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Ras and Rap1: A Tale of Two GTPases

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Abstract

Ras oncoproteins play pivotal roles in both the development and maintenance of many tumor types. Unfortunately, these proteins are difficult to directly target using traditional pharmacological strategies, in part due to their lack of obvious binding pockets or allosteric sites. This obstacle has driven a considerable amount of research into pursuing alternative ways to effectively inhibit Ras, examples of which include inducing mislocalization to prevent Ras maturation and inactivating downstream proteins in Ras-driven signaling pathways. Ras proteins are archetypes of a superfamily of small GTPases that play specific roles in the regulation of many cellular processes, including vesicle trafficking, nuclear transport, cytoskeletal rearrangement, and cell cycle progression. Several other superfamily members have also been linked to the control of normal and cancer cell growth and survival. For example, Rap1 has high sequence similarity to Ras, has overlapping binding partners, and has been demonstrated to both oppose and mimic Ras-driven cancer phenotypes. Rap1 plays an important role in cell adhesion and integrin function in a variety of cell types. Mechanistically, Ras and Rap1 cooperate to initiate and sustain ERK signaling, which is activated in many malignancies and is the target of successful therapeutics. Here we review the role activated Rap1 in ERK signaling and other downstream pathways to promote invasion and cell migration and metastasis in various cancer types.

Keywords

Ras; Rap1; integrins; cell-adhesion; EMT; metabolism; Rap1Gap; ERK/MAPK; FTI

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Introduction

Ras isoforms

The Ras family of GTPases transduce signals from various receptors, including receptor tyrosine kinases, G protein-coupled receptors and cytokine receptors, to modulate multiple signaling pathways leading to cell proliferation, survival, and gene expression [1–3]. There are three human Ras genes that code for four distinct proteins: H-Ras, N-Ras, and two splice variants of K-Ras — K-Ras4A and K-Ras4B [4, 5]. Ras isoforms are functionally distinct [6–10]. For example, in mouse embryo fibroblasts (MEFs) deficient for particular Ras isoforms, N-Ras regulates adhesion through Raf and RhoA, while K-Ras coordinates motility by signaling through Protein Kinase B (AKT) and Cdc42 [11]. The three isoforms exhibit differences in their localization *in vivo,* which alters access to regulatory proteins and downstream effectors [12, 13]. H-Ras and N-Ras are both localized at the plasma membrane as well as the Golgi and are found in lipid rafts [14, 15], whereas K-Ras is predominantly in disordered regions of the plasma membrane [16]. The main structural differences between Ras isoforms occur in the short hypervariable region upstream of the C-terminus [17] and may explain why these isoforms exhibit specific subcellular localizations [18, 19]. Ras isoforms are also subjected to distinct post-translational modifications (such as ubiquitination and phosphorylation), which could lead to differences in their activity, effector interactions, and signaling output [10, 20]. These dissimilarities likely contribute to the *in situ* selectivity of their activation and deactivation [21], as well as isoform specific downstream signaling [22]. Presumably, this at least partially explains why mutations in these isoforms are concentrated in certain cancer types (Figure 1).

Ras mutations

Mutated Ras proteins play pivotal roles in the development [23] and maintenance of tumors [24, 25]. *RAS* genes were the first oncogenes identified in human cancer cells [26]. In a series of classic experiments, several groups independently identified the transforming gene from T24/EJ bladder cells as *HRAS* [27–30]. *RAS* is well established as the most frequently mutated oncogene in human cancer and is a major driver of the disease [26] (Figure 1). This is particularly true for lung, colorectal, and pancreatic cancers, which were the top three causes of cancer-related death for U.S. men and women in 2016 [31]. Mutated Ras proteins are present in approximately 30% of tumors, appearing in 98% of pancreatic, 52% of colorectal, and up to 35% of lung adenocarcinomas (Figure 1). Among the isoforms, K-Ras is mutated most often, and is present in more than 20% [32] of cancers, especially pancreatic, intestinal, cholangio, and lung carcinomas. N-Ras mutations have an 8% prevalence rate [32] and are concentrated in thyroid as well as certain skin and blood cancers (Figure 1). H-Ras mutations are less common, with a 3% prevalence rate [32], and are found most often in head and neck, salivary, urinary tract [33], bladder, and thyroid carcinomas [34] (Figure 1).

Ras oncogenes play distinct roles in the development of different cancers. In colorectal cancers they promote tumor progression after mutational loss of the APC tumor suppressor gene [35]. In contrast, *KRAS* mutations are a required initiating genetic alteration in pancreatic cancer and leads to activation of downstream pathways [36]. Nearly 95% of

precancerous pancreatic lesions harbor these mutations [37], and *in vivo* proof of concept studies have demonstrated that while induction of *RAS* led to the appearance of precancerous lesions, inactivation of the gene caused regression—indicating that it is required for tumor maintenance and survival [36].

There is a broad spectrum of *RAS* mutations found in human patient samples [38], but *RAS* oncogenes most often harbor single missense mutations that are located in one of three known hotspots: glycine 12 (G12); glycine 13 (G13); and glutamine 61 (Q61) [33, 39]. These mutations result in amino acid substitutions that impair intrinsic and GTPase activating protein (GAP)-stimulated GTP hydrolysis activity. One result is a constitutively active GTP-bound Ras protein. G12 mutations comprise 83% of all K-Ras mutations, while G13 mutations make up 14% of the profile, and Q61 mutations are less frequent (2%). In N-Ras, Q61 mutations are predominant (62%), followed by G12 (23%) and G13 (12%). In H-Ras, G12 Q61 and G13 mutations are distributed more evenly (35%, 34%, and 27%, respectively) [40].

