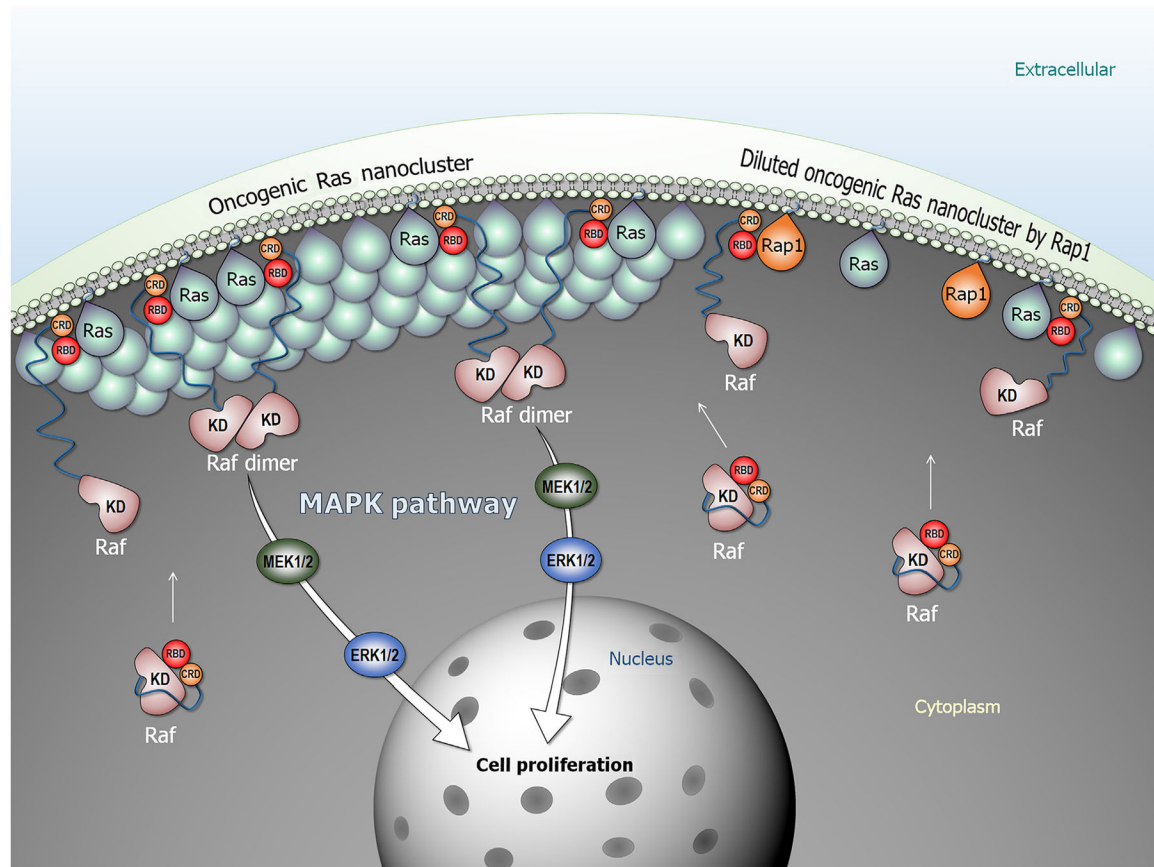
Ras and Rap1 Signaling Pathways

Figure 1.
Oncogenic Ras forms nanoclusters and promotes Raf dimerization, activation, and signaling in the mitogen-activated protein kinase (MAPK) pathway (Raf/MEK/ERK). In the cytosol, Raf is autoinhibited, but the high-affinity Ras–(RBD) Ras-binding domain interaction releases the autoinhibition. Raf is activated through side-by-side dimerization. Rap1 dilutes oncogenic Ras nanoclusters, suppressing Raf's activation. Abbreviations: CRD, cysteine-rich domain; KD, kinase domain.

