ONCOGENIC RAS-DEPENDENT REPROGRAMMING OF CELLULAR PLASTICITY

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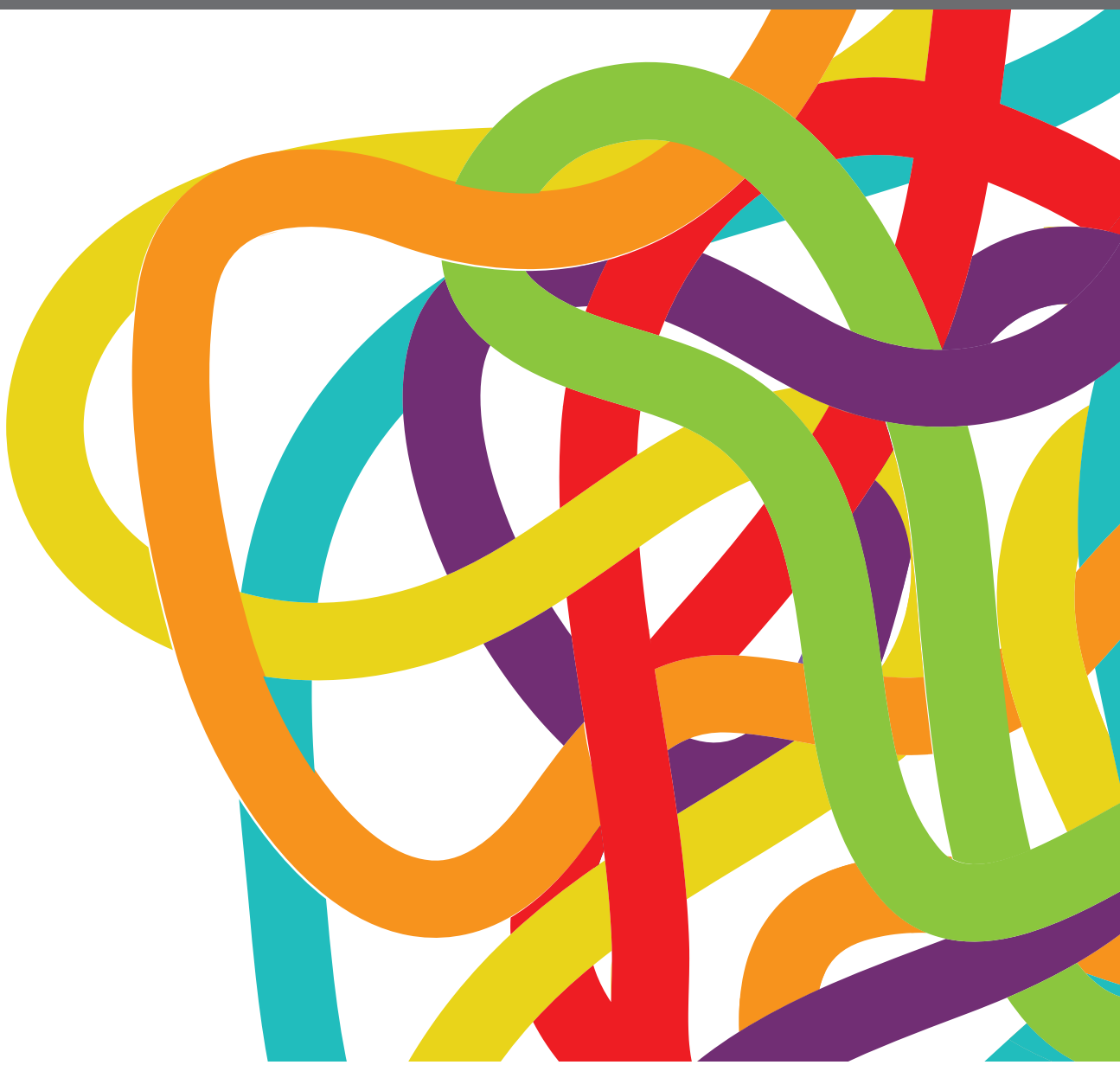

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ONCOGENIC RAS-DEPENDENT REPROGRAMMING OF CELLULAR PLASTICITY

Topic Editors:
Alessandro Rimessi, University of Ferrara, Italy
Georgia Konstantinidou, University of Bern, Switzerland

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EDITORIAL
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Editorial: Oncogenic RAS-Dependent Reprogramming of Cellular Plasticity

Georgia Konstantinidou[1]* *and Alessandro Rimessi*[2]*

[1] Institute of Pharmacology, University of Bern, Bern, Switzerland, [2] Section of Pathology, Oncology and Experimental Biology, Laboratory for Technologies of Advanced Therapies, Department of Medical Sciences, University of Ferrara, Ferrara, Italy

Keywords: oncogenic Ras, cellular plasticity, cancer hallmarks, therapy resistance, oncogenesis

Editorial on the Research Topic

Oncogenic RAS-Dependent Reprogramming of Cellular Plasticity

In human cells, three RAS genes, named HRAS, KRAS, and NRAS, encode four highly homologous small GTPases (H-RAS, K-RAS4A, K-RAS4B, and N-RAS). Gain-of-function mutations occur in ∼30% of all human cancers, including non-small cell lung cancer, pancreatic, colorectal and breast cancer, and are associated with poor clinical prognosis and resistance to treatment. Since 1982, when activated and transforming human *RAS* genes were discovered, there have been many unsuccessful attempts to target RAS oncogenes. RAS oncogenes have thus long been considered to be undruggable.

