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New insights into RAS biology reinvigorate interest in mathematical modeling of RAS signaling

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Abstract

RAS is the most frequently mutated gene across human cancers, but developing inhibitors of mutant RAS has proven to be challenging. Given the difficulties of targeting RAS directly, drugs that impact the other components of pathways where mutant RAS operates may potentially be effective. However, the system-level features, including different localizations of RAS isoforms, competition between downstream effectors, and interlocking feedback and feed-forward loops, must be understood to fully grasp the opportunities and limitations of inhibiting specific targets. Mathematical modeling can help us discern the system-level impacts of these features in normal and cancer cells. New technologies enable the acquisition of experimental data that will facilitate development of realistic models of oncogenic RAS behavior. In light of the wealth of empirical data accumulated over decades of study and the advancement of experimental methods for gathering new data, modelers now have the opportunity to advance progress toward realization of targeted treatment for mutant RAS-driven cancers.

Keywords

RAS; ERK cascade; mechanistic modeling; mathematical modeling; systems biology

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Conflict of Interest

The authors declare that they have no conflict of interest.

1. Introduction

Following the discovery that mutant RAS genes can cause oncogenic transformation (reviewed by Barbacid in 1987 [1]), RAS signaling has been in the limelight of scientific interest for over three decades [2]. RAS proteins are GTPases and key transducers of receptor tyrosine kinase (RTK) signaling, which is central for cell proliferation and survival. Although more than 170 RAS-related proteins have been identified in humans, three RAS family members are recognized as important oncogenes: HRAS, NRAS, and KRAS (with splice isoforms KRAS4A and KRAS4B) [3]. Variants in these three RAS genes are the most prevalent mutations across human cancers, appearing in more than 30% of all cases, yet therapies to selectively inhibit mutant RAS are only recently emerging [4,5].

RAS receives activating inputs from guanine nucleotide exchange factors (GEFs) and deactivating inputs from GTPase activating proteins (GAPs) (Fig. 1). GEFs (e.g., SOS1-2, RASGRP1-4, RASGRF1-2) facilitate RAS conformational change from the inactive GDP-bound form to the active GTP-bound form by promoting nucleotide release. As intracellular GTP/GDP ratios are estimated to be greater than 10 under nutrient replete conditions [6,7], a free RAS G domain is more likely to bind GTP than GDP. GAPs (e.g., RASA1, NF1, SYNGAP1, RASAL1, RASA3-4) prompt the reverse transition by accelerating GTP hydrolysis [8-13]. Thus, RAS GAPs have the potential to function as tumor suppressors, and loss-of-function mutations affecting RAS GAPs have the potential to be oncogenic. Indeed, suppression of NF1 is found in a variety of cancers including glioblastoma, non-small cell lung cancer, and melanoma, and mutations in NF1 are found in neurofibromatosis type 1 [14]. Similarly, inactivation of RASA1 can promote melanoma tumorigenesis via RAS activation, and lower expression of RASA1 is associated with decreased survival in melanoma patients harboring BRAF mutations [15]. RAS has similar affinities for GTP and GDP, but because of intrinsic GTPase activity and a low GDP/GTP exchange rate in the absence of GEF activity, inactive RAS-GDP is the prevailing form in unstimulated cells [16].

RAS interacts with a number of downstream effectors, which become activated when recruited to the GTP-bound form of RAS. These effectors include RAF kinase family proteins (ARAF, BRAF, and CRAF), phosphoinositide 3-kinase (PI3K), RAS-like (RAL) small GTPases (RALGDS, RGL, RGL2, RAGL3), phospholipase C epsilon (PLCε), the RNA-silencing effector Argonaute 2 (AGO2), and RAS association domain family (RASSF) proteins (NORE1, RASSF1A) [11,17,18]. The pathways emanating from RAS effectors influence a wide variety of cellular processes, including mitogenic, survival, pro-apoptotic, inflammation, DNA-damage repair, and senescence pathways [19] (Fig. 2). Furthermore, GTP-bound KRAS4B proteins may form RAS-RAS dimers that promote dimerization and activation of RAF kinases while hiding other RAS effector binding regions [20].

Most oncogenic *RAS* mutations disrupt the GTPase activity of RAS isoforms, locking RAS in the GTP-bound state and resulting in constitutive activation of downstream cell signaling pathways. Over 99% of all oncogenic *RAS* mutations occur in codons 12, 13, and 61 [21]. Codons 12 and 13 are located in one of four primary sequence regions critical for GTP-binding. Codon 61 falls in a region that is important for both GTP-binding and GEF-binding

(the Switch II region) [22]. Although codons 12, 13, and 61 are in regions that are identical for all RAS isoforms, the distribution of oncogenic mutations differs between these isoforms [21]. Constitutive activation of NRAS by mutation at codon 61 is more common in melanoma [23], whereas mutations in *KRAS* codons 12 and 13 are common in colorectal, lung, and pancreatic cancers [24]. Interestingly, 80% of oncogenic *KRAS* mutations occur in codon 12 [21].

The prevalence of *RAS* mutations in cancers, availability of empirical data accumulated over decades of study, and the complexity of RAS signaling networks render RAS a promising candidate for investigation via mathematical modeling. Models have proven useful in simulating both the RAS activation cycle as well as the larger network surrounding RAS, including the extracellular signal-related (ERK) cascade [25]. In 2000, Brightman and Fell published an an ordinary differential equation (ODE) model describing regulation of ERK that considered RAS activation and GEF/GAP activity [26]. This model revealed the importance of feedback regulation in achieving either sustained or transient activation of RAS, MEK, and ERK. In 2002, Schoeberl et al. [27] produced an ODE model of the ERK pathway, consisting of 101 reactions and 94 species, many of which were included in Kholodenko et al.'s [28] 1999 model of signal transduction from the epidermal growth factor receptor (EGFR) through SOS. This model was applied to predict how dynamics of growth factor binding impact ERK activation. However, it lacked GAP regulation and considered GAP activity as a constant factor (reviewed by Orton et al. [29]). In 2004, Markevich et al. described an early mechanistic model focused on RAS activation by RTKs [16]. This model captured the regulation of wildtype RAS by GEFs and GAPs as well as the consequences of changes in RAS intrinsic nucleotide exchange activity and GTPase activity. Importantly, the model demonstrated that RAS activation patterns can be explained by delays between the activation of GEFs and GAPs by RTKs, resulting in transient RAS activation in response to epidermal growth factor (EGF) treatment. In 2007, mechanistic models began to be used to study the impact of mutations on RAS signaling, with the model of Stites et al. [30] comparing wildtype and oncogenic mutant RAS to infer strategies for selectively inhibiting the oncogenic network. In 2009, Orton et. al. modeled the ERK pathway to predict the result of EGFR overexpression or mutations in RAS, BRAF, and EGFR [31]. In 2015, the model of Stites et al. (2007) was expanded to simulate random mutagenesis throughout the network, leading to the conclusion that mutations in the tumor suppressor gene *NF1* work in concert with mutations in RAS signaling to drive cancer [32].