A Nanoclusters-Centric View Can Explain Puzzling Ras Observations

Ras signaling plays a critical role in cancer initiation and cell proliferation [37]. To signal, Ras needs to be GTP bound and membrane anchored [31,38–47]. Attachment to the membrane is required for Ras activation and nanocluster formation [43]. However, nanoclustering is not essential for all oncogenic Ras pathways: it is needed for Raf's activation and MAPK signaling but not for PI3Kα activation and signaling through the PI3Kα/Akt/mTOR pathway [30,31]. This is because Raf's activation requires side-to-side dimerization (homo- or hetero-) of two kinase domains [48–57] (Figure 2). As the figure shows, the two domains are contributed by two Raf molecules, each is bound to active Ras through its Ras-binding domain (RBD) (Figure 1). For the two kinase domains to interact efficiently, they need to be nearby, a requirement that is fulfilled when the Ras molecules are spatially adjacent in the nanocluster (or exist as dimers, oligomers). To activate Raf

efficiently, the population of active Ras in the nanocluster should be of sufficient size. If sparse, the probability of dimerization of two Raf kinase domains decreases. This explains the necessity of Ras anchorage in the membrane. In solution, the chance of Ras molecules, thus Raf kinase domains, getting into proximity is low. Thus, if there are other small GTPases in the nanocluster that can bind – but do not activate – Raf, the outcome is essentially tantamount to 'Ras suppression.' Later, we suggest that Rap1 falls into this rubric. Nanoclusters are not needed for activation of PI3Kα, where the kinase domain from the p110 subunit exists as a dimer with the regulatory p85 subunit. p85 covers the active site, and activation involves a conformational change that exposes it at the membrane to its PIP_2 lipid substrate [58,59].

As in the case of Rap1, decades ago another puzzling observation was made: wild-type HRas can suppress cancer [60,61]. Subsequent work clarified that this holds for KRas as well: wild-type KRas can inhibit lung cancer. Additional crucial information was obtained subsequently [62]: wild-type NRas can suppress the malignant phenotype in the presence or absence of its mutant, and importantly, this is particularly the case for KRas [63]. Wild-type KRas inhibited cancer initiation and progression in cell lines expressing KRas, with stronger effect than for NRas [62]. Follow-up experiments confirmed these results [64,65] and provided further crucial data: wild-type Ras proteins can suppress their mutant isoforms [66]; however, it is still unclear exactly how. It was hypothesized that alternative pathways, or complex feedback loops, for the mutants and wild type could be involved. Further work extended these seemingly perplexing data [67–69]. Explaining these tantalizing observations was challenging but deemed essential if we are to harness them toward Ras drug discovery [66].

So, why does wild-type Ras inhibit its oncogenic variants, and why KRas more so than NRas? A Ras nanoclustering-centric outlook can help in resolving this question [30] (Figure 1). It can explain why wild-type KRas can inhibit its oncogenic variant, by simply considering that the presence of inactive wild-type Ras diminishes the concentration of the oncogenic isoform in the nanocluster, thus 'suppressing' oncogenic Ras. It also explains why suppression is stronger for KRas than for NRas by simply considering their relative abundance. In the cell line in which the experiments were conducted KRas is more abundant than NRas. It can also explain why a wild-type Ras isoform does not suppress a mutant isoform of a different type [65,70]. This is because different Ras isoforms favor distinct membrane compositions, a preference which is dictated largely by their hypervariable region (HVR) membrane-binding domains [31]. KRas favors acidic disordered membranes; HRas and NRas prefer neutral ones. Consequently, they are unlikely to share clusters.

A nanocluster-centric view can provide a simple explanation as to why wild-type Ras suppresses its oncogenic mutant: wild-type Ras typically exists in an inactive state. To get activated it requires a signal from receptor tyrosine kinase (RTK). The concentration, or population, of the nanocluster is mixed, containing oncogenic (constitutively active) and inactive Ras molecules, thus depressing Raf kinase domain dimerization and activation, and MAPK signaling. Here, we suggest that the presence of Rap1 in oncogenic Ras nanoclusters lowers the concentration of active Ras molecules (Figure 1), thus weakening MAPK signaling, in much the same way the wild-type inactive Ras tampers signaling by its

oncogenic isoform. This scenario rests on three notions. First, Ras nanoclustering plays a critical role in Raf activation, an increasingly accepted view in the Ras community, supported by experiments. Second, Rap1 (Rap1A) nestles in KRas nanoclusters. Even though to date there is no direct experimental data, considering the experimental evidence that HRas, NRas, and KRas do not share the same nanocluster due to the distinct chemical properties of their respective HVR membrane-attaching domains [71–73], it is reasonable that Rap1, that shares KRas HVR properties, will join its nanocluster. Third, Rap1 and Ras interact with BRAF and Raf-1 differently. Even though there is some experimental evidence in support of this, further confirmation and conformational details are essential. Our ongoing modeling and simulations are exploring this notion.

Consistent with this idea, we found that in the Cancer Genome Atlas (TCGA), pancreatic cancer and lung adenocarcinoma oncogenic *RAS* mutations co-occur with a shallow deletion of *RAP1A*. Importantly, shallow deletions of *RAP1A* are associated with its decreased expression in both cancer types (www.cbioportal.org) (Figure 3). These genetic data suggest that decreased Rap1 expression might favor development and progression of *KRAS* mutated tumors.