Approaches to target RAS oncogenes and RAS-driven cancers are underway, all the efforts to design therapeutics that selectively target the oncogene or its downstream effectors are justified by the degree to which RAS-driven tumors remain dependent on oncogenic RAS, making it a crucial target (1). At the clinical level, the complexity and the signaling redundancy of RAS function and of its downstream pathways have restrained the successful targeting of RAS-mediated oncogene addiction. Although recent discoveries have generated interest in the development of KRAS inhibitors either targeting directly mutant KRAS or targeting the crucial steps required for KRAS activation, these developments can be beneficial only to a small subset of human tumors (2, 3).

RAS proteins principally localize in close proximity to plasma membrane, which participate to the transduction of extracellular growth factor-dependent signaling triggering the activation of different intracellular pathways, such as MAPK and PI3K pathways (4). The lack of functional redundancy between the 3 different RAS isoforms is due to their distinctive intracellular localization and redistribution, generating specific compartmentalized signals (5, 6). Oncogenic RAS signaling establishes cancer hallmark traits that support cancer plasticity, evade immune attack and enhance cancer cell migration and metastasis (7, 8). Moreover, RAS proteins promote metabolic reprogramming of tumor cells, shifting them toward an anabolic metabolism necessary to produce biomass to support their needs (9–12). The specific rewiring depends on the subcellular, cellular, and tissue environments within which oncogenic RAS operates (13).

This Research Topic entitled "*Oncogenic RAS-dependent reprogramming of cellular plasticity*" aimed to contribute to a better understanding of oncogenic RAS signaling in several traits of cancer hallmarks, which are the basis of the reprogramming of cancer cells. The published original research and review articles are briefly described below:

- Muñoz-Maldonado et al. focused on the differences of individual RAS-mutated variants related to signaling and phenotype, as well as on transcriptomics, proteomics, and metabolomics profiles and discussed the association of these mutations with particular therapeutic patient outcomes.

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Daniel Christian Hoessli, University of Karachi, Pakistan

***Correspondence:**
Georgia Konstantinidou
georgia.konstantinidou@pki.unibe.ch
Alessandro Rimessi
alessandro.rimessi@unife.it

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- Galiè reviewed the studies that explored the controversial role of Ras proteins and their mutational status in breast cancer, revealing their role as supporting actors.
- Gimple and Wang reviewed the role of oncogenic RAS and its downstream effectors in different cancer types and grades, focusing on the new strategy of targeting RAS recently emerged and their therapeutic potential.
- Arner et al. reviewed the role of KRAS signaling in epithelial-to-mesenchymal transition (EMT) and cellular plasticity, and discussed the contribution of cellular plasticity in cancer progression, metastasis, and therapy resistance.
- Yang et al. reviewed the recent advances in KRAS-mutant lung cancer with a particular focus on mechanistic insights into tumor heterogeneity, clinic implications, and new therapies.
- Roncarati et al. reviewed the role of microRNAs in RAS oncogenic activation in human cancers, resulting to a potentially useful approach to control RAS oncogenic activation.
- Maffeis et al. reviewed the role of RAS in colorectal cancer and its link with cellular plasticity, invasion, and migration at both molecular and morphological levels.
- Nussinov et al. reviewed the mechanisms through which oncogenic RAS activates its effectors MAPK (Raf/MEK/ERK) and PI3K (PI3K/Akt/mTOR), shedding light on the implications for their pharmacological targeting.
- Pupo et al. reviewed the interplay between KRAS and metabolism focusing on metabolic dependencies of mutant KRAS-driven lung and pancreatic cancers that could be attractive therapeutic targets.

There has been a tremendous progress in the understanding of the genetic architecture, the biological heterogeneity, and the distinct molecular pathways driven by RAS oncogenes that raised new hopes for personalized cancer treatment. More extensive understanding of the RAS pathway in human cancer will guide the future development of precision therapies.

AUTHOR CONTRIBUTIONS

GK and AR conceived the idea and wrote the manuscript.

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MINI REVIEW
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KRAS-Driven Metabolic Rewiring Reveals Novel Actionable Targets in Cancer

Emanuela Pupo[1,2], *Daniele Avanzato*[1,2], *Emanuele Middonti*[1,2], *Federico Bussolino*[1,2] *and Letizia Lanzetti*[1,2]*

[1] Department of Oncology, University of Torino Medical School, Turin, Italy, [2] Candiolo Cancer Institute, FPO-IRCCS, Turin, Italy

Tumors driven by mutant KRAS are among the most aggressive and refractory to treatment. Unfortunately, despite the efforts, targeting alterations of this GTPase, either directly or by acting on the downstream signaling cascades, has been, so far, largely unsuccessful. However, recently, novel therapeutic opportunities are emerging based on the effect that this oncogenic lesion exerts in rewiring the cancer cell metabolism. Cancer cells that become dependent on KRAS-driven metabolic adaptations are sensitive to the inhibition of these metabolic routes, revealing novel therapeutic windows of intervention. In general, mutant KRAS fosters tumor growth by shifting cancer cell metabolism toward anabolic pathways. Depending on the tumor, KRAS-driven metabolic rewiring occurs by up-regulating rate-limiting enzymes involved in amino acid, fatty acid, or nucleotide biosynthesis, and by stimulating scavenging pathways such as macropinocytosis and autophagy, which, in turn, provide building blocks to the anabolic routes, also maintaining the energy levels and the cell redox potential (1). This review will discuss the most recent findings on mutant KRAS metabolic reliance in tumor models of pancreatic and non-small-cell lung cancer, also highlighting the role that these metabolic adaptations play in resistance to target therapy. The effects of constitutive KRAS activation in glycolysis elevation, amino acids metabolism reprogramming, fatty acid turnover, and nucleotide biosynthesis will be discussed also in the context of different genetic landscapes.