Mathematical modeling promises to help us understand distinct RAS signaling patterns in the context of different adaptive topologies of the RAS network and diverse cellular backgrounds [33]. Yet, existing models have mostly focused on RAS activation within a single RTK pathway, neglecting to consider the impacts of intricate feedback and feedforward interactions between multiple RAS effector pathways. Furthermore, there is an unmet need for modeling studies that evaluate the phenotypic consequences of the broad spectrum of RAS mutations and that consider differential localization of RAS isoforms. In this review, we describe several new technologies that can generate the data needed to develop more sophisticated models of RAS signaling. We summarize complex and nonlinear phenomena involved in RAS signaling, which provide novel opportunities for mathematical modeling studies. In light of these developments, the future application of improved

mathematical models of RAS signaling could enable prediction of clinical responses to drugs and their combinations and to eventually aid in the rational design of cancer therapies.

2. New technologies enable development of improved mathematical models

2.1 Measuring equilibrium and rate constants for mutant forms of RAS

RAS mutations associated with cancer, such as mutations at codons 12, 13, and 61, result in impaired RAS GTPase activity and a decrease in the binding and/or catalytic activities of GAPs [2,34,35]. Integrating experimental data from cells harboring oncogenic *RAS* into mathematical models provides insight into the aberrant signaling underlying RAS-driven cancers.

One technique commonly used to measure rate constants for RAS activation involves N-methylanthraniloyl derivatives of GTP/GDP that can be tracked with fluorescence spectroscopy [36,37]. Using this methodology, rate constants for tens of variants of mutant RAS have been reported [38-41] and incorporated into mechanistic models [30,42-44]. Obtaining rate constants for RAS variants as well as for other mutant enzymes involved in RAS signaling will continue to be important for understanding RAS-driven diseases. Going forward, the application of advanced techniques leveraging nuclear magnetic resonance (NMR) spectroscopy may well provide new information on signaling pathways, with NMR being capable of simultaneous measurement of many relevant processes, including binding, catalysis, post-translational modification, and conformational change [45-47]. NMR has been used to study hydrolysis and nucleotide exchange rates of oncogenic RAS mutants in real-time [48]. It may also be possible to approximate rate constants in cases where a specific variant has not been measured, as there is good correlation between structure-derived energy values and rate constants across libraries of mutant RAS [44]. Table 1 lists many of the available experimentally determined parameters for wildtype and oncogenic mutant RAS.

With recent technological advances, binding affinities can be measured in high-throughput fashion, which will assist in the development of more accurate and comprehensive mechanistic models. Techniques using protein domain microarrays and fluorescence-polarization (FP) allow for the measurement of thousands of equilibrium dissociation constants [49,50]. Of particular importance when the goal is to model cell signaling networks, these techniques measure binding affinities in a domain-specific manner. Other high-throughput techniques for quantifying binding affinities are based on chromatography or deep sequencing [51-53]. Affinities measured by high-throughput proteomics are already contributing to the development of new mechanistic models of cell signaling [54]. For RAS-related models, effort is still needed to quantify affinities for the various RAS-effector interactions. A summary of available equilibrium dissociation constants for RAS isoforms and 13 effectors is presented in Table 2.

Finally, techniques enabling real-time activity quantification for single molecules allow for insight into the degree of intracellular variation in reaction rates. For instance, Iversen et al. [55] measured nucleotide exchange rates on single SOS molecules with a novel assay involving a synthetic membrane partitioned into micrometer-scale compartments.

Interestingly, they observed a very wide distribution of catalytic activities, with a small proportion of SOS molecules transiently entering a state of very high catalytic activity. From a modeling perspective, this observation suggests that ODE-based models may not appropriately capture some aspects of RAS signaling. Stochastic modeling approaches are applicable when molecular species with low copy numbers, such as rare highly active SOS molecules, contribute significantly to system behavior.

2.2 Protein copy number measurements

Protein copy number measurements are key data necessary for the development of realistic mechanistic models. Techniques for measuring the proteome are becoming increasingly complete, fast, and cost-effective [56]. Mass-spectrometry (MS)-based approaches have demonstrated the ability to quantify abundances of thousands of mammalian proteins in parallel [57-60]. New proteomics methods have been introduced that eliminate the need for spiking-in reference peptides, counting cells, or protein concentration measurements, which simplifies protocols for large-scale proteomics studies [61] (see the excellent recent review on MS-based proteomic technologies by Aebersold and Mann [62]).

Improvements in targeted proteomics techniques expedite quantification of specific subsets of proteins across varying conditions, such as across cells types, or in response to external stimuli [54,63]. In contrast to shotgun proteomics, targeted proteomics techniques are capable of detecting proteins expressed at nanomolar levels (corresponding to a detection threshold of approximately 1000 cytosolic molecules per cell) [64]. A recent study used ultra-sensitive targeted proteomics to measure abundances of 26 proteins in the ERK pathway in normal human mammary epithelial cells (HMECs), breast cancer cell lines, and normal human fibroblasts [65]. This analysis revealed that the majority of proteins in the pathway have similar expression levels across cell types, and helped to quantify relative abundances between members of the pathway. Proteomics raises awareness of previous modeling assumptions that may not be valid across cell types, thereby improving the accuracy of future models. For instance, the assumption that the EGFR adapter molecules SHC1, GRB2, and GAB1 are present in excess [28] would be incorrect in many cell lines, as these proteins have been found to have per-cell quantities ranging from 3,000 to 55,000, whereas EGFR was found to have a median per-cell copy number of 210,000 across seven normal and cancer cell lines [65].

Protein phosphorylation plays an important role in many cellular regulatory processes, including signaling downstream of RAS. Thus, new and developing phosphoproteomic techniques for quantitatively monitoring changes in protein phosphorylation can potentially be applied to gain insights into RAS signaling dynamics and to support modeling studies of RAS-related pathways. Sudhir et al. [66] identified phosphorylation events in human bronchial epithelial cells (HBECs) with and without expression of the oncogenic mutant KRAS G12V. A total of 52 proteins were differentially phosphorylated upon transformation with mutant *KRAS*. This analysis allowed for identification of new pathways by which oncogenic and wildtype RAS activation influence human cells. Others have used phosphoproteomics to study alterations in phosphorylation arising from the presence of oncogenic KRAS G12V, activated CDC42 G12V, or knockout of the downstream effector

PAK4 [67]. Phosphoproteomics was also applied to study serine-threonine family kinases, identifying proteins with decreased phosphorylation following addition of EGFR, PI3K, mTOR, and MEK inhibitors [68].