Rap1–Raf Interaction: Why the Difference between Raf-1 and BRAF and How Does It Relate to Rap1 Depressing Oncogenic Ras Signaling?

Why is it experimentally observed that Ras suppression is incomplete? This is because a certain extent of signaling will go through. Later, we discuss the apparent perplexing observations relating to the interactions of Rap1 with Raf. Early experiments indicated that Rap1 tampers with Raf-1 activation but not with BRAF's.

HRas Activates Raf Via a Shift in the Equilibrium toward Kinase Domain-Exposed States

Raf is autoinhibited [48,74]. In the autoinhibited state the kinase domain is unable to establish a side-to-side dimer, which is an absolute requirement for wild-type Raf autophosphorylation. Occlusion of the kinase domain is accomplished by the RBD and CRD domains [75,76], with involvement of the linker connecting CRD with the kinase domain [the serine/threonine-rich conserved region (CR)2] [77] (Figure 2). Active, GTP-bound Ras activates Raf through its high-affinity binding to its RBD. This shifts the equilibrium toward the open, kinase domain-exposed state [74,78]. Likewise, binding of active Rap1 to Raf's RBD is essential for Rap1-dependent Raf activation. The data on Rap1–Raf interactions via two Raf domains [32,33] were not entirely unexpected, given the earlier observations. Those observations showed that Raf-1 contains two distinct HRas-interaction regions, CRD and RBD, each recognizes distinct Ras sites [79–82], and only active Ras can bind and activate Raf [83]. Arguably, shifting the equilibrium in favor of conformations where both domains are away from the kinase domain would relieve the autoinhibition, thus facilitating the kinase domain dimerization. Those works were, however, confined to Raf-1. By contrast, the Rap1 works compared the interactions of Rap1 with Raf-1 with those of Rap1–BRAF and their consequences. Surprisingly, they discovered that whereas active Rap1 activates BRAF, it does not activate Raf-1.

Rap1 Activation Scenarios of Raf-1 Versus BRAF

Rap1 has an identical effector-binding domain to that of Ras (Figure 4A). However, whereas Raf-1 can bind active or inactive Rap1, active Rap1 does not activate Raf-1 and *in vivo* it even antagonizes some Ras functions [32,33]. To explore this puzzling observation, HRas protein residues, N26G and V45E, were replaced by those in Rap1. The mutations abolished Raf-1 CRD binding and activation, suggesting that HRas$^{N26G/V45E}$ impaired binding to both CRD and RBD. On the contrary, Rap1, with those residues, binds Raf-1 CRD more strongly than HRas does, even though it still does not activate it, indicating that those residues are not responsible for the Rap1–Raf-1 CRD interaction. E31K, another Rap1-mimic substitution, strengthened (tenfold) Raf-1 CRD binding; however, HRas^{E31K} was incapable of Raf-1 activation. The failure of the HRas^{E31K} mutant to activate Raf-1 led the authors to propose that K31 of Rap1 may play a role in the loss of activation. This hypothesis was confirmed by structural studies that observed that Raf-1 RBD binds HRas about 100-fold tighter than Rap1 does, and importantly, the lower Rap1 affinity was attributed to electrostatic repulsion by the interaction of K84 of Raf-1 RBD and K31 of Rap1 (which is Glu in HRas and can form a salt bridge) [84–86] (Figure 4B).

BRAF was also tested [33]. The experiments indicated that BRAF is activated by both HRas and Rap1. The authors concluded that Rap1 not only obstructs HRas-dependent activation of Raf; but also, fails to activate Raf-1 in contrast to BRAF. Their measurements of the strengths of the respective CRD interactions to Rap1 indicated that, different from Raf-1, BRAF's CRD interacted only weakly, leading them to conclude that the interactions of the CRDs hold the clue to the differential activation of Raf-1 versus BRAF. Domain-shuffling between Raf-1 and BRAF confirmed their conclusion: with BRAF's CRD, Raf-1 was activated by Rap1, whereas with Raf-1 CRD, BRAF was not. BRAF CRD double mutant BRAF$^{K253E/M279T}$, that strengthened the interaction, was not activated by Rap1 either, but it was still activated by Ras. Importantly, CRD is a membrane-interacting domain; but all experiments were carried out *in vitro*; thus, in the absence of the cell membrane.

Why the Distinct Rap1 Activation Scenarios of Raf-1 versus BRAF?