Keywords: KRAS, PDAC, metabolic rewiring, metabolic adaptability in cancer, NSCLC, gluocose metabolism in cancer, glycolysis

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Edited by:
Alessandro Rimessi,
University of Ferrara, Italy

Reviewed by:
Chiara Ambrogio,
Dana–Farber Cancer Institute, United States
Nikolaos Patsoukis,
Beth Israel Deaconess Medical Center and Harvard Medical School, United States

***Correspondence:**
Letizia Lanzetti
letizia.lanzetti@ircc.it

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INTRODUCTION

KRAS mutations can promote all the key aspects of cancer cell metabolism. It elevates glucose, glutamine and fatty acids uptake and consumption to sustain biosynthetic pathways and the cell redox potential. All these functions are regulated by a number of events, here summarized in three major points, that cooperate with mutant Kras in metabolic reprogramming and specify metabolic adaptation in different tumor types.

(i) Similarly to other oncogenic lesions (2), the effect of *KRAS* mutations in metabolic adaptation can differ in distinct tumor types depending on the tissue of origin. This has been revealed by comparing the metabolic adaptations of non-small cell lung carcinoma (NSCLC) and pancreatic ductal adenocarcinoma (PDAC) driven by *Kras* mutations and *Trp53* deletion in mice. These

two cancer types, despite sharing the same genetic alteration, use branched-chain amino acids differently. While NSCLCs incorporate free branched-chain amino acids into tissue protein and use them as nitrogen source, uptake of these amino acids and expression of key enzymes responsible for their catabolism are decreased in PDACs (3).

(ii) Cancer cells carrying mutant Kras crosstalk with the microenvironment, exchanging cytokines, growth factors, and metabolites to improve metabolic adaptation and overcome low nutrients availability (4–6).

(iii) Finally, a number of concomitant genetic alterations have been shown to cooperate with *KRAS* mutations in sustaining specific metabolic adaptations (7–10).

In this framework, the purpose of this review is to discuss the most recent findings on the interplay between Kras and metabolism focusing on metabolic dependencies of mutant Kras-driven lung and pancreatic cancers that could be attractive as therapeutic targets.

MUTANT KRAS AND GLUCOSE METABOLISM

The involvement of the Ras oncogene in metabolic reprogramming has been initially revealed by its ability to promote glycolysis (11). In pancreatic cancer, *KRAS* mutations are an early event being detectable in the initial lesions known as pancreatic intraepithelial neoplasias (PanIN), which can progress in infiltrating ductal carcinomas through the acquisition of additional genetic alterations (12). In mouse models, PanIN lesions rapidly evolve in aggressive PDACs when *Kras* mutations are combined with *Trp53* loss (13). Elevation of glycolysis is a distinguishing feature of Kras-driven tumorigenesis. Indeed, in the Kras mutant NSCLC model, inhibition of increased lactate production, which results from high rates of glycolysis, severely impacts on disease progression (14). Moreover, increased expression of the facilitative glucose transporter GLUT1, which fosters glycolysis by increasing glucose uptake (15), can be invariably detected in Kras mutant pancreatic lesions (16, 17) (**Figure 1**). The major outcome of increased glycolysis is the generation of intermediates that can be used as building blocks by other metabolic routes to synthetize nucleotides, amino acids, and fatty acids which are required by the rapidly dividing cells to generate the tumor mass (20). Indeed, elevation of glycolysis by Kras channels glucose intermediates in the pentose phosphate pathway (PPP) and in the hexosamine biosynthesis pathway (21). Using a $Kras^{G12D}$ inducible PDAC murine model (also carrying deletion of p53), abrogation of $Kras^{G12D}$ expression causes tumor regression that is accompanied by severe reduction of the expression of GLUT1 and rate-limiting glycolytic enzymes, and of the amount of glycolytic intermediates as revealed by both metabolomics and transcriptomic studies (21). These metabolites fuel the non-oxidative arm of PPP whose primary function is to produce the nucleotide precursor ribose-5-phosphate. Mechanistically, activation of MAPK by Kras up-regulates Myc-directed transcription. In turn, this increases the expression of the glycolytic enzymes that promote glucose uptake and consumption, and of the PPP enzyme RPIA. RPIA catalyzes the conversion of ribose-5-phosphate in ribulose-5-phosphate, thus fueling nucleotides biosynthesis (21, 22). In agreement, inhibition of PPP suppresses xenograft tumor growth indicating that mutant Kras, by increasing glucose uptake and consumption, sustains biosynthetic pathways leading to nucleotide production finally maintaining tumor growth (21). Interestingly, nucleosides supplementation can rescue cell death caused by Kras knockdown in mutant Kras-addicted PDAC cell lines without promoting cell proliferation suggesting that the metabolic function of Kras can be uncoupled from its functions in proliferation (22).