2.3 Genetically engineered cell lines to study effects of RAS network mutations

The ability to genetically alter human cell lines is an exciting avenue by which to study the biological basis for disease. The introduction and maturation of CRISPR/Cas9 and related CRISPR technologies has resulted in significant improvements in genome engineering efficiency with reduced off-target effects [69-72]. CRISPR/Cas9 is exceptionally versatile and can provide the means for controlled studies of heterozygous and homozygous mutations, as well as gene amplification or overexpression, gene deletion or repression, and isoform effects. To our knowledge, CRISPR-Cas9 has not yet been used to directly support mechanistic RAS modeling, but findings from the application of this technology are already offering intriguing results that can be further analyzed computationally. Related to RAS signaling, CRISPR-libraries have been used to engineer cells for identifying mechanisms of resistance to the BRAF inhibitor vemurafenib [69,73]. Vemurafenib resistance was observed with CRISPR-induced overexpression of *BCAR3, EGFR*, and a number of G protein-coupled receptors (*GPR35, LPAR1, LPAR5*, and P2RY8), and following deletion of *NF1, NF2*, and *MED12*.

Genome-wide screening with CRISPR can be used to identify important pathway components that may have been neglected in prior modeling efforts. For instance, Wang et al. [74] used CRISPR to engineer acute myeloid leukemia (AML) cell lines to examine gene essentiality. One of the genes determined to be essential for the proliferation of RAS-driven AML was *SHOC2*. A complex of SHOC2, protein phosphatase 1 (PP1), the scaffold protein SCRIB, and the RAS-family member MRAS is capable of promoting ERK activation by dephosphorylating the inhibitory S259 site on CRAF [75,76]. However, SCRIB has also been noted to have inhibitory effects on ERK activation through interactions with MEK and ERK [76]. No mathematical model has yet accounted for the cooperative role of MRAS in RAS signaling. The presence of both positive and negative effects on ERK by proteins involved in MRAS regulation could contribute to observed temporal pulses in ERK [33], which is certainly worthy of model-guided investigation.

2.4 Real-time monitoring and control

Recent advances in real-time cellular monitoring enable direct observation of how input signals propagate through RAS signaling networks and contribute to resulting cellular phenotypes, providing mechanistic insights and empirical data useful for defining and parameterizing mechanistic models. For instance, Harvey et al. [77] examined neuronal RAS signaling by applying a Förster (or fluorescence) resonance energy transfer (FRET)-based sensor. HRAS was tagged with a green fluorescent protein and the RAS-binding domain of RAF was tagged with two red fluorescent proteins. Two-photon fluorescence lifetime imaging (2pFLIM) was used to generate spatial profiles of RAS activation by tracking FRET arising from RAS/RAF interaction. RAS activation was observed to be reduced with inhibition of calcium/calmodulin dependent protein kinase II (CaMKII), PI3K, or protein kinase C (PKC). The spatial data was used to parameterize a model accounting for one-

dimensional diffusion and the rate of RAS deactivation, leading to the conclusion that rapid diffusion of active RAS was the primary mechanism for the observed spreading of RAS activation along dendrites and to neighboring dendritic spines. However, these data [77] might call for an alternative explanation. Diffusion distance is proportional to the square root of time, and diffusion is a notoriously slow mechanism for propagating signals over large distances [78]. A positive feedback loop in the RAS pathway induced by RAS-mediated activation of SOS (see Section 3.1.2) can endow excitability and/or bistability features to the spatial propagation along dendrites [79]. The wave-like propagation of RAS activation can be expected to transmit RAS signals more effectively than diffusion [80].

Numerous other studies providing real-time data have not yet received attention from modelers. Verissimo et al. [81] applied real-time confocal imaging to scrutinize differences in response to afatinib (an EGFR/HER2 inhibitor) and selumetinib (a MEK1/MEK2 inhibitor) treatment between patient-derived colorectal cancer (CRC) organoids with or without mutations in KRAS. KRAS mutant organoids entered a state of cell-cycle arrest upon treatment, whereas the CRC organoids with wildtype KRAS exhibited cell death. A similar FRET sensor was applied to study ERK activation and localization dynamics [82,83] and different cell-fate decisions resulting from distinct dynamics, as well as to quantify cell-to-cell variability in ERK activation within a population [84]. Improved FRET-based ERK activity sensors with larger dynamic range have been described recently [85], as was a method using single fluorescent-proteins to measure kinase activity in live single cells [86]. Time-course data generated from these and related technologies will be useful for developing accurate models of decision-making networks.

Related to recording observations in real-time is the ability to control cellular networks and measure the response to varying input patterns. Toettcher et al. [87] designed a system that allows for light-activated RAS signaling based on the plant Phy-PIF system. The Phy-PIF module is recruited to the membrane when red light is detected [88]. Because the SOS catalytic domain is fused to PIF, PIF-SOS localizes to the membrane and activates RAS when cells are exposed to a specific wavelength of light. Studies using this technique helped elucidate how the RAS signaling network can modulate a range of cellular behaviors, because differing sets of downstream factors are activated in response to changes in the duration of RAS activation [87]. This intriguing experimental result has not yet been recapitulated through mechanistic modeling.

2.5 Molecularly targeted drugs

Drugs that target critical signaling enzymes, thereby inhibiting specific pathways driving disease manifestation, might improve treatment efficacy and reduce negative side effects. A number of these agents, deemed “molecularly targeted” drugs, are now applied to treat various cancers. The tyrosine kinase inhibitor imatinib, FDA-approved in 2001, was a revolutionary molecularly targeted drug for treatment of chronic myeloid leukemia (CML) [89,90]. Imatinib blocks ATP binding to the kinases ABL, BCR-ABL, PDGFRA, and c-KIT, thereby inhibiting activation and preventing signaling to the downstream pathways controlling leukemogenesis. The success of imatinib paved the way for additional drugs targeting products of mutated cell signaling genes, including those involved in RAS

signaling (Fig. 2). Vemurafenib, FDA-approved in 2011, is applied to treat BRAF V600E metastatic melanoma [91]. Approximately half of subcutaneous and cutaneous melanomas have a mutation in BRAF [92,93]. Sorafenib and dabrafenib are other examples of FDA-approved RAF inhibitors. Gefitinib, erlotinib, and afatinib are tyrosine kinase inhibitors targeting EGFR, approved for use in treating advanced non-small cell lung cancer [94-96]. Vandetanib and lapatanib also target EGFR [97], with lapatanib additionally capable of inhibiting ERK and AKT [98,99]. Trametinib inhibits MEK, and was approved for treating BRAF V600E melanoma when used in combination with dabrafenib [100]. Selumetinib is another MEK inhibitor for treatment of differentiated thyroid cancer [101]. Trastuzumab targets the epidermal growth factor receptor HER2 (aka ErbB2) and is approved for treating breast, gastric, and gastro-oesophageal junction cancers [102,103]. Other agents are approved for targeting related pathways, such as the macrolides sirolimus and everolimus, which inhibit mTOR [104].