In the case of Raf-1, the tight binding of CRD to Rap1 is expected to interfere with optimized interaction of its RBD to Rap1's catalytic domain, thus retaining the largely inactive state (Figure 4C). The figure provides a structural model of Rap1 interaction with the CR1 of Raf-1, which is based on our modeling of KRas4B with Raf-1 RBD and CRD [87]. In the model of Rap1–Raf-1 CRD interaction, we observed formation of a possible salt bridge between K31 and E174, residues that are Rap1 and Raf-1 specific, respectively. We have also modeled Rap1 with BRAF, based on our previous KRas4B-Raf-1 RBD–CRD model [87]. Rap1 interacts with BRAF RBD (Figure 4B) and activates it as Ras does. In the autoinhibited states of BRAF and Raf-1, both RBD and CRD are bound to the kinase domain (Figure 4D). BRAF is activated by Rap1, with the equilibrium shifting toward the active BRAF state. The higher affinity BRAF RBD binding to the GTPase catalytic domain, facilitates the weaker binding of BRAF CRD to Rap1, relieving the autoinhibition. By contrast, the strong Raf-1 CRD interaction with Rap1 [32,33] prevents optimal Raf-1 RBD interactions, retaining Raf-1 in the inactive state. As to Ras [87,88], binding is expected to be isoform dependent, in line with membrane composition and HVR preference.

The membrane is absent in some early experiments but is present in others [89]. Notably, these also include solution NMR. The experiments showed that the Raf-1 CRD–HRas interactions are nucleotide independent but are enhanced by Ras farnesylation. An impaired Raf-1 CRD variant helped identify the key interacting residues in Ras-mediated activation of Raf-1. These residues, 149, 150, 151, 152, 158, and 159, form a hydrophobic patch on Raf-1 CRD. Mutant Raf-1 CRD$^{F151Q/L149T}$ abolishes the HRas–Raf-1 interaction, and consequently Raf-1 activation. Guided by this observation, our inspection of Rap1 surface suggests the presence of several hydrophobic patches with which CRD may form a strong association. To date, there is no direct measurement of the dissociation constant between Rap1 and Raf-1 CRD domains.

How Does the Difference between Raf-1 and BRAF Relate to Rap1 Depressing Oncogenic Ras Signaling?

Ras-dependent Raf activation requires that Ras molecules be spatially close to each other (Figure 1). If they are too sparse, the chance of Raf's kinase domains to dimerize is low. This is what happens if Rap1 is present in the Ras nanocluster. If Rap1 is active, BRAF will be activated; if inactive, it will not be. For Raf-1, in neither case will it be. In the absence of incoming activation signals, Rap1 is likely to be inactive in the oncogenic Ras nanoclusters. The reduced number of oncogenic molecules in the nanocluster, diminishes – but not abolishes – Ras signaling.

Would Rap1 Suppress All Ras Isoforms?

Experimentally, Rap1A inhibits HRas activation of Raf-1. However, those early experiments were carried out in the absence of the cell membrane. Distinct Ras isoforms favor different membrane compositions and assemble in distinct nanoclusters. Since the HVR of Rap1A is highly positively charged, we expect that in the membrane it colocalizes with and suppresses KRas (KRas4A or KRas4B). By contrast, the HVR of Rap1B is fairly neutral; thus, we expect it to colocalize with and suppress NRas or HRas.

Concluding Remarks

So why does Rap1 interfere with Ras downstream MAPK signaling and revert its transformation? This baffling observation has stymied Ras biology for decades. Possible explanations were proposed early on [1], advanced again a few years later [2], and have been unchallenged since. These argued that Rap1 competes with a Ras target. Even though the similarity in the effector-binding domain lends support to such a hypothesis, no candidate target protein has been identified in the decades since. In addition, this hypothesis cannot explain why, if the two effector-binding surfaces are so similar, and the relative expression levels had no clear effects, there would be such an outcome. The same arguments would apply to interference by inhibiting a pathway protein. Tissue specificity is not an argument here either, since, if that was the case, Rap1 would not depress Ras. Here we take it up from a structural standpoint. This permits us to propose a coherent view that is consistent with available data, albeit one that still needs direct testing (see Outstanding Questions). In essence, we suggest that the key rests with small GTPases organized in the membrane in nanoclusters, an organization which is required for activating those effectors that need to

dimerize in order to get activated, like Raf or Rassf5/NORE1A [90], although not for other pathways, like PI3K/Akt that do not [30].