The genetic landscape of the tumor cooperates with *KRAS* mutations in the elevation of glycolysis to promote cancer growth and dissemination. In pancreatic cancer, overexpression of paraoxonase 2 (PON2), a target of p53 transcriptional repression, has been found to join forces with mutant Kras to elevate glycolysis. PON2 increases glucose uptake by binding to GLUT1 thus preventing interaction of the latter with the inhibitory protein STOM (7). PON2 overexpression controls the cell starvation response and increases glucose uptake to protect pancreatic cancer cells from detachment-induced cell death, which, in part, occurs through suppression of the AMPK/FOXO3A/PUMA signaling pathway (7). AMPK is a highly conserved kinase that works as a sensor of low cellular energy and that can either repress or promote tumor growth depending on tumor type and context (23). Here, pharmacological activation of the AMPK pathway inhibits growth of tumors generated by subcutaneous injection of PDAC cancer cells revealing a potential metabolic druggable vulnerability (7).

SCAVENGING PATHWAYS AND AMINO ACID METABOLISM IN KRAS MUTANT CANCER CELLS

KRAS mutations are known to stimulate processes such as macropinocytosis and autophagy that can scavenge nutrients from, respectively, external and internal compartments to sustain cancer cell survival under condition of nutrient deprivation [reviewed in Kimmelman (1)]. Both these two scavenging pathways generate vesicles, macropinosomes, and autophagosomes, which ultimately fuse with lysosomes to release their cargoes for degradation. In the lysosomes, breakdown of nutrients provides the cell with pools of free amino acids, lipids, nucleotides and glucose that can be used by the anabolic pathways for synthetizing novel macromolecules (1, 24). Interestingly, both in Kras mutant lung and pancreatic cancers, the lysosomal compartment undergoes expansion thanks to the increased activity of the transcription factors Tfeb/Tfe3 (25, 26), which are responsible for lysosomal biogenesis (27, 28). In Kras-driven NSCLC, glucose starvation activates AMPK that promotes dephosphorylation and nuclear translocation of Tfeb and Tfe3 (25). Accordingly, Tfe3 activity is required for growth of mouse lung tumors and increased expression of lysosomal genes correlates with accelerated disease recurrence in human

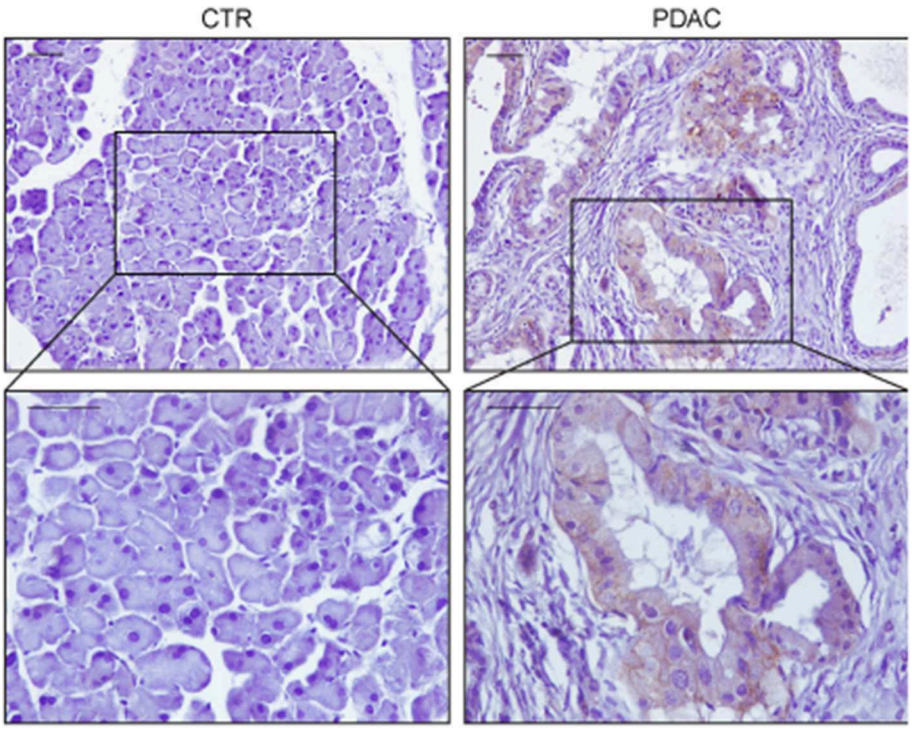

FIGURE 1 | Representative immunohistochemistry stainings of GLUT1 in sections of pancreas from a wild type mouse (CTR) or from a mouse expressing $Kras^{G12V}$ in the acinar/centroacinar lineages (Elas-tTA/tetOFF-Cre;K-$Ras^{+/LSL\ G12V\ Geo}$) (18). GLUT1 is up-regulated specifically in most tumor cells, with mixed membranous/intracellular localization. In each case pancreas was formalin fixed, paraffin embedded and slices were processed as described in Pupo et al. (19). Briefly, paraffin removal was performed with two 10 min steps in Xylene, rehydrated in decreasing concentration of ethanol, and antigen retrieval was performed using 2100 Antigen Retriever/R-Universal buffer (Aptum Biologics). Slices were permeabilized with 0.2% TritonX, saturated in 5% goat serum/BSA and endogenous peroxidase was inhibited by H_2O_2 incubation. Staining was performed with anti-GLUT1 antibody (AbCam, 1:200) and secondary antibody anti-Rabbit-HRP (Dako). Immunoreactivity was developed using DAB chromogen (Dako). Scale bars are 50 μm.

lung adenocarcinoma patients (25). Similarly, upregulation and increased nuclear residence of Tfe3 sustain pancreatic tumor growth (26). Of note, overexpression of Mitf, which belongs to this family of transcription factors, promotes progression of Kras mutant PanIN lesions in PDAC indicating that increased lysosomal activity plays a driver function in mutant Kras tumors (26).