Recently, methods to inhibit RAS, once thought of as an “undruggable” target, are showing promise [105]. Molecules have been developed that prevent oncogenic RAS signaling by selectively binding KRAS G12C [106,107], interfering with KRAS plasma membrane localization by inhibiting PDEδ [108,109], disrupting palmitoylation-driven localization of HRAS by binding APT1 [110], or disrupting KRAS4B membrane organization by preventing calmodulin binding to the C-terminal hypervariable region [111].

Clinical and experimental observations show that resistance to molecularly targeted cancer drugs occurs frequently [112,113]. One means of resistance is the generation of secondary mutations affecting the drug target site, as is observed in *EGFR* following gefitinib or erlotinib treatment [114]. Resistance can also emerge through re-activation of inhibited pathways, either bypassing the targeted enzyme or through activation of parallel, alternate pathways. For instance, resistance to BRAF inhibitors can be conveyed by re-activation of ERK or activation of the parallel PI3K/AKT/mTOR pathway [93,115]. ERK re-activation can be achieved via drug-induced allosteric activation of BRAF/CRAF heterodimers, or by the kinase COT, which activates MEK1/2 [116-118]. Mechanistic models of cell signaling can be used to further characterize mechanisms of resistance to molecularly targeted therapeutics. For instance, Iadevaia et al. [119] developed an ODE model of the insulin-like growth factor 1 receptor (IGF1R) signaling network using mass action kinetics parameterized with particle swarm optimization against experimental time-course data. This model was able to predict resistance pathways in a breast cancer cell line after treatment with inhibitors targeting molecules in the network. A model of RAS signaling revealed a basis for differences in EGFR inhibitor efficacy in cells encoding different oncogenic RAS mutants [43].

3. Complex features of RAS signaling continue to necessitate application of mathematical modeling

3.1 Feedback in RAS-associated signaling pathways

Serum starved cells expressing wildtype EGFR and RAS usually respond to EGF stimulation by transient RAS activation lasting 10 30 minutes [120]. Temporal activation

patterns of RAS are influenced not only by delays in activation of RAS-GEFs and RAS-GAPs [16], but also by positive and negative feedbacks from RAS downstream effectors to GEFs and GAPs of RAS [121,122]. In the following sections, we discuss feedback loops impacting RAS signaling.

3.1.1 Negative Feedback—Within the ERK cascade, negative feedback loops from ERK to SOS, RAF, and MEK have been discovered and characterized (Fig. 2). A unifying mechanism for these feedback loops is phosphorylation: ERK phosphorylates MEK1 as well as CRAF, BRAF and SOS. MEK1 possesses a threonine residue at position 292 in the protein kinase domain that is phosphorylated by ERK; this residue is not present in MEK2 [123,124]. SOS1 is phosphorylated by ERK at four serine residues (S1132, S1167, S1178, and S1193), yet SOS2 is only phosphorylated at one location, which may indicate that negative feedback from ERK impairs the activities of these isoforms to varying degrees [125]. Phosphorylated SOS is recruited to the membrane less efficiently due to impaired interaction with GRB2 [126,127]. CRAF kinase activity is attenuated upon phosphorylation at six sites, five of which are phosphorylated by ERK (S29, S296, S301, and S642) [128]. ERK phosphorylates BRAF on four sites (S151, T401, S750, and T753), which inhibits BRAF/CRAF dimerization and BRAF binding to RAS-GTP [129].

Negative regulators of RAS signaling can enable the preferential activation of downstream effector pathways. For instance, consider the Sprouty family of proteins, which are versatile regulators of ERK signaling [130-132]. Upon growth factor stimulation, Sprouty localizes to the plasma membrane and suppresses signaling from RAS to ERK, promoting instead RAS signaling via PI3K [130,131]. Decreased Sprouty expression, and therefore decreased ability to reduce ERK activation, has been linked to several cancer types, including melanoma, breast, liver, lung, metastatic prostate, and other cancers [132]. Interestingly, Sprouty's mechanism of action does not appear to be consistent across isoforms or across cell types; Sprouty proteins have been shown to interact with many partners in the ERK cascade, including GRB2, GAP1, and BRAF [133-136].

3.1.2 Positive Feedback—Intriguingly, in addition to negative feedback loops, the RAS/ERK pathway also contains positive feedback loops (Fig. 2), including positive feedback from RAS to its activator SOS [37]. Both structural and kinetic data suggest that RAS is able to bind to SOS at two distinct sites, such that RAS may act not only as a substrate, but also as an allosteric activator of SOS (Fig. 3) [37,137]. When RAS is bound to the SOS allosteric site, the dissociation rate of GDP from RAS bound to the SOS GEF domain is increased by up to 75-fold [138]. The enhancement of SOS GEF activity by binding the SOS allosteric site is more pronounced for RAS-GTP than for RAS-GDP [138,139]. In addition to allosteric activation, binding of RAS contributes to recruitment of SOS to the plasma membrane, which is known to increase SOS GEF activity by co-localizing SOS and RAS molecules [55, 140].

Positive feedback from active RAS to its GEF SOS may result in bistability in RAS-GTP regulation and hysteresis in RAS-GTP levels in response to stimuli. Das et al. [141] developed a model of bistable RAS behavior suggesting that positive RAS-SOS feedback might account for a digital all-or-nothing response of RAS-GTP to external stimuli.

Following transfection of Jurkat cells with the SOS catalytic domain, which has higher catalytic activity than the full-length SOS, the authors observed bimodal expression of the cell-surface marker CD69 (e.g., CD69 expression was correlated with ERK activity). Other indirect evidence of bistability came from observations that populations of HEK293 cells split into subpopulations with either high or low levels of active ERK (ppERK) after transfection with oncogenic KRAS G12V [142] (although the emergence of a bimodal cell population response can be alternatively explained, see [143-145]).

Whereas ERK-mediated phosphorylation of SOS and CRAF/BRAF generates negative feedback, ERK- phosphorylation of the RAF kinase inhibitor protein (RKIP) creates a positive feedback loop, because it results in enhanced CRAF activity [146]. This positive feedback loop was not considered in a recent modeling effort focused on feedback in the ERK cascade, although the negative feedback from ERK to RAF was included [147]. Historically, ERK pathway models have not analyzed cases where both positive and negative feedback loops have been identified between two signaling proteins. This situation may occur for several enzyme pairs in the ERK pathway where so-called hidden negative feedback is brought about by the sequestration of an upstream kinase by a downstream kinase within a protein-protein complex [148]. Such feedback combinations can generate intricate dynamic behaviors, calling for use of computational models to aid in reasoning about these behaviors [147,149].