Mutation frequencies within one Ras isoform can vary by cancer type. In melanoma, N-Ras^{Q61} mutations are prevalent, but G12 mutations are not [9]. However, in acute myeloid leukemia, N-Ras^{G12} mutations are relatively frequent. In pancreatic cancers, K-Ras^{G12} mutations are very frequent compared to G13 and Q61 mutations, and G12D can be a prognostic factor in advanced pancreatic adenocarcinoma [41]. The K-Ras^{G12} mutation occurs in 20% of lung cancers and is the most common *RAS* mutation, accounting for one-half of all RAS mutations in lung cancer overall [42]. Colorectal cancers also harbor a high frequency of K-Ras^{G12} mutations, but exhibit an increase in G13 mutations by comparison [40]. Understanding the mechanistic differences in Ras mutation profiles could provide the evolutionary reason behind the propensity of certain cancers to harbor specific hotspots. For example, studies have already aimed to determine the differences in the oncogenicity of K-Ras4B mutations [43]. Knowledge of mutation frequency and type could be crucial to the intuitive design of specific inhibitors for Ras-addicted cancers.

The role of Ras proteins in human cancers is more complex than whether they are present as mutated, driving oncogenes in certain tumors. There is significant evidence that the wild-type Ras isoforms also contribute to the malignant phenotype [44]. For example, oncogenic K-Ras activity may require functional, wild-type H-Ras or N-Ras to drive its effects in some systems [45, 46]. Type 1 neurofibromatosis (NF1) is a common tumor disposition syndrome in which loss of expression of neurofibromin, a GAP and negative regulator of Ras, leads to aberrant activation of N-Ras [47]. Sporadic, non-syndromic loss of neurofibromin expression is found in many additional human cancers, notably, melanoma, lung adenocarcinoma, and glioblastoma [48]. In breast carcinoma, for example, there is a common theme of Ras pathway activation [49] through multiple mechanisms, including neurofibromin loss [50] and over-expressed growth factor receptors, although Ras mutations themselves are rarely found. Activation of K-Ras signaling in basal and luminal breast lesions plays a significant role in the maintenance of metastatic characteristics and is associated with poor prognosis [51, 52].

The role of Ras in EMT and metabolic reprogramming

Epithelial-to-mesenchymal transition (EMT) is a characteristic of some aggressive cancers, correlates with poor prognosis, and is proposed to play a role in metastasis [53, 54]. It is a complex, transient, and reversible process, characterized by the loss of epithelial characteristics (such as cell–cell attachments, adhesion, and apical–basal polarity) and the gain of mesenchymal characteristics (such as increased motility, invasive properties, and a spindle-like morphology) [55]. K-Ras activation serves as a critical inducer of mesenchymal characteristics in basal-type breast cancer cells [51] and, along with N-Ras, likely induces mesenchymal characteristics through effects on the cytoskeleton [10].

In addition to its role in metastasis, EMT has been linked to metabolic reprogramming [56]; in particular, the Warburg effect, which consists of excess glucose uptake with increased lactate production even in the presence of oxygen [57]. This process involves an energetic preference towards anabolic processes that produce building blocks such as amino acids, nucleic acids, lipids, and cofactors such as NADPH for redox balance and reductive biosynthesis [58–60]. Assays performed on three-dimensional (3D) models of breast cancer have shown that inhibition of glucose utilization suppressed oncogenic pathways and resulted in reversion to a normalized phenotype [61, 62], thus revealing a link between glucose metabolism and regulation of oncogenic pathways. Further studies were able to link the presence of mesenchymal characteristics in cancer cell lines to changes in the expression profiles of metabolic genes [63, 64]. This is in line with studies that have shown that mesenchymal cells exhibit a high rate of glycolysis, which fuels cytoskeletal remodeling, a hallmark of aggressive cancers [63].

K-Ras activation plays a role in the acquisition of EMT characteristics [51], which is linked to metabolic programming. Oncogenic K-Ras increases glucose uptake and promotes a transcriptional program that leads to alterations in key rate-limiting enzymes of anabolic glucose metabolism [65] through the Raf-MEK-ERK pathway [65, 66]. Conversely, low glucose conditions can select for tumor cells with *KRAS* mutations [67]. A recent study in yeast has directly linked glycolytic metabolism to Ras activation, by showing that fructose-1,6 bisphosphate enhances stimulation of Ras by the cdc25 guanine nucleotide exchange factor (GEF). The authors also show that an analogous pathway of fructose-1,6 bisphosphate-induced H-Ras activation can occur in mammalian cells through binding to the Sos GEF, with consequent activation of MEK and ERK signaling [68]. Complementary to these findings, *SNAI1* (which codes for Snail1, an important transcription factor in EMT) was found to repress fructose-1,6-bisphosphatase in basal breast cancer, creating a shift towards glucose uptake and diversion of glycolytic carbons towards biosynthetic pathways [69]. The result of these changes is increased flux of glycolytic intermediates through pathways such as the hexosamine biosynthesis pathway and the non-oxidative arm of the pentose phosphate pathway. This leads to increased production of precursors used for glycosylation and ribose used in DNA and RNA synthesis [40]. Alterations in metabolism are now included as one of the hallmarks of cancer [70].