A nanocluster organization implies that Ras molecules are assembled in the membrane at favored membrane locations (such as certain lipid composition [91] and membrane geometric features [92]). Its aim is signaling efficiency. In the nanocluster, dynamic Ras molecules are spatially proximal. However, the presence of Rap1 within the cluster 'dilutes' active Ras content, making it sparser, thus, essentially 'suppressing' Raf's activation. If Rap1 is inactive, which is likely to be the case in oncogenic Ras clusters since no incoming cue, neither Raf-1 nor BRAF, will be activated. If Rap1 is active, Raf-1 still wouldn't be, but BRAF would. Notably, the Rap1–Raf data comes from *in vitro* experiments [32,33]. These should be repeated in the presence of the membrane to capture the *in vivo* environment.

The search is on for new drug targets and novelty is often the key [93,94]. Mechanism complexity is, however, often difficult to untangle and overlooked factors can be at play [95,96]. Nanoclustering is a fundamental organization in the cell. However, it is often not considered in explaining *in vivo* phenomena. We hope that this work as well as an earlier one, which similarly showed how the presence can explain puzzling observations [30], will help with making strides in this direction. However, the underlying assumption, that Rap1's suppression action on oncogenic Ras can be captured by inhibitors, does not appear as a viable model for Ras inhibition.

Acknowledgments

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Highlights

Rap1, a small GTPase very similar to Ras, suppresses oncogenic Ras pheno-type; albeit not completely. The initial hypothesis was that Ras and Rap1 compete for a common target, but none has been found.

One reported function of Rap1 is to modulate the activity of Raf kinases, thus MAPK signaling. Based on published observations, we propose that Rap1 suppresses MAPK signaling via Raf-1 but not via BRAF. We suggest that Rap1 high-affinity binding to Raf-1's cysteine-rich domain (CRD) disfavors Rap1 interaction with Raf-1's Ras-binding domain (RBD). BRAF's CRD has lower affinity to Rap1; its RBD can bind to Rap1.

The presence of active Rap1 in oncogenic Ras nanoclusters reduces (dilutes) the number of the oncogenic Ras molecules in the cluster, thus suppressing Ras activation of Raf-1 but not of BRAF.

We suggest that Rap1 suppression of Ras is likely to be Ras and Rap1 isoform specific.

Outstanding Questions

Experiments discovered that active Rap1 interacts with BRAF RBD and activates it as Ras does, but it does not activate Raf-1. Structural determination of the Rap1–BRAF RBD would address the essential question of whether those early results are relevant to oncogenic cell signaling, as we suggest here.

Experimentally, in the absence of the cell membrane, Rap1 (Rap1A) inhibits activation of Raf-1 by HRas. However, the hypervariable region (HVR) of Rap1A is KRas-like, thus, it can colocalize and suppress KRas, and Rap1B can colocalize with and suppress NRas or HRas, whose HVRs it resembles. Thus, do these isoform distinction scenarios hold *in vivo*? This requires experimental verification.

RAP1A loss of heterozygosity is associated with *KRAS*-mutated lung and pancreatic tumors. Functional studies should address the question of whether decreased *RAP1A* expression triggers *KRAS*-mediated transformation.