Transcriptional feedback loops in the RAS network include not only negative regulators, such as Sprouty [130,133,134], but also positive transcriptional regulators. In response to growth factor stimulation, the scaffold protein, KSR1, localizes to the plasma membrane and facilitates activation of the RAS-to-ERK pathway [150,151]. In quiescent cells, KSR1 is phosphorylated on S392 and localized in the cytoplasm. The RhoGEF GEF-H1 is necessary for recruitment of the phosphatase PP2A to KSR1 and dephosphorylation of KSR1 on S392 [151]. Because ERK signaling markedly enhances the GEF-H1 level, its expression creates a positive transcriptional feedback loop in the ERK pathway.

3.1.3 Interlocked negative and positive feedback loops—Mathematical models have not yet considered all negative and possible feedbacks involved in RAS signaling [65,152], and it remains an open challenge to discern the feedbacks that are most significant for clinical applications. However, models have begun to address the implications of interlocking sets of feedback and feedforward connections between signaling pathways emanating from RAS. For instance, RAS regulation works in concert with feedback connections from RAS effector pathways such as ERK. When both negative feedback from ERK to SOS as well as positive feedback from RAS to SOS are considered, models predict the occurrence of relaxation oscillations consistent with experimental observations [147]. Negative feedback between ERK and RAF and between ERK and MEK further determines the shape of the oscillating waveforms. Taking into account spatial heterogeneity, one might also consider the possibility of the formation of traveling phosphorylation waves [153,79].

Interlocked feedback loops originating from RAS downstream effector pathways influence RAS activation patterns. For instance, a mathematical model of the EGFR signaling network and validating experiments show that the RAS/PI3K/AKT and ERK signaling pathways

interact through the scaffold protein GAB1 [154]. A positive feedback from PI3K to GAB1 strongly activates RAS in response to weak EGF stimuli in HEK293 cells and also accounts for the increase in RAS-to-ERK signaling by insulin in these cells [121]. Experiments on HeLa cells have shown that transient RAS activation in response to EGF stimulation is substantially prolonged if ERK is inhibited [122]. This effect is mainly explained by the ability of ERK or its downstream effectors to inactivate SOS (RAS-GEF) and activate NF1 (RAS-GAP).

3.2 Trafficking and co-localization

The amino acid sequences of the RAS isoforms KRAS4A, KRAS4B, HRAS, and NRAS are 80% the same, but these isoforms possess distinct, non-redundant functionalities [22]. All RAS isoforms are translated in the cytosol and then transported to membranes after post-translational modifications in the C-terminal hypervariable region (HVR) that differ for each isoform. Because all differences in sequence identity are found in the HVR rather than in the regions involved in binding GTP/GDP or effectors, RAS isoform localization may underlie isoform-specific activity [155]. RAS activation can occur at the plasma membrane (PM) and endomembranes of the endoplasmic reticulum (ER)/Golgi complex, mitochondria, and endosomes [155], although the localization for each isoform may differ between cell types. In neurons it has been shown that KRAS, but not HRAS, can reversibly transfer between the PM and intracellular membranes through an interaction with Ca^{2+} and calmodulin [156]. Localization can be specifically controlled to occur at certain regions of larger cellular membranes. For instance, NRAS was observed at both caveolin-positive and caveolin-negative regions of the PM, but KRAS was preferentially located at caveolin-positive domains [157].

Localization of RAS has implications for the associated signaling pathways by impacting which effectors are activated. One study found that HRAS localized to the ER or to lipid rafts in the PM activated ERK and AKT more readily than HRAS localized at the bulk membrane or the Golgi apparatus [158]. In contrast, HRAS located at the Golgi apparatus was more effective in activating RAL-GDS than HRAS located at the ER or lipid rafts in the PM. Mathematical models are only beginning to consider localization and trafficking of RAS isoforms. Schmick et al. [159] provided one such model, which used cellular automata to simulate KRAS trafficking. The model reproduced experimental observations, including a decrease in plasma-membrane bound KRAS upon inhibition of PDEδ or upon introduction of a phosphomimetic mutant KRAS, but it did not examine the effects of differential KRAS localization on downstream effector activation. Future models of RAS signaling that include isoform-specific RAS trafficking and localization will provide a more complete picture of RAS influence on cellular phenotype.

3.3 Competition between RAS effectors

Available structures of RAS in complex with GEFs, GAPs and other effectors show that binding interfaces overlap on the RAS proteins [160]. Therefore, despite having multiple direct interaction partners, a single RAS molecule can bind only one effector, GEF, or GAP at a time. Competition between multiple RAS binding partners implies that any change in the abundance or affinity of a single downstream effector not only impacts its binding to

RAS, but can also affect the RAS-GTP level and interaction of RAS with other effectors. As RAS effector pathways crosstalk through feedback loops, inhibition of one pathway can activate or inhibit other signaling branches. In addition, oncogenic RAS variants also influence interactions with effectors; KRAS G12V and G12C preferentially activate RAL-GDS, but KRAS G12D preferentially activates RAF and PI3K [35].

Mechanistic models can provide insights into the dynamics of effector competition. Stites et al. [54] presented a rule-based model of EGFR signaling to identify important protein-protein interactions across 11 cell lines. This model simultaneously considered experimentally determined protein copy numbers and protein binding affinities and found potential cell-line specific differences in the rank order of recruited EGFR binding partners. Such an approach could also be applied to evaluate effector binding to each of the RAS isoforms and to make predictions regarding the abundances of different RAS-effector complexes in disparate cellular backgrounds.

4. Conclusions and outlook

Integration of mathematical modeling into the clinical setting has the potential to revolutionize treatments by informing therapeutic strategies in a patient- and tumor-specific manner [161]. While models specifically incorporating RAS-activation have not, to our knowledge, been directly used to inform clinical practice, there are many promising studies that indicate how mathematical modeling is already being applied to generate relevant predictions. For instance, Chmielecki et al. [162] suggested that the dosing of EGFR tyrosine kinase inhibitors (TKIs) for non-small cell lung cancers was not optimized for mutant *EGFR.* By incorporating data from engineered cell lines into evolutionary models of cancer, the authors were able to predict the frequency of resistant cells in a population treated with varying doses and schedules of TKI as well as the time necessary to reestablish drug sensitivity in treated populations. A dosing schedule found to reduce the emergence of resistance was validated in the human lung adenocarcinoma cell line PC-9 [162], but has not yet been applied in the clinic. Numerous other recent computational attempts simulate therapeutic effects or dosing schedules with the goal of improving efficacy and/or evading resistance [163-166]. Other computational studies examine the potential effects from combination therapies [118,167-169]. Future efforts toward this end will benefit from the availability of the NCI-ALMANAC database, which contains a systematic evaluation of interactions between 104 anticancer drugs across 60 human cancer cell lines [170]. Models have also been used to guide the design or predict the outcome of clinical trials [168,171].