Direct targeting of Ras

Small molecule inhibitors

Ras is regulated through the cycle of GTP binding for activation (mediated by GEFs) and GTP hydrolysis to GDP for deactivation (facilitated by GAPs) [71]. Early structural analyses of Ras indicated that it is not likely possible to design a small molecule that could restore the lost GTPase activity and sensitivity to GAPs [26, 40, 72, 73]. One reason is because Ras binds to GTP at picomolar levels, which makes it difficult to design a small molecule that can displace the activating nucleotide [74]. This is significantly different from targeting protein kinases, where ATP binding occurs at a micromolar affinity [4]. As a result, small molecule nucleotide analogues effectively block ATP binding to kinases [75], but it has been considerably more challenging to disrupt GTP binding to Ras. This strategy is still in active consideration for inhibition of other members of the Ras superfamily. For example, a new class of compounds that block nucleotide binding to Rac1 and prevent its interaction with effectors has recently been described [76].

Since K-Ras^{G12} mutations drive oncogenesis, they pose as an attractive target as their inhibitors would provide some degree of selectivity over normal tissues [42]. Shokat and colleagues designed small molecule inhibitors that covalently target K-Ras with the activating G12C mutation following binding to an allosteric pocket (Switch II) located close to the nucleotide binding site [77]. This led to the impairment of K-Ras G12C association with effectors. Unfortunately, this first generation of covalent inhibitors failed to exert its effects in cells [78]. However, further optimization has led to next-generation covalent Ras inhibitors such as ARS-853 [79]. The effects of this compound were promising, as it led to reduced levels of GTP-bound K-Ras in multiple K-Ras^{G12C} expressing lung cancer cell lines, reduction in activated ERK/MAPK, AKT and c-Raf kinases, reduced proliferation, induced apoptosis [78, 79], and decreased growth in 2D and 3D assays [78]. Mechanistically, it was discovered that ARS-853 preferentially binds to GDP-bound K-Ras^{G12C} [78, 79] – and that K-Ras^{G12C} still rapidly cycles between active and inactive forms. These findings challenge the view that all activating mutations in *KRAS* function like the G12D mutation and lock Ras in the active state (i.e., the GTP-bound form) [80]. Adding to these observations, ARS-853 was also found to reduce the interaction between K-Ras^{G12C} and Sos, thus reducing Sos mediated nucleotide exchange [78]. Furthermore, while treatment of cells with epidermal growth factor (EGF) reduced the inhibitory effects of ARS-853, addition of EGF receptor tyrosine kinase inhibitors enhanced its effects to increase cell death and inhibit the PI3 kinase pathway [78, 79]. Therefore, the authors suggest that ARS-853 competes with GEFs for K-Ras^{G12C}, which is still responsive to upstream signaling pathways. These interesting discoveries suggest that ARS-853 could be combined with other inhibitors of upstream signaling pathways to disrupt signaling via K-Ras^{G12C} and achieve clinical efficacy. The compatibility of ARS-853 with *in vivo* use has yet to be confirmed, since it has low metabolic stability and the potential to engage in off-target effects [81]. Despite the need for further optimization, the creation of ARS-853 represents a significant step forward and can be used as a robust benchmark for the design of future small molecule inhibitors.

Meanwhile, the synthesis of compound 3144 by Stockwell and colleagues has advanced the prospect of targeting Ras in a different way. This compound targets the K-Ras^{G12D} mutation, which is the most common found in human colorectal carcinoma [38]. In contrast to the K-Ras ^{G12C} mutation, the K-Ras^{G12D} mutation keeps K-Ras in the constitutively active position [42]. This group showed that simultaneous binding of two adjacent sites on the Ras protein could inhibit both tumor growth and Ras signaling in mouse cancer models. In addition to K-Ras^{G12D}, 3144 was shown to interact with wild-type K-Ras, N-Ras, and H-Ras, but not with other small GTPases in the Ras superfamily, save for a weak interaction with RRas2. Biophysical assays and mutagenesis experiments suggested that 3144 had an affinity for its targets in the micromolar range. Viability studies confirmed that the compound acted in a Ras-dependent manner by using cultures with varying amounts of Ras addiction, as well as MEFs where *KRAS* could be removed and *HRAS* and *NRAS* had been deleted (*KRAS*$^{lox/lox}$, *HRAS*$^{-/-}$, *NRAS*$^{-/-}$, *RERTn*$^{ert/ert}$). Treatment of fibrosarcoma cells with compound 3144 resulted in moderately decreased ERK and AKT phosphorylation. In MEFs, the decrease in viability seen in the absence of *RAS* genes could be reversed with the introduction of membrane targeted BRAF^{V600E}-CaaX. Efficacy was evaluated in xenograft and pancreatic cancer models and revealed reduction in tumor growth, likely associated with ERK and AKT inhibition [82]. While analyses of the compound's activity revealed some toxicity and off-target effects *in vitro* and *in vivo*, it also laid the groundwork for more selective molecules to be generated, providing another path toward targeting Ras proteins in cancer.