Are there additional small GTPases that may interfere with oncogenic Ras signaling?
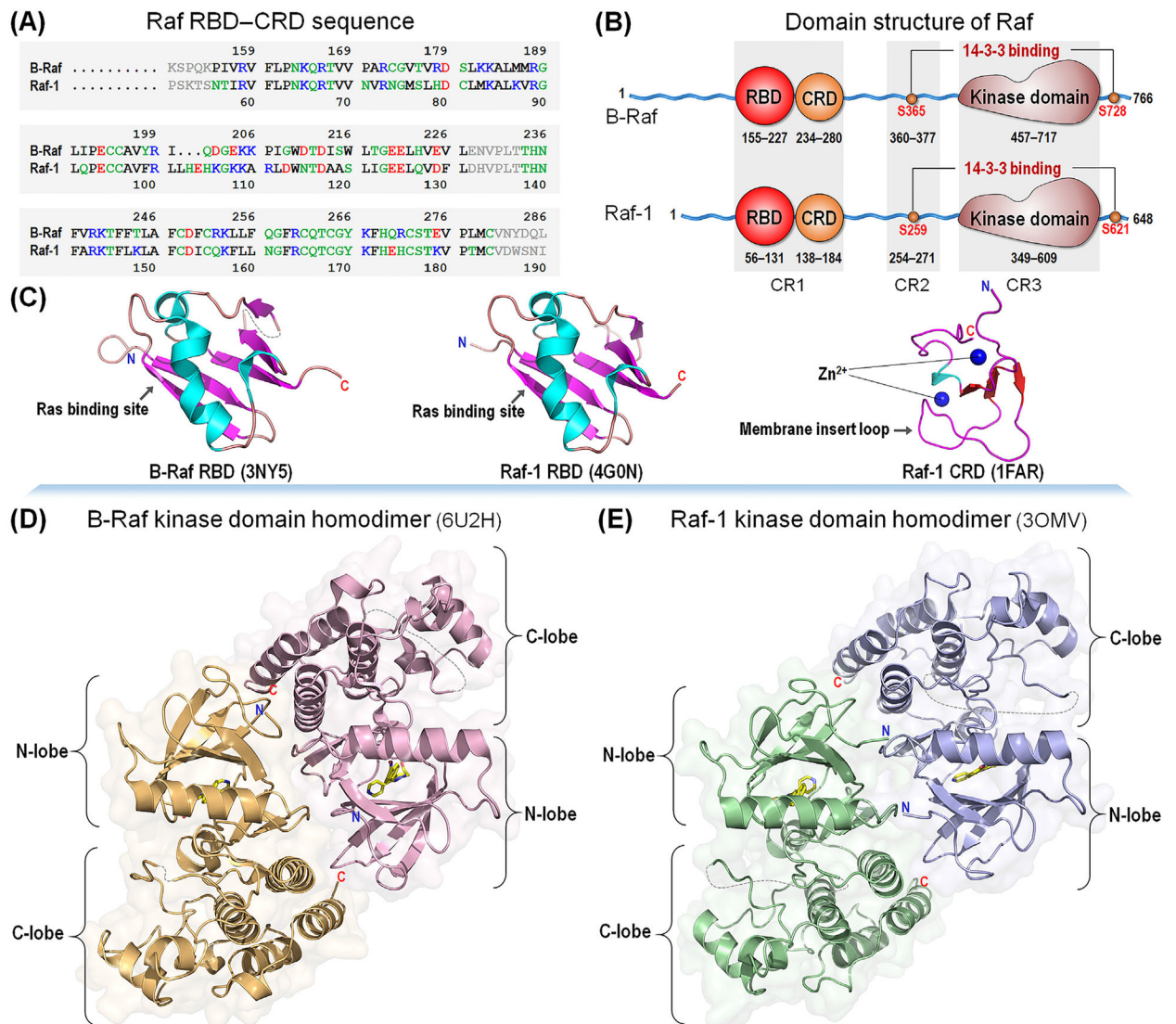
(A) Raf RBD–CRD sequence

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              159         169         179         189
B-Raf .......... KSPQKPIVRV FLPNKQRTVV PARCGVTVRD SLKKALMMRG
Raf-1 .......... PSKTSNTIRV FLPNKQRTVV NVRNGMSLHD CLMKALKVRG
                 60          70          80          90

         199       206         216         226         236
B-Raf LIPECCAVYR I...QDGEKK PIGWDTDISW LTGEELHVEV LENVPLTTHN
Raf-1 LQPECCAVFR LLHEHKGKKA RLDWNTDAAS LIGEELQVDF LDHVPLTTHN
      100         110         120         130         140