Mathematical models of RAS signaling and the MAPK pathway include many potential targets for therapeutic agents, including drugs targeting upstream RTKs or downstream RAS effectors. Unfortunately, single drug therapies often fail because of molecular interactions that bypass the block, almost inevitably resulting in resistance [172]. Patients with cancers driven by *RAS* mutations are commonly excluded from treatment with RTK inhibitors, although combination therapy that suppresses both RTKs and RAS downstream effectors, such as RAF or MEK, might be more effective than RAF/MEK therapy alone [81]. However, selecting the best drug combinations for patients with diverse expression and mutation profiles remains an open challenge. The number of intuitively promising drug

combinations applicable to treatment of RAS-driven cancer is large, and mathematical, mechanism-based modeling offers an avenue by which to predict effective combinatorial treatments that exhibit synergy, overcome/prevent resistance, or avoid toxicity. Inferring optimal treatment for RAS-driven cancers will be made possible through improvements in understanding of the biology underlying RAS signaling. Accurate predictive models should capture the complexities of the network described here, including the myriad positive and negative feedbacks described, or the possibility of effector competition, or localization specific differences between RAS isoforms, all of which have ramifications for downstream signaling. We foresee an improvement in the clinical relevance of mathematical models of RAS signaling as our comprehension of the mechanisms underlying disease is bolstered by technological advances in quantification and monitoring of biological systems.

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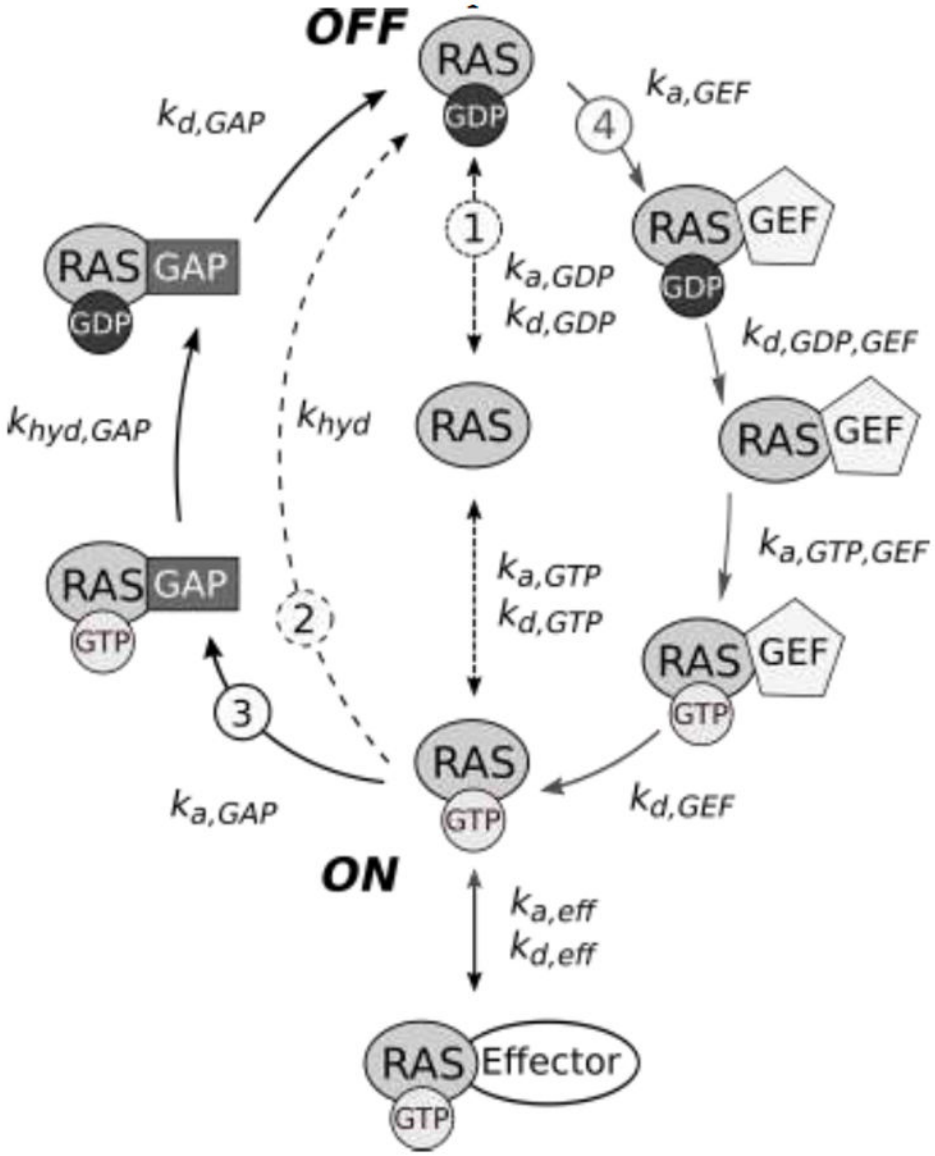

Fig. 1.

The RAS activation cycle. RAS can bind either GTP or GDP, and is active when bound to GTP. In the active configuration, it is able to interact with downstream effectors. RAS activation/deactivation can occur through multiple processes. Processes of interest are labeled with circled numbers: 1 shows free nucleotide exchange, 2 describes RAS-catalyzed hydrolysis of GTP to GDP, 3 depicts GTP hydrolysis stimulated by GAP, and 4 is GEF-induced GDP release from RAS to facilitate GTP binding. Rate constants for each step are labeled; variable names correspond to those in Table 1.