There have been many efforts to screen and design small molecule inhibitors that disrupt Ras interactions with Sos and other GEFs [83–89], and Ras interactions with its various effectors [90–92]. Results from screens can be difficult to define mechanistically, however. This is exemplified by the initial characterization of rigosertib, a small molecule inhibitor that is in phase III clinical trials for myelodysplastic syndrome [93]. Rigosertib was originally identified in screens of non-ATP competitive kinase inhibitors that induce mitotic arrest and block polo-like kinase 1 activity [94] and was found to induce apoptosis in a broad spectrum of human breast cancer cell lines [95]. A recent study, using a different screening method that combined CRISPR and chemical genetic screens revealed that rigosertib exerts its activity as a microtubule destabilizing agent [96]. Rigosertib has also been reported to achieve part of its inhibitory effect through disruption of Ras binding to Raf [91]. Rigosertib binds directly to the Ras binding domain (RBD), resulting in a dose-dependent inhibition of Ras-Raf, Ras-PI3Kα,β, γ and Ras-RalGDS interactions, which translates into downstream inhibition of MEK, ERK and AKT phosphorylation [81]. However, another study has shown that its effects on the Raf/ERK pathway are mediated indirectly by JNK cascade activation [97]. Thus, promising small molecular inhibitors may have to go through various screens to elucidate the possible mechanisms of action and discover downstream targets.

Anti-Ras antibodies

Soon after the discovery of Ras, proof-of-concept studies regarding Ras inhibition with antibodies were performed. Feramisco and colleagues showed anti-Ras antibodies could be used to phenotypically revert cells to a normal state by directly inhibiting Ras GTP binding [98, 99]. In order to maximize their effects in cells, antibodies have to be delivered and their

structural integrity maintained despite the reducing environment of the cytosol [100]. Stable antibody fragments were synthesized and successfully inhibited Ras signaling in oocytes and fibroblasts leading to apoptosis [101]. Further progress towards pre-clinical antibody inhibitors of Ras was made when Tanaka and colleagues, using *in vivo* mouse models, demonstrated that an adenoviral vector expressing anti-Ras antibodies induced regression of tumors formed by colon cancer cells [102]. Subsequent studies from the same group revealed that blockage of K-Ras function in mouse models by anti-Ras antibodies prevented tumor initiation and controlled tumor growth [103]. Building upon previous challenges, other groups successfully engineered a cell-permeable antibody (called RT11) that entered mammalian cells by endocytosis and selectively bound to active Ras mutant proteins and blocked their effector interactions. This antibody had anti-proliferative effects on a variety of Ras mutant tumor cells. Subsequent promising *in vivo* studies further revealed that an RT11 variant containing a tumor-targeting moiety significantly inhibited the growth of Ras-mutant tumor xenografts, but not of control tumors harboring wild-type Ras [104].

Induction of Ras mislocalization

Ras function requires membrane localization that is conferred by post-translational modification, particularly the covalent addition of an isoprenoid farnesyl lipid to a cysteine residue in a tetra amino acid CaaX (Cysteine-aliphatic-aliphatic-other) motif at the C-terminal tail of the protein [105, 106]. Recognition of the essential role for this prenyl modification led to the hypothesis that inhibition of the enzyme responsible, farnesyl transferase (FTase), would provide a therapeutic approach to cancer treatment [107, 108]. Several small molecule FTase inhibitors (FTIs) were developed that competed with the CaaX peptide motif of the substrate protein, and preclinical studies on tumor xenografts in mice were promising [109]. The CaaX-competitive inhibitor tipifarnib advanced the farthest and was tested in stage III clinical trials for colorectal cancers and in combination with gemcitabine for pancreatic cancers [110, 111]. Unfortunately, no significant anti-tumor activities above placebo control were observed.

There are likely several inter-related reasons for the failure of FTIs to achieve clinically meaningful responses, one of which is the lack of evidence that target inhibition was achieved in the human tumors [4]. Further, most of the pre-clinical work was performed with mutant *HRAS* driven cancer models, whereas the focus of the clinical trials was to test in the human cancers that have the highest total mutant Ras burden. Pancreatic and colorectal carcinomas have prevalent *KRAS* mutations but negligible *HRAS* mutations (Figure 1). This discrepancy was compounded by the fact that, in the presence of an FTI, N-Ras and K-Ras, but not H-Ras, could still be prenylated. The continued modification of K-Ras and N-Ras and their consequent membrane localization is because they can also be substrates for geranylgeranyl transferases [112–114].

At least two potential strategies have been proposed to overcome the alternative prenylation of Ras proteins that contributes to FTI resistance. Both of these approaches are based on compounds that mimic the prenyl co-factor of FTase rather than the CaaX peptide substrate. FTIs that are analogues of the prenyl moiety show enhanced activity with low doses of statins that are by themselves insufficient in blocking prenylation [115, 116]. The rationale

for this effect is that statins will reduce the endogenous pool of prenyl precursors and so make prenyl-competitive inhibitors more effective [117, 118]. A similar sensitization occurs with a combination of low-dose statin and a prenyl-competitive inhibitor of geranylgeranyl transferase [119]. The combination of low-dose statin plus prenyl-competitive FTI is able to inhibit Ras modification in malignant peripheral nerve sheath tumor cells, which express N-Ras and K-Ras but little or no H-Ras, suggesting that it does overcome alternative prenylation [120]. Another strategy is to use the prenyl analog as a modified co-factor for the enzyme and thus cause it to be covalently attached to Ras by FTase. This exciting approach has recently been described to cause mislocalization of mutated K-Ras in a pancreatic adenocarcinoma cell model [121].