Macropinocytosis is a non-selective actin-dependent endocytic process that uptakes nutrients from the extracellular environment in large intracytoplasmatic vesicles (29). In tumors, macropinocytosis works as a feeding mechanism to overcome high nutrients demand and support metabolic flexibility and adaptation. *KRAS* mutations have been shown to stimulate macropinocytosis allowing for large uptake of albumin, the most abundant serum protein, which is degraded in lysosomes to increase the intracellular pool of amino acids (30, 31). Breakdown of albumin provides amino acids that feed the central carbon metabolism (30) and, among them, glutamine, is avidly used by Kras transformed cells for anaplerosis and nucleotide production (30, 32) (**Figure 2**). Indeed, in Kras mutant pancreatic cancer cells, glutamine is the major carbon source and is consumed via a non-canonical pathway. In the majority of non-transformed cells, in mitochondria, glutamine-derived glutamate is converted, by the enzyme glutamate dehydrogenase (GLUD1), in α-ketoglutarate to fuel the tricarboxylic acid (TCA) cycle. Instead, in PDAC cells, glutamate is used by the mitochondrial aspartate transaminase GOT2 to produce aspartate and α-ketoglutarate. Aspartate is transported in the cytoplasm where it is converted to oxaloacetate, by the aspartate transaminase GOT1, then into malate and pyruvate thus elevating the $NADPH/NADP^+$ ratio, which, in turn, sustains the cell redox potential (33) (**Figure 2**). In agreement, genetic deletion of any enzyme in the pathway elevates production of reactive oxygen species, diminishes the amount of reduced glutathione, and results in suppression of PDAC growth both *in vitro* and *in vivo* (33). Kras drives the alternative glutamine consumption pathway by up regulating transcription of GOT1 and reducing expression of GLUD1. While this pathway is essential for PDAC growth, it seems to be dispensable in non-transformed cells. This offers a therapeutic option to this type of tumors also considering that its inhibition might synergize with therapies that increase intracellular reactive oxygen species such as chemotherapy and radiation (33). Along this line, Kras mutant cells that have become resistant to cisplatin, a compound that works by increasing the reactive oxygen species in the cytoplasm, display elevation of glutamine consumption and anti-oxidant