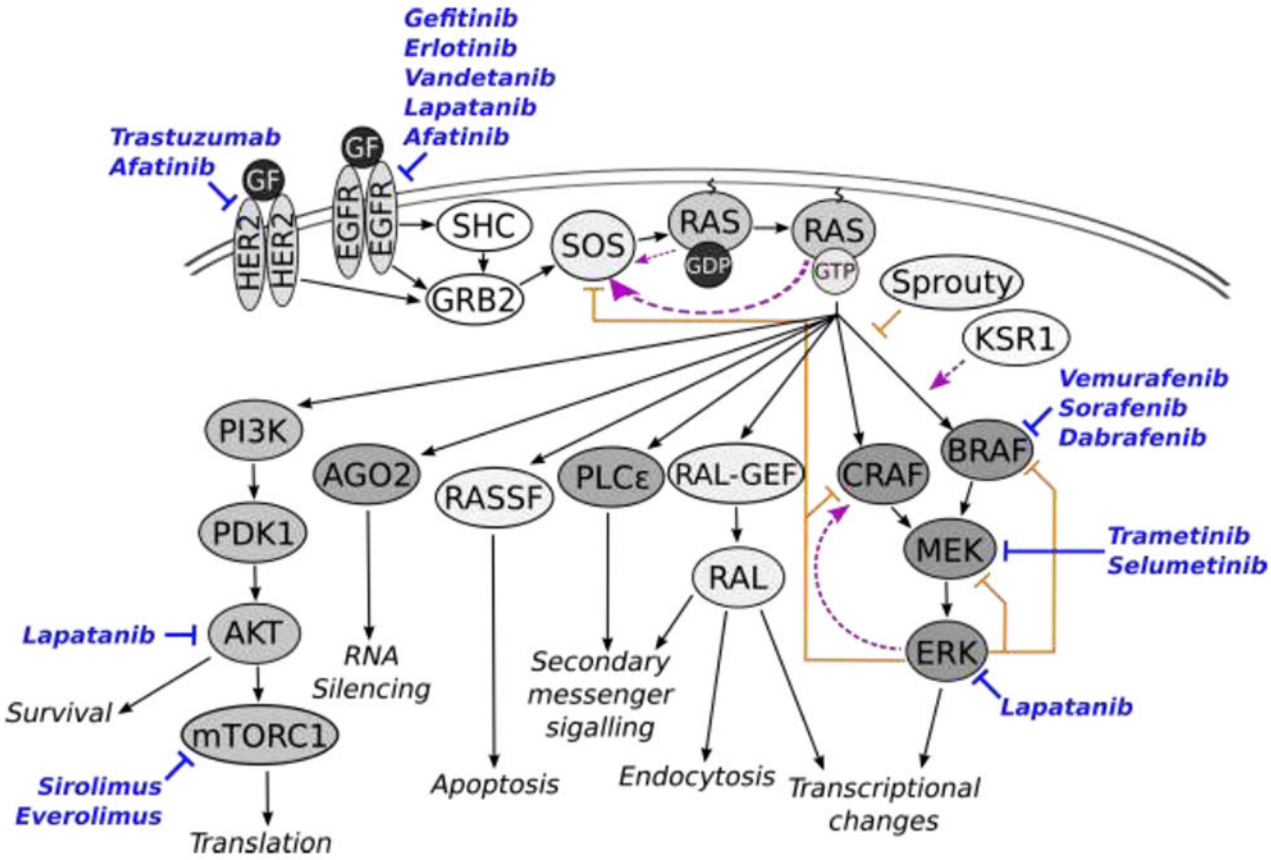

Fig. 2.

Molecularly targeted drugs impacting networks related to RAS signaling. RAS receives activating inputs from EGFR and HER2 triggered by growth factors. Signals propagate from RAS to downstream effectors including RASSF, RAF, RAL-GEF, PLCε, and PI3K, resulting in varied impacts on cellular phenotype, shown with gray text and arrows. Positive feedbacks are indicated with dashed lines and arrows in magenta. Note that the positive feedback from RAS-GTP to SOS is stronger than that of RAS-GDP, as indicated by the thickness of the line. Negative feedbacks are shown with orange lines with blunt ends. Inhibitory drugs are shown in blue with their target indicated by a blue line with a blunt end.

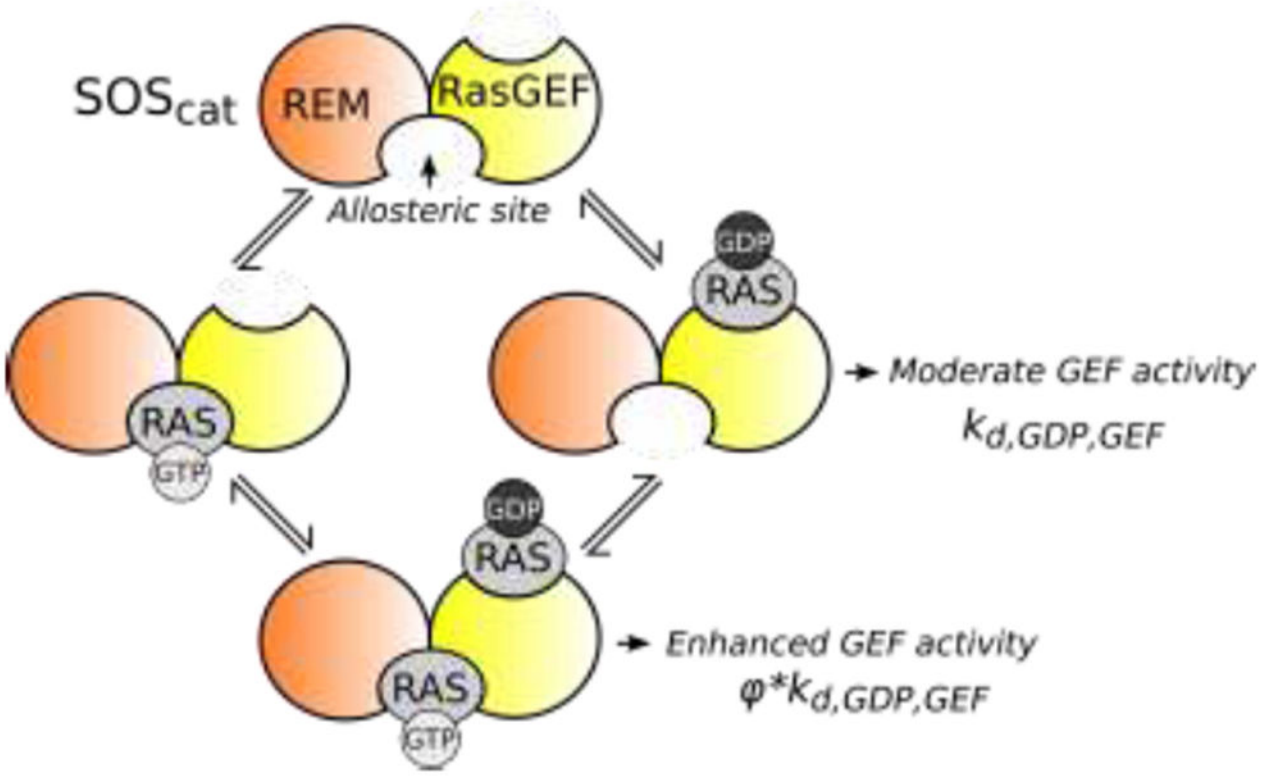

Fig. 3.

Positive feedback from RAS to SOS is achieved via binding of RAS to the SOS allosteric site that bridges the RAS exchanger motif (REM) and RAS-GEF domains, which together are responsible for SOS GEF activity. When the allosteric site is empty, SOS has moderate GEF activity, facilitating release of GDP from RAS-GDP bound at the RAS-GEF site with a rate constant $k_{d,GDP,GEF}$. When RAS is bound to the REM domain, GEF activity increases to a rate characterized by the rate constant $\varphi * k_{d,GDP,GEF}$, where $\varphi > 1$.