Another reason to reconsider FTIs is that H-Ras does not undergo alternative prenyation for membrane targeting [44, 122]. Multiple studies have reported that FTI treatments have been effective against H-Ras transformed cells and H-Ras driven murine tumors [123, 124]. H-Ras mutations are less common in human cancers (Figure 1), but tumor profiling is already identifying patients with oncogenic H-Ras [125–127] in different tumor types. Profiling could be coupled with bioinformatic analysis to identify driving oncogenic pathways [128, 129]. Even though H-Ras driven cancers are less prevalent, they are found in a significant fraction of some cancer types, including thyroid and head and neck squamous cell carcinoma (Figure 1). The latter comprises the majority of head and neck cancers diagnosed worldwide [130]. The vast majority of these patients present with advanced stage disease [131], their prognosis has not changed over the past decade [132], and new approaches are needed. For example, there is a Phase II clinical trial to evaluate tipifarnib in patients with *HRAS* mutant relapsed or refractory head and neck squamous cell carcinomas and malignant thyroid tumors with *HRAS* mutations [NCT02383927].

There are some potential problems that should be considered when evaluating the results from Ras related clinical trials. For example, Ras is only one of many proteins in the cell that are normally prenylated, which could lead to two complicating factors. One is whether any future positive trial results could actually be due to a non-Ras target of the approach. Another concern is whether significant side effects could be expected from compounds that inhibit maturation of many proteins in addition to Ras isoforms. Nevertheless, clinical trial results indicate that FTIs are typically well tolerated [133], even in pediatric populations [134]. In view of the likely role for H-Ras in neuronal signaling, synaptic remodeling, and memory formation [135–139] drugs that impact wild-type H-Ras function could be expected to impair learning and memory [44]. Many chemotherapeutic approaches also impair cognition [140], so combinations of FTIs with such traditional anti-cancer drugs should be evaluated very carefully for potential effects on the central nervous system [44].

Inhibiting Activated Ras by targeting downstream Ras effectors

ERK/MAPK

Given the difficulty with directly targeting Ras or its association with membranes, most efforts have shifted to the development of selective inhibitors of downstream pathways that are driven by activated Ras [32, 141]. Ras effectors are a diverse group of signaling proteins that are characterized by the presence of Ras-binding (RBD) or Ras association (RA)

domains. In many cases the Raf serine/threonine kinases (CRAF/RAF1, ARAF and BRAF) are the best validated effectors for driving the oncogenic function of mutant Ras proteins through the MAP kinase (MEK/ERK) cascade [142], but the phosphoinositide 3-kinase (PI3K) cascade and Ral-GDS pathway may also be important [143, 144] and the most relevant downstream effectors likely vary by cancer type [145, 146].

Raf-MEK-ERK signaling is required for Ras-mediated transformation and tumorigenesis [147–149], and inhibition of this pathway has become the main focus for targeting Ras-driven cancers. This approach was vindicated by the FDA's approval of two MEK1/2 inhibitors: trametinib in 2013 [150] and cobimetinib in 2015 [151] (in combination with dabrafenib and vemurafenib, respectively) for the treatment of advanced melanoma with a BRAF V600E or V600K mutation. Another MEK inhibitor, binimetinib or MEK162, is also under investigation and has shown promising results in patients with N-Ras mutated melanoma [152].

Although the results obtained thus far from using MEK inhibitors have been encouraging, responses are typically transient due to emergence of resistance. Selectively blocking signal transduction at one point in a cascade often presents the opportunity for therapeutic resistance to occur, and this can result from releasing feedback inhibition on upstream kinases or through the utilization of alternative pathways [153, 154]. In addition, the further downstream the target, the more likely multiple drugs will be needed to prevent the selection of a resistant cell population. A pre-clinical example of this can be seen in melanomas driven by N-Ras, where MEK and mTOR inhibition combined to produce a synergistic benefit [155]. Ultimately, while effective in some cases, such as melanoma, increasing the number of drugs has the potential to be detrimental for the patient, both in regard to tolerance and treatment cost.

PI3K

Two PI3K inhibitors, idelalisib and copanlisib appear promising for the treatment of leukemia and lymphoma. When used in combination with rituximab, idelalisib showed significant improvements in progression-free survival, response rate, and overall survival for patients with relapsed chronic lymphocytic leukemia (CLL) whose coexisting medical conditions made them less able to undergo standard chemotherapy (NCT01539512, [156]). This combination was likely potent for two reasons. First, rituximab targets the B-lymphocyte antigen (CD20) [157] and B-cell receptor signaling is known to play an important role in the pathogenesis of CLL [158–161]. Additionally, this signaling is partially mediated by the delta isoform of PI3K (the target of idelalisib) which is highly expressed in lymphoid cells [162]. Copanlisib is a pan-class I PI3K inhibitor that mainly shows activity against PI3K alpha and delta isoforms, and so can take advantage of some of the same biology as idelalisib. During clinical trials it demonstrated significant efficacy and a manageable safety profile for patients who had previously received treatment and continued to suffer from relapsed or refractory indolent lymphoma [163]. Despite some concern about the side-effects of these drugs, both idelalisib and copanlisib were approved by the US Food and Drug Administration [164].

Rap1

Rap1 (Ras-associated protein 1) is not a direct effector of Ras, but rather has the demonstrated potential to be a significant regulator and mediator of Ras functions and is linked to many of the hallmarks of cancer (Figure 2). It is a small GTPase that belongs to the Ras family of GTPases [165] and was first discovered by Kitayama and colleagues in 1989 as a gene product that normalized a malignant phenotype of *KRAS* transformed fibroblasts [166]. It was later found that this reversal might be due to the ability of Rap1 to compete with Ras for Raf1, thus antagonizing Ras activity [167]. Furthermore, dominant active Rap1 mutants (Rap1V12) attenuate Ras-mediated ERK activation, via competitive interference with c-Raf activation by Ras [168, 169]. Despite the great sequence similarity between Ras and Rap proteins, their activators and effector pathways are generally distinct [170].