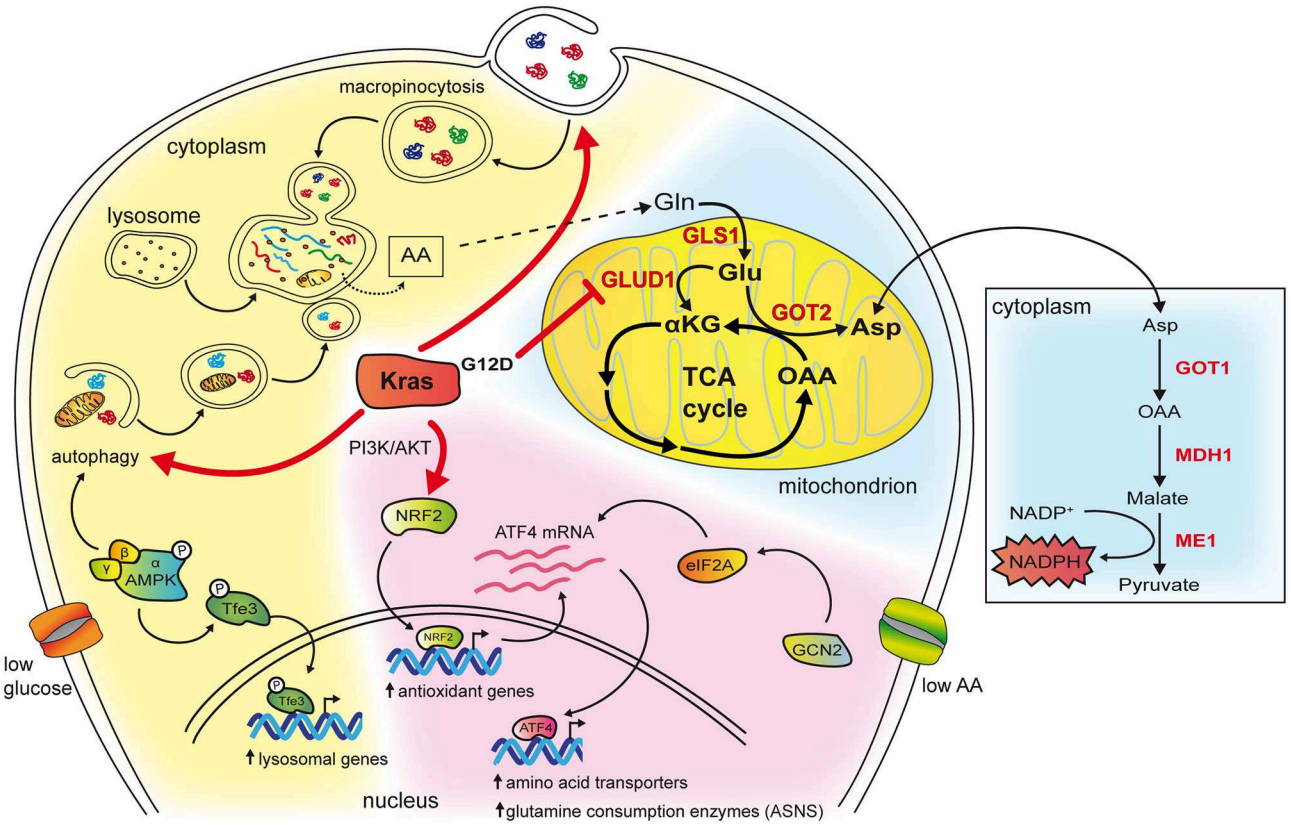

FIGURE 2 | The cartoon schematizes some of the effects of mutant Kras in reprogramming amino acid metabolism. The cytoplasm of the cell has been colored in blue in the background to highlight the role of mutant Kras in PDAC, in pink to represent pathways revealed in NSCLC, in yellow when the mechanisms are common to both tumor types. Kras potentiates both macropinocytosis and autophagy whose vesicles end up in lysosomes, a compartment that is frequently found enlarged in Kras mutant cancer cells. In, NSCLC, in condition of low glucose, this occurs by activating AMPK that phosphorylates Tfe3 resulting in its nuclear translocation and transcription of lysosomal genes. Moreover, AMPK activation increases autophagy initiation and maturation. Breakdown of macromolecules in lysosomes produce free amino acids available to biosynthetic and energy pathways. Among them glutamine can enter the TCA cycle in mitochondria (depicted on the right). In PDAC glutamine is consumed through an alternative pathway (highlighted in red and in the box on the right). In NSCLC, mutant Kras activates the PI3K/AKT pathway that, in condition of low glutamine, favors mRNA expression of the ATF4 transcription factor via the NRF2 factor. In addition, NRF2 is also a key regulator of genes involved in the antioxidant response. Under condition of asparagine deprivation, the GCN2-eIF2 pathway prompts transduction of the ATF4 mRNA into protein, which, in turn, activates the transcription of amino acids transporters and glutamine consuming enzymes. Among them, asparagine synthetase ASNS catalyzes the synthesis of asparagine from glutamine. Asparagine levels and ASNS control proliferation, mTORC1 activation and suppress apoptosis.

capacity (34). Knock down of GOT1 in the resistant cells reduces their proliferation suggesting that Kras-mediated metabolic reprogramming of glutamine consumption contributes to the acquired resistance to platinum-based drugs (34).

The role of Kras in detoxification is also reported in advanced lung cancer, where high frequency of $Kras^{G12D}$ copy gain is observed. This enrichment in mutant alleles promote channeling of glucose-derived metabolites in the TCA cycle and glutathione biosynthesis enhancing the management of reactive oxygen species and increasing the metastatic potential (35). It is of note that upregulation of glutathione is specifically associated with increased mutant gene copy number highlighting a "dose" effect and suggesting therapeutic vulnerability (35).

Macroautophagy (here referred as autophagy) promotes survival under metabolic stress conditions by directing intracellular components to lysosomes via the formation of vesicles known as autophagosomes (24). Even if autophagy does not increment the biomass, as it re-utilizes pre-existing molecules to generate new ones, it supports cell survival under stress condition allowing tumor persistence (36). Autophagy is known to sustain several aspects of Ras transformation, from maintenance of the cell glycolytic capacity (37), of the mitochondrial oxidative metabolism (38), of energy charge and nucleotide pool (39), to the secretion of pro-migratory cytokines (40). Autophagy has complex functions in cancer, being both pro-tumorigenic and tumor suppressive (24), but increasing evidence in mouse models of pancreatic cancer indicates that, especially at later stages of tumorigenesis, autophagy sustains tumor growth [reviewed in Amaravadi and Debnath (41)]. Indeed, pancreatic deletion of the autophagy gene *Atg5* in a model of pancreatic cancer driven by oncogenic *Kras* and the stochastic loss of heterozygosity of *Trp53* ($Kras^{G12D}$; $Trp53^{lox/+}$), a condition that reproduces the stepwise human development of pancreatic cancer, increases the number of PanIN lesions,

but impairs the progression of PanIN to PDAC, prolonging mice survival (42). Moreover, inhibition of autophagy by treatment with hydroxycloroquine causes tumor reduction in *KRAS* mutant *TP53* mutant patients-derived pancreatic cancer xenografts (42). In addition, the effects of intermittent autophagy inhibition, which would mimic patients treatment, have been recently tested using an inducible transgenic PDAC mouse model generated by crossing mice carrying the inducible dominant-negative mutant of the autophagic gene *Atg4B* with the $Kras^{G12D}$; $Trp53^{lox/+}$, mice. In these animals, metronomic impairment of autophagy has been found to delay tumor growth via both cell autonomous, by decreasing proliferation and sensitizing apoptosis in nutrient-restricted areas of the tumor, and non-autonomous, macrophage-mediated, mechanisms (5).

Notably, two recent studies have shown that autophagy inhibition synergizes with pharmacological targeting of the KRAS downstream effectors MEK1/2 or ERK, preventing growth of KRAS-driven pancreatic adenocarcinomas (43, 44). The efficacy of combining these two treatments appears to rely on the fact that inhibition of the MAPK pathway, one of the major pathways downstream KRAS, potentiates autophagy, suggesting that this treatment causes addiction to autophagy. Concomitant treatment with MAPK and autophagy inhibitors might therefore represent a novel strategy to target KRAS-driven cancers (43, 44).