Table 1

Parameters relevant for modeling the RAS activation cycle, for wildtype (WT) RAS and 15 oncogenic RAS variants. Rate constants correspond to those in Fig. 1. Variables have units s^{-1} for first-order reactions and $M^{-1}s^{-1}$ for second-order reactions. Values are formatted for the RAS isoform for which the measurement was taken. Values for KRAS are in bold, values for HRAS are italic, and values for NRAS are underlineds

RAS Mutation	$k_{a,GTP}$ (1/M/s)		$k_{d,GTP}$ (1/s)		$k_{a,GDP}$ (1/M/s)		$k_{d,GDP}$(1/s)		k_{hyd} (1/s)	
			2.05E-4	[37]			*6.33E-5*	[48]	*1.47E-4*	[48]
			1.00E-4	[174]			4.20E-4	[174]	3.40E-4	[174]
			2.00E-3	[175]			**2.00E-3**	[175]	**6.80E-4**	[175]
	1.40E8	[174]	*9.00E-5*	[176]	5.10E7	[174]	*1.20E-4*	[176]	*2.10E-4*	[176]
None (WT)	*2.20E6*	[173]	*2.50E-4*	[177]	*2.30E6*	[173]	*1.08E-4*	[177]	*3.49E-4*	[177]
			2.33E-4	[180]			**3.00E-5**	[38]	**9.30E-3**	[38]
							2.17E-4	[180]	**2.17E-4**	[180]
							1.60E-5	[173]		
							0.013	[181]		
G12A			**200E-3**	[175]			**2.00E-3**	[175]	**1.30E-5**	[175]
			9.00E-5	[176]			*5.00E-5*	[176]	*5.00E-5*	[176]
G12C			**2.00E-3**	[175]			**2.00E-3**	[175]	**4.90E-4**	[175]
			6.00E-5	[176]			*2.20E-4*	[176]	*1.80E-4*	[176]
			5.00E-4	[174]			2.00E-4	[174]	1.50E-4	[174]
			2.00E-3	[175]			**2.00E-3**	[175]	**1.90E-4**	[175]
G12D	4.80E8	[174]	*9.50E-4*	[176]	7.00E7	[174]	*1.60E-4*	[176]	*1.40E-4*	[176]
	7.54E6	[30]	*1.25E-3*	[30]	*3.16E6*	[30]	*5.16E-5*	[30]	*1.40E-4*	[30]
			6.00E-4	[180]			**1.67E-4**	[180]	**9.33E-5**	[180]
G12E			*4.90E-4*	[176]			*1.50E-4*	[176]	*1.60E-4*	[176]
G12R			**2.00E-3**	[175]			**2.00E-3**	[175]	**1.80E-5**	[175]
							6.17E-3	[181]		
G12S			*2.00E-5*	[176]			*4.80E-4*	[176]	*1.20E-4*	[176]
G12V			8.00E-5	[174]			*3.33E-5*	[48]	*1.48E-5*	[48]

RAS Mutation	$k_{a,GTP}$ (1/M/s)		$k_{d,GTP}$ (1/s)		$k_{a,GDP}$ (1/M/s)		$k_{d,GDP}$(1/s)		k_{hyd} (1/s)	
	5.80E8	[174]	**2.00E-3**	[175]			1.30E-4	[174]	4.00E-5	[174]
	9.11E6	[30]	*9.00E-5*	[176]	1.16E8	[174]	**2.00E-3**	[175]	**4.20E-5**	[175]
			2.00E-4	[30]	*5.23E6*	[30]	*2.00E-5*	[176]	*5.00E-6*	[176]
							2.00E-5	[38]	**1.50E-3**	[38]
							3.35E-5	[30]	*5.23E-5*	[30]
							3.83E-3	[181]		
G13C			*8.00E-05*	[176]			*2.50E-4*	[176]	*1.10E-4*	[176]
G13D			**0.018**	[175]			*9.45E-4*	[48]	*5.17E-5*	[48]
			6.30E-04	[176]			**0.027**	[175]	**9.60E-5**	[175]
							1.60E-4	[176]	*1.90E-4*	[176]
G13S			*7.00E-5*	[176]			*3.60E-4*	[176]	*3.20E-4*	[176]
G13V			*1.20E-4*	[176]			*3.40E-4*	[176]	*2.00E-4*	[176]
Q61H			**2.00E-3**	[175]			**2.00E-3**	[175]	**1.30E-5**	[175]
			1.53E-4	[177]			*1.60E-4*	[177]	*3.05E-5*	[177]
							0.012	[181]		
Q61K			*1.50E-4*	[177]			*1.52E-4*	[177]	*7.07E-4*	[177]
Q61L			**2.00E-3**	[175]			*1.50E-4*	[48]	1.83E-6	[48]
			2.67E-4	[177]			**2.00E-3**	[175]	8.00E-6	[175]
							2.67E-4	[177]	7.58E-6	[177]
Q61P			*3.17E-4*	[177]			*1.13E-4*	[177]	*2.72E-5*	[177]
Q61R			*1.12E-4*	[177]			*1.23E-4*	[177]	*6.10E-6*	[177]
Q61W			*2.00E-4*	[177]			*1.08E-4*	[177]	*2.53E-5*	[177]
10G11			**5.50E-4**	[180]			**1.18E-3**	[180]	**1.83E-5**	[180]

^{G}GEF is RASGRF1. ^{S}GEF is SOS1. ^{R}GAP is RASA1. ^{N}GAP is NF1. *calculated from Lenzen et al. [173] using nucleotide dissociation rate as $k_r = 3.9\ s^{-1} = k_{d,GDP,GEF}$ and K_d ($K_d = K_{d4}$), such that $k_f = k_{a,GTP,GEF} = k_r/K_d$. **calculated from Lenzen et al. [173], $k_r = K_{d3}k_{f3}$ = (0.6e-3)(3.4e4), assuming the same dissociation rate of GEF from RAS-GTP and RAS-GDP.

Table 2

Equilibrium dissociation constants (K_D) for RAS effectors. $K_D = k_{d,eff}/k_{a,eff}$ as in Fig. 1. Values have units of μM. A dash indicates that no value for the isoform was reported in the cited study

Effector	HRAS	NRAS	KRAS	Reference
CRAF	0.094	0.048	0.142	
RASSF5	0.238	0.442	0.421	
RALGDS	2.5	2.84	1.39	
PLCE1	3.7	5.36	8.9	[160]
PIK3CA	84.3	145	204.7	
PIK3CG	-	2.9	-	
RASSF1	39	-	-	
MLLT4	17.8	-	-	[182]
RGL1	1.73	-	-	[183]
ARAF	0.81	-	-	
RGS14	14	-	-	
RIN1	0.8	-	-	
BRAF	0.04	-	-	