Rap1 is activated in response to upstream signaling, such as growth factors, cytokines and chemokines that act on receptor tyrosine kinases and G-protein coupled receptors [171]. Initial functional studies on Rap1 in testes of *Drosophila melanogaster* showed that Rap1 signaling regulates morphogenic processes through the proper positioning of adherens junctions [172, 173], implicating Rap1 as a regulator of cell-cell attachment. These results were corroborated by studies of Rap1 in the control of barrier function in endothelial cells [174, 175]. Rap1 is now recognized as a central regulator of cell adhesion and motility [176] and asymmetrical distribution of activated Rap1 promotes cell polarity and migration by remodeling the actin cytoskeleton at the leading edge of the cell [176–181]. Research has shown that while the activity of Rap1 plays a role in the organization of polarity in normal human breast epithelial cells, increased and aberrant activation of Rap1 can lead to tumor formation and progression to malignancy [182].

Rap1 also plays a major role in various integrin-mediated biological processes, such as immunological synapse formation, macrophage phagocytosis, chemokine-induced adhesion and transmigration of leukocytes, lymphocyte and dendritic cell homing to peripheral organs, platelet adhesion and aggregation, as well as adhesion of cells to various extracellular proteins such as fibronectin, fibrinogen, collagen, and laminin[183]. This is in line with studies that show that, in normal and malignant conditions, the functional coordination of E-cadherin and the integrins is essential for maintenance of cellular architecture and dissemination into the stroma, especially during ductal branching in mammary gland development [184]. Rap1 also promotes vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) activation and angiogenesis through the integrins [185]. Rap1 regulates recycling, avidity and affinity of integrins that are associated with the actin cytoskeleton [183] and regulates integrin activation either directly through their polarized spatial distribution or via cytoskeleton dynamics [165, 176, 186]. The Rap-GEF Epac activates Rap1 to control integrin-mediated cellular functions by modulating inside-out activation processes [187, 188]. Rap1 controls T-cell receptor, CD31 and CD98 induced activation of $\alpha_1\beta_2$ [189–193]. Thus, Rap1 controls integrin-mediated cellular functions by modulating inside-out activation processes, such as modulation of cytoskeletal dynamics.

It is likely that the antagonistic actions of Rap1 are more relevant to the early events in Ras-induced transformation. Rap1 and Ras share similar binding partners, including Ral-GDS,

phosphoinositol-3 kinase, B-Raf and Raf1 [194]. The downstream effects of Rap activation depends on the binding partner and its cellular localization. For example, Raf1 binds to GTP-loaded Rap1 but is not activated by this interaction, whereas B-Raf can be activated by both GTP-loaded Ras and Rap1 [195]. When it comes to location, specific GEFs activate certain pools of Rap1, which then activate certain effectors. This is illustrated by studies that have reported that Epac activates a perinuclear pool of Rap1 and does not result in ERK activation; C3G (another Rap-GEF) mediates activation of Rap1 that is localized to the plasma membrane and leads to B-Raf and ERK activation [196].

A plethora of studies have implicated Rap1 activation in a variety of cancers, including leukemias [197] and solid tumors, but Rap1 mutations are rarely reported in cancer [198]. Rap1 plays a role in invasion and metastasis in various cell types due to its regulation of adherens junctions and remodeling of the cytoskeleton. In colon cancer cells, Rap1 activation results in impairment of cell adhesion and increased cell-matrix adhesion [199], inducing dissemination (Figure 2). Blockade of Rap1 cycling and activation in melanoma cells alters adhesion and cytoskeletal dynamics and prevents metastasis to the lungs [200]. Additionally, Rap1 activity is increased in progressively metastatic prostate cancer cell lines and promotes metastasis in *in vivo* models through the integrins [201]. These findings are complemented by evidence of cancer cell migration and invasion [201, 202], as well as an enhanced rate of tumor incidence in mouse xenograft models following Rap1 activation [201]. In pancreatic cancer systems, decreases in Rap1 activity (via over-expression of Rap1Gap) inhibit cell proliferation and survival [203]. Complementary studies in melanoma models show that increases in Rap1 activation (via the loss of Rap1Gap) lead to proliferation, survival, and migration (Figure 2, [204]). Activated Rap1 is highly expressed in human oral cancer [205] and in squamous cancer cells compared to non-malignant keratinocytes. In 3D models, forced increases in glucose uptake and metabolism activated multiple oncogenic pathways through Rap1, leading to acquisition of a cancer phenotype in non-malignant breast cells [61].

Rap1 exists in two isoforms with 95% sequence homology – Rap1A and Rap1B, which are the products of two separate genes on chromosomes 1 and 12 [206, 207]. Relatively little is known of the functional differences between Rap1A and Rap1B in normal conditions. Studies have suggested that while Rap1A maintains cell-cell junctions, Rap1B regulates dynamic changes in cell-cell junctions [208–210]. There has been emphasis on illuminating the role of Rap1A or Rap1B in malignancy, with little reported differences in the role of the two isoforms. In gastric cancer, expression of Rap1B is associated with poor prognosis and aggressive phenotypes [211]. Rap1B has also been shown to play a role in angiogenesis and migration [212]. In ovarian cancer, upregulation of Rap1B promoted the migration and metastasis of ovarian cancer cells and is regulated by miR-708 [213]. Rap1B expression is associated with invasion in esophageal squamous cell carcinomas and is regulated by miR-518b [214]. Some studies in other cancer models have shown that while Rap1A is robustly expressed in basal breast cancer cell lines, it is reduced in non-malignant breast epithelial cells [215]. These studies complement analyses of Rap1 in breast tissues, where Rap1 is expressed at higher levels in ductal carcinoma *in situ* and invasive breast cancers than in normal mammary ductal cells [215]. Furthermore, depletion of Rap1A inhibits

invasion and migration of cells from breast [215], prostate [201] and squamous cell carcinomas [216]. Thus, it appears that Rap1A and B may have over-lapping functions.