         246       256         266         276         286
B-Raf FVRKTFFTLA FCDFCRKLLF QGFRCQTCGY KFHQRCSTEV PLMCVNYDQL
Raf-1 FARKTFLKLA FCDICQKFLL NGFRCQTCGY KFHEHCSTKV PTMCVDWSNI
      150         160         170         180         190
```

(B) Domain structure of Raf

(C) B-Raf RBD (3NY5) — Ras binding site; Raf-1 RBD (4G0N) — Ras binding site; Raf-1 CRD (1FAR) — Zn²⁺, Membrane insert loop →

(D) B-Raf kinase domain homodimer (6U2H)

(E) Raf-1 kinase domain homodimer (3OMV)

Figure 2. Comparison between BRAF and Raf-1.
(A) Sequences of conserved region (CR)1 and (B) domain structures of BRAF and Raf-1. In the sequence, hydrophobic, polar/glycine, positively charged, and negatively charged residues are colored black, green, blue, and red, respectively. Gray denotes the unstructured loop region. All Raf kinases share three conserved regions; CR1 involves the Ras-binding domain (RBD) and cysteine-rich domain (CRD), CR2 contains the Ser/Thr-rich region at the flexible linker, and CR3 is the kinase domain. (C) Crystal structures of BRAF RBD (PDB: 3NY5) and Raf-1 RBD (PDB: 4G0N), and solution structure of Raf-1 CRD (PDB: 1FAR). (D) Crystal structures of the kinase domain homodimer for BRAF (PDB: 6U2H) and (E) Raf-1 (PDB: 3OMV). Raf domain structures are highly homologous among the isoforms.

(A)

Pancreatic adenocarcinoma (TCGA, PanCancer Atlas)

RAS status (A)	CNV (B)	Neither	A not B	B not A	Both	Log2 odds ratio	p-value	q-value	Tendency
Mutant KRAS	RAP1A shallow deletion	50	82	8	35	1.416	0.014	0.042	Co-occurrence
Wild-type KRAS	KRAS shallow deletion	55	105	3	12	1.067	0.202	0.303	Co-occurrence

Lung adenocarcinoma (TCGA, PanCancer Atlas)

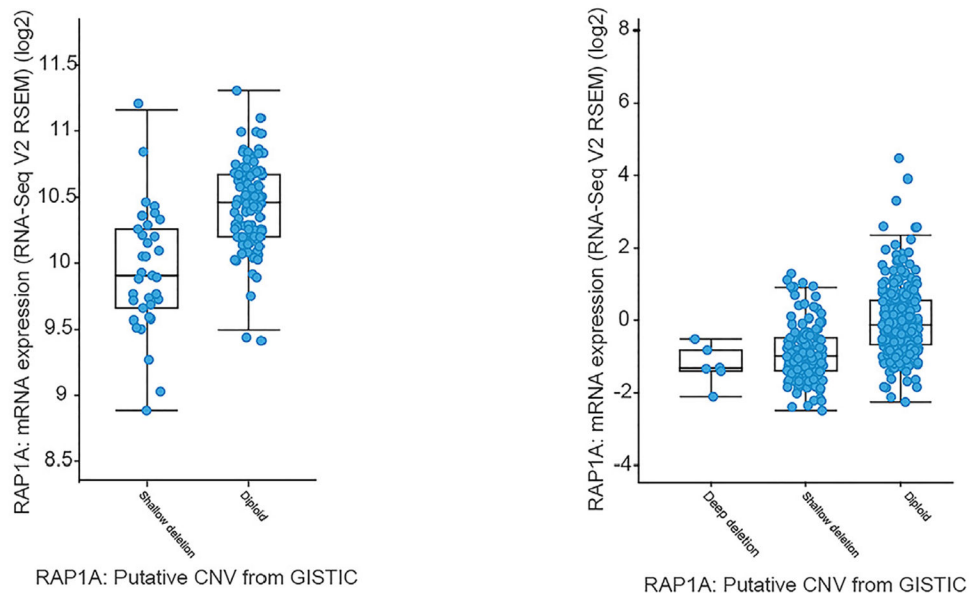| RAS status (A)	CNV (B)	Neither	A not B	B not A	Both	Log2 odds ratio	p-value	q-value	Tendency
Mutant KRAS	RAP1A shallow deletion	261	100	93	53	0.573	0.037	0.055	Co-occurrence
Wild-type KRAS	KRAS shallow deletion	278	134	76	19	-0.947	0.010	0.030	Mutual exclusivity