The ability of mutant Kras to model the microenvironment is a long standing observation in PDACs where abrogation of $Kras^{G12D}$ expression, not only affects tumor growth, but also reduces the desmoplastic stroma, which is typical of this type of cancer (18). In PDACs, mutant Kras instructs the microenvironment to sustain tumor growth both by engaging stromal cells that instigate reciprocal signaling (4), and by exploiting stroma-derived alternative fuels (6). This latter function relies on the stroma-associated pancreatic stellate cells that, following stimulation by the cancer cells, activate autophagy and secrete their breakdown products mainly consisting of non-essential amino acids. Among them, alanine, the second most abundant amino acid in proteins, is up-taken by the cancer cells and used as carbon source to run the TCA cycle, and to synthetize other non-essential amino acids and lipids (6).

The role of Kras in mediating the nutrient stress response to reduced amino acid availability has been recently elucidated in NSCLC. Gene expression profiles of lung cancer cell lines with different genetic background have been analyzed in presence of high or low glutamine concentrations with or without concomitant Kras knockdown, to identify a set of genes that are differentially regulated by Kras signaling in response to glutamine availability (45). In low glutamine, Kras regulates over 100 genes. Among them, 39 are controlled by the transcription factor ATF4. Kras increases the expression of ATF4 mRNA through PI3K-AKT-mediated upregulation of the NRF2 transcription factor, which drives the expression of a number of genes mainly involved in the antioxidant response [reviewed in Sullivan et al. (46)]. During nutrient deprivation, activation of the GCN2-p-eIF2 pathway stimulates translation of the ATF4 mRNA, resulting in increased ATF4 protein levels and transcription of target genes responsible for amino acids uptake and metabolism thus regulating cell proliferation and mTORC1 activation (45). Among the ATF4 targets, the enzyme asparagine synthetase (ASNS), which transfers the γ amino group of glutamine to aspartate, yielding asparagine and glutamate, uncovers a key role because it contributes to apoptotic suppression, protein biosynthesis and mTORC1 activation. Consistently, inhibition of AKT impairs Kras-dependent activation of ASNS therefore sensitizing NSCLC tumors to depletion of extracellular asparagine (45). Overall these findings identify KRAS as a master regulator of the transcriptional response to nutrient deprivation that controls amino acids uptake and consumption (schematized in **Figure 2**). ATF4 has been shown to exert both pro- and anti-oncogenic effects depending on the genetic context and nutrient availability (45). In condition of low glutamine, ATF4 has a protective role toward apoptosis in Kras mutant NSCLC cell lines that carry loss of *KEAP1* (45), a deletion that, in humans, affects approximately 20% of Kras-mutant lung adenocarcinomas (8). Keap1 is a ubiquitin ligase that causes degradation of NRF2 [reviewed in Sullivan et al. (46)]. Its loss cooperates with *KRAS* mutations in lung adenocarcinoma progression by opposing to the oxidative stress barriers during tumorigenesis (8). Of note, Kras mutant Keap1 deficient cancers are dependent on the glutamine anaplerotic pathway as their growth rate in mice is reduced by pharmacological inhibition of the enzyme glutaminase. This suggests that increased NRF2 activation in Kras mutant lung cancer might be exploited as a stratification tool to identify patients that benefit from glutaminase inhibition (8).

In NSCLC, *KRAS* mutations are often accompanied by loss of the tumor suppressor *STK11*, which encodes the LKB1 kinase, leading to the formation of aggressive tumors characterized by perturbed nitrogen handling (9). LKB1, through AMPK, suppresses transcription of CPS1 (carbamoyl phosphate synthetase-1), a mitochondrial enzyme that catalyzes the rate-limiting step of the urea cycle. In non-pathological settings, expression of CPS1 is restricted to the liver where robust urea production from ammonia takes place (47). In NSCLC cells bearing both mutant Kras and LKB1 loss, expression of CPS1 produces carbamoyl phosphate in the mitochondria from ammonia and bicarbonate, initiating pyrimidine synthesis (9). Depletion of CPS1 in these cells results in pyrimidine depletion, replication fork stalling and DNA damage finally reducing their ability to grow tumors. Interestingly, wild type Kras cells carrying LKB1 loss express CPS1, but do not depend on it. Thus oncogenic Kras is required to generate CPS1 "addiction." This addiction might result from the ability of mutant Kras to increase glutaminolysis in mitochondria (33) thus locally generating ammonia that would support carbamoyl phosphate production by CPS1 (9).

MUTANT KRAS IN LIPID METABOLISM

Lipid metabolism, in particular the synthesis of fatty acids, is required for membrane biosynthesis, signaling molecules production and energy storage (48). Recently, it is also emerging as a mechanism to cope with oncogenic stress (49). Mutant Kras has been shown to control both β-oxidation and *de novo* lipogenesis in NSCLC (49, 50). The role of mutant Kras in fatty

TABLE 1 | Summary of potential metabolic targets in PDAC and NSCLC.

Cancer type	Potential metabolic targets	Proposed mechanism	Proposed inhibitor	References
PDAC	Penthose phosphate pathway (PPP)	MAPK through Kras leads to an increase of glycolytic enzymes expression	PPP inhibition	(21)
PDAC	PON2	Suppresses cell detachment-induced cell death (anoikis) by inhibiting the AMPK/FOXO3A/PUMA pathway	Pharmacological inhibition of PON2 or activation of AMPK	(7, 23)
PDAC NSCLC	Tfeb/Tfe3	Tfe3 sustains tumor growth through increased lysosomal activity	Inhibition of lysosomal function	(25, 26)
PDAC	GOT1 and GOT2	Elevating the NADPH/$NADP^+$ ratio leading to higher antioxidant capacity of tumor cells	GOT1 inhibition	(33, 34)
PDAC	MAPK (MEK1/2, ERK) and autophagy pathway	MAPK inhibition leads to tumor cell addiction to autophagy	Combined inhibition of autophagy and MAPK in cells addicted to autophagy	(43, 44)
NSCLC	ATF4 transcription factor	Amino acid dependency	Inhibition of glutamine utilization	(45)
NSCLC	Carbamoyl phosphate synthetase-1 (CPS1)	KRAS/LKB1 mutant enhances CPS1 expression, pyrimidine synthesis and glutaminolysis	Inhibition of CPS1 or glutamine utilization	(9, 33)
NSCLC	Acsl3	Kras enhances Acsl3 activity and lipid metabolism	Silencing or inhibition of Acsl3	(49)
PDAC	GNAS	Promotes cAMP/PKA signaling and metabolism rewiring	Inhibitors of the cAMP/PKA pathway and lipid metabolism	(10)

acid oxidation has been reported in a transgenic mouse model that expresses the (doxy)-inducible Kras transgene ($Kras^{G12D}$) in the respiratory epithelium (49). These mice, when fed with doxy, develop lung tumors that completely regress when doxycycline is removed with concomitant significant decrease in the expression of enzymes that control glycolysis and lipid metabolism (49). Among the latters, Acyl-coenzyme A synthetase long chain family member 3 and 4 (*Acsl3* and *Acsl4*) are significantly down regulated in tumors undergoing $Kras^{G12D}$ extinction and *Acsl3* seems to contribute the most to the oncogenic phenotype both *in vitro* and *in vivo* (49). *Acsl3* promotes uptake, retention, and β-oxidation of fatty acids converting them into Acyl-CoA esters. Genetic deletion of *Acsl3* in mice does not cause any morphological defects neither during development nor adult life, but impairs mutant Kras tumorigenesis. *Acsl3* silencing has likely similar effects as fatty acid synthase pharmacological inhibition opening to new possible therapeutic strategies in NSCLS (49).

The role of Kras in lipogenesis is highlighted by the upregulation of enzymes that control fatty acid metabolism such as ATP citrate lyase, fatty acid synthase and acetyl coenzyme A carboxylase in the $Kras^{G12D}$ lung cancer model (50). Overexpression of both ATP citrate lyase and fatty acid synthase correlates with poor survival and with increased lipogenesis as shown by the higher levels of newly synthetized palmitate and oleate (48, 50).

As for other metabolic adaptations, *KRAS* mutations work synergistically with additional genetic alterations in reprogramming lipid metabolism. In PDAC arising from intraductal papillary mucinous neoplasm (IPMNs), *KRAS* mutations are associated to a gain of function mutation on the gene *GNAS* ($GNAS^{R201C}$) which encodes $G\alpha_s$, the stimulatory subunit of heterotrimeric G proteins (10). *GNAS* mediates G-protein-coupled receptor (GPCR)-stimulated cAMP signaling, and its mutation has been identified in different tumor types (10). In double mutant mice carrying inducible $Gnas^{R201C}$ expression and $Kras^{G12D}$ mutation, $Gnas^{R201C}$ promotes IPMN initiation and sustains tumor formation. Mechanistically, using tumor-derived organoids, $Gnas^{R201C}$ has been found to support pancreatic cancer growth via cAMP-PKA signaling that suppresses the salt-inducible kinases (SIKs) (10). Proteomics reveals that this pathway is overall correlated with lipid metabolism and with components of the peroxisome, an organelle required for long-chain fatty acids processing and the generation of ether lipids suggesting that concurrent *GNAS* and *KRAS* mutations cooperate in lipid metabolism rewiring (10).

CONCLUSIONS

Studies on the role of mutant Kras in rewiring cancer cell metabolism are blooming and the approaches to exploit Kras-driven metabolic vulnerabilities that stem from these findings hold promises, at least in pre-clinical settings, as we summarized in **Table 1**. A take home message is that metabolic interfering drugs can be attempted, preferentially in combination with other therapies, to tackle Kras mutant cancers but, to be successful, these strategies have to consider the genetic mutational background, the tissue of origin and the crosstalk between the tumor and the microenvironment. It is of note that some of the putative targets including AMPK and autophagy have, depending on the context, pro-tumorigenic functions, while others, such as ATF4, by regulating transcription of distinct set of genes, are endowed with a wide range of downstream functions. This could pose limits to their exploitation as therapeutic targets (23, 51, 52). Moreover, findings on the role of AMPK in $Kras^{G12D}$-driven lung cancer during glucose starvation (25), and on the KRAS-dependent transcriptional response to nutrient deprivation (45), reveal that the effects of *KRAS* mutations on metabolic reprogramming are also strongly influenced by the availability of nutrients which can be heterogeneously distributed within the tumor and change over time. There is a lot more to be learned, there are still big research gaps in the field that need to be addressed in future studies. Moreover the interplay with other pathways, such as PPARγ and WNT/β-catenin, involved in metabolic

enzymes changes in other cancers (53, 54) should be further investigated. This growing body of knowledge points to the complexity of this system and suggests that analysis of the genetic context and the metabolic activity of the tumor should be combined to identify KRAS-driven metabolic vulnerabilities and stratify patients.

AUTHOR CONTRIBUTIONS

EP and LL wrote the manuscript. EP and EM performed the staining showed in **Figure 1**. DA designed **Figure 2**. FB and LL reviewed the final version of the manuscript.

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GLOSSARY

The use of capital letters or the italic to indicate KRAS reflects the nomenclature guidelines here reported.
KRAS human protein.
KRAS human gene.
Kras murine protein.
Kras murine gene.