Synergistic Activation of ERK via Rap1 and Ras

Early studies showed that in response to nerve growth factor stimulation, C3G–activated Rap1 binds to B-Raf to sustain activated ERK activation that is initiated by Ras [217]. Subsequent studies have shown that this cooperation also occurs in response to ERK activation that is mediated through the second messenger cyclic AMP (cAMP) [218] and requires Protein Kinase A phosphorylation of Rap1 to induce binding to the scaffold protein KSR (kinase suppressor of Ras) [219]. In pituitary cells, it was found that Ras and Rap1 control distinct pools of ERK. Vasoactive intestinal peptide (VIP), which signals through cAMP, activates nuclear ERK solely through Rap1, whereas Ras contributes to both cytosolic and nuclear ERK activation by VIP. In contrast activation of nuclear ERK in response to EGF stimulation requires Ras but not Rap1 [220]. These findings compliment FRET studies that show simultaneous Ras and Rap1 activation at the peripheral plasma membrane and the endomembrane compartment, respectively, in response to a multitude of growth factors, which suggests that Ras and Rap1 cooperate in growth factor signaling [221]. Thus, the combined spatio-temporal activation of Ras and Rap1 in response to growth factor stimulation provides a physiological mechanism to regulate the kinetics of ERK signaling that is critical to many cellular processes such as differentiation and regulation of hormone secretion [217, 220, 222].

Rap1 activation of ERK has also been the subject of interest in endothelial cells, especially in response to VEGF. Rap1 is involved in the processes of proliferation and migration via activation of ERK and AKT pathways. This phenomenon was demonstrated by experiments with Rap1Gap overexpression and expression of a dominant-negative form of Rap1 (Rap1N17), which resulted in reduction of ERK and AKT activation and a concomitant decrease in proliferation and migration [223]. These data build upon previous studies that have shown that endothelial cells derived from Rap1b-deficient mice exhibit reduced ERK activation in response to VEGF stimulation [212] and that Rap1a and Rap1b knockdown in endothelial cells impaired cell migration and reduced levels of fibroblast growth factor-induced ERK activation [224].

Rap1 Activation of ERK and Other Effectors in Cancer

In melanoma, Rap1 plays a role in progression by promoting increased cell migration and metastasis via hepatocyte growth factor-induced activation of ERK and β integrins [225]. This is particularly important since *N-Ras* and *B-Raf* mutations are prevalent in melanoma and that ERK activation has been reported in a large portion of human melanomas and cell lines [226]. Further evidence for the role of Rap1 in ERK activation in melanoma is that Rap1Gap has been shown to be downregulated by promoter hypermethylation [204].

In ovarian cancer models, Rap1 activates Notch pathways, in conjunction with ERK, leading to enhanced expression of several EMT proteins such as Slug, Zeb1, vimentin, fibronectin and MMP9 that is associated with increased migration, invasion, tumorigenesis and metastasis. Treatment with an ERK inhibitor reversed the effects of Rap1over-expression, as

evidenced by decrease in protein expression of EMT markers and reduction in migration [227]. In colon and pancreatic cancer cells, activation of Rap1 leads to Src and focal adhesion kinase (FAK) phosphorylation [199, 203], leading to cell-cell spreading and invasion. In H-Ras driven head and neck cancer and in esophageal squamous cell carcinoma respectively, Rap1A and Rap1B play important roles in invasion through stabilization of beta-catenin and promotion of Wnt signaling. The potential importance of these findings is emphasized by the demonstration that the prognostic value of Rap1 is tied to the intensity of beta-catenin expression [216, 228].

Ras and Rap1 have sequence similarities, and their spatio-temporal activation plays a role in the fine tuning of downstream pathways, such as ERK signaling. In addition to its central role in cell-adhesion, significant evidence demonstrating the ability of Rap1 to promote tumorigenesis in various systems has emerged. Studies in multiple model systems have outlined a role for Rap1 activation in various cellular processes such as cellular metabolism, cytoskeletal remodeling, cell proliferation, migration and metastasis via its regulation of downstream pathways such as ERK, AKT, FAK and Wnt signaling. Thus, Rap1 is a potentially significant modulator of oncogenic pathways in some Ras driven cancers and it will likely be critical to further determine its functions in the context of therapeutic strategies that are being developed to target Ras.

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Abbreviations

EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal regulated-kinase
FTase	farnesyl transferase (FTase)
FTI	FTase inhibitor
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
MAPK	mitogen-activated kinase
MEFs	mouse embryo fibroblasts
MEK	Mitogen-activated protein kinase kinase
NF1	Type 1 neurofibromatosis
RA	Ras association domain
RBD	Ras binding domain

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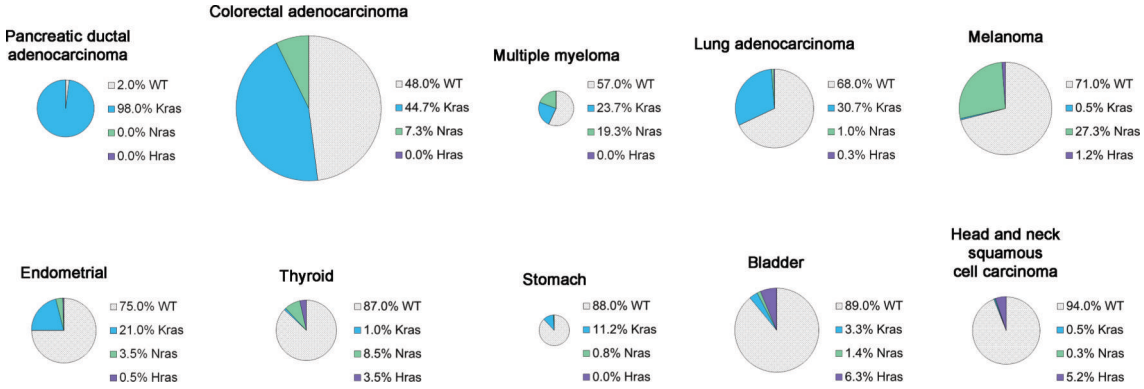

Figure 1.

Ras mutation frequency in cancer

Cancers with frequent rates of *RAS* missense mutation are shown. Charts are ranked by decreasing *RAS* mutational burden (left to right). Isoform percentages are adapted from supplementary figures S2 and S3 found in reference [40]. The radius of each chart is proportional to the incidence rates released in the American Cancer Society's 2017 Cancer Facts & Figures report. Lung adenocarcinoma incidence was calculated as 33% of lung and bronchus cancers, as reported rates range from 30–35%.

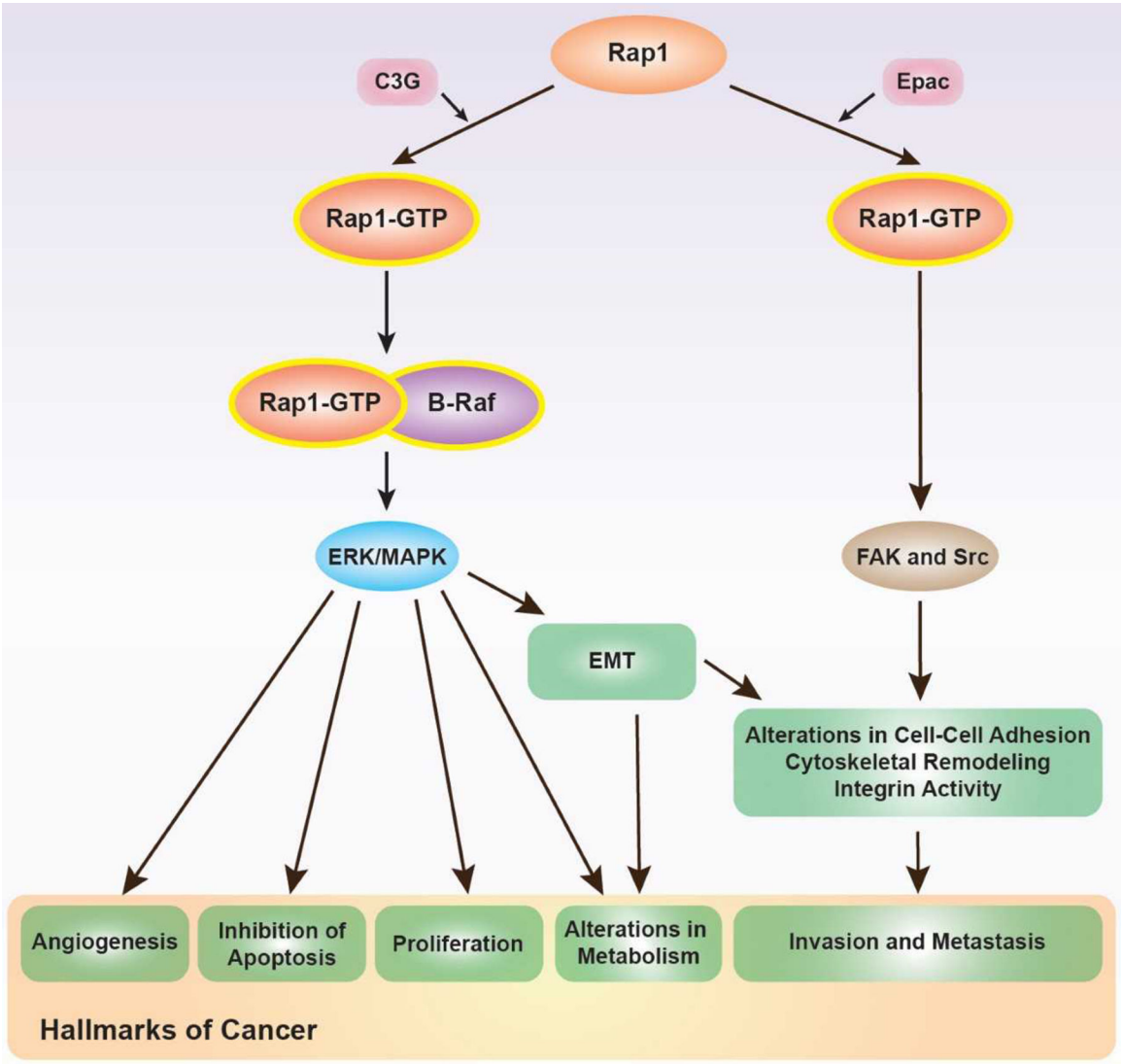

Figure 2.
Rap1 plays a role in some of the hallmarks of cancer.