(B)

Figure 3. Decreased Rap1 Expression is Associated with KRas Mutation.
(A) Shallow deletion of *RAP1A* and *KRAS* mutation co-occur in pancreatic and lung cancers. The distribution of *KRAS* somatic mutations and *RAP1A* copy number variations (CNV) in the Cancer Genome Atlas (TCGA) pancreatic and lung cancers obtained from cBioPortal. cBioPortal was queried over all completed tumors from the PanCancer Atlas datasets using the Onco Query Language (OQL), '*RAP1A*:HETLOSS; KRAS: G12, G13, K61.' (B) Shallow deletion of *RAP1A* leads to decreased mRNA in pancreatic and lung cancers. The plots are generated by cBioPortal (www.cbioportal.org) [97,98]. Abbreviation: GISTIC, Genomic Identification of Significant Targets in Cancer.
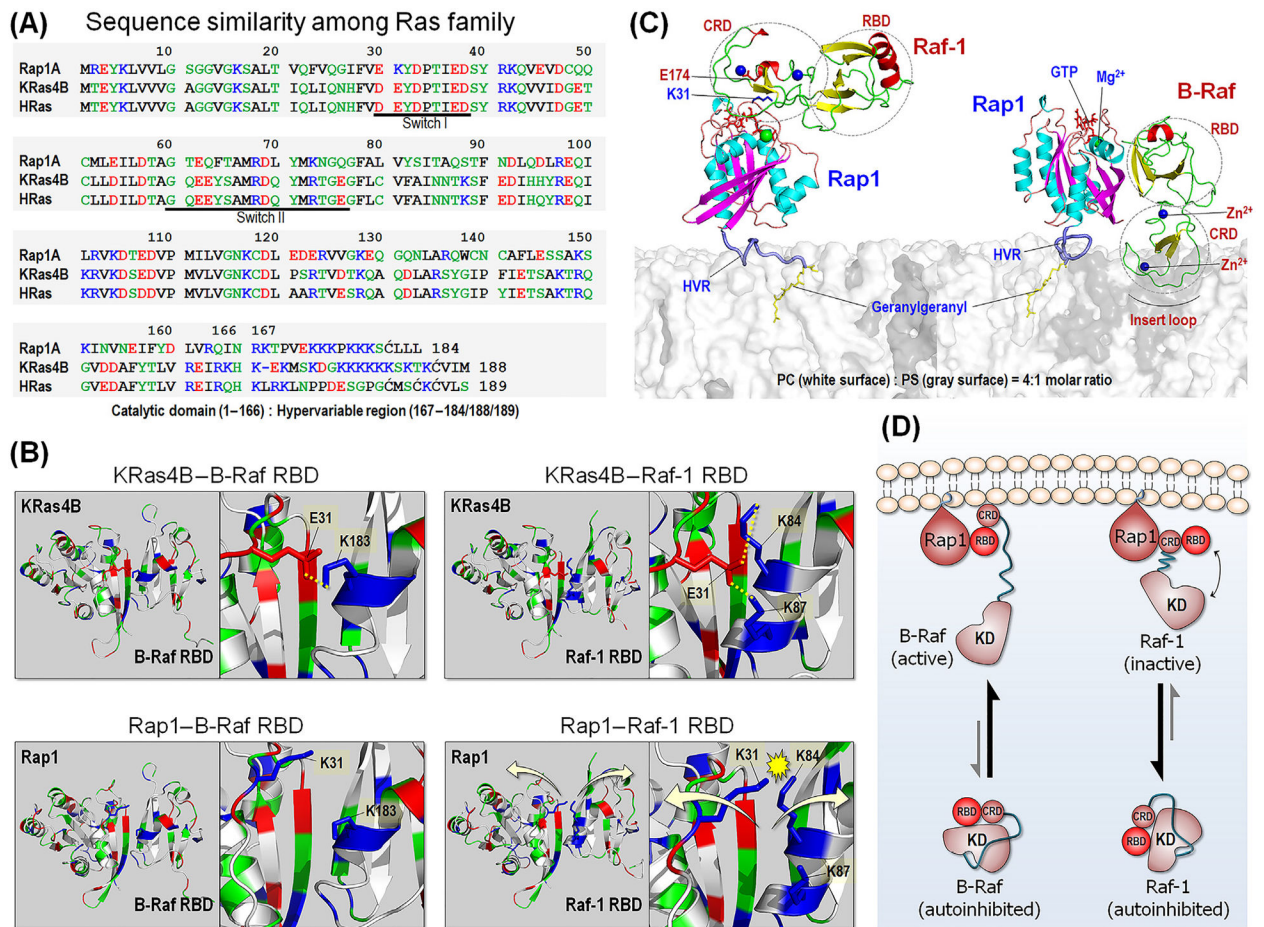
Figure 4. Comparison between Rap1 and Ras.

(A) Sequence similarity among Ras family, including Rap1, KRas4B, and HRas. In the sequence, hydrophobic, polar/glycine, positively charged, and negatively charged residues are colored black, green, blue, and red, respectively. (B) Model structures of KRas4B catalytic domain interacting with BRAF Ras-binding domain (RBD) (upper left) and Raf-1 RBD (upper right), based on our previous studies [87]. In addition to the strong β-sheet interaction, Ras adds stability to the interaction with the RBDs through the salt bridges between E31 and K183 for BRAF, and between E31 and K84/K87 for Raf-1. Rap1 can interact with BRAF RBD due to distant repulsive force between K31 and K183 (lower left). However, in the interaction with Raf-1 RBD, Rap1 K31 electrostatically crashes with K84/K87, leading to dissociation. (C) Model structures of Rap1 interacting with Raf-1 conserved region (CR)1 (left) and BRAF CR1 at the anionic membrane. Rap1 prefers to bind Raf-1 cysteine-rich domain (CRD), while it binds to BRAF RBD. (D) Schematic diagram of Raf activation by Rap1. Abbreviations: KD, kinase domain; HVR, hypervariable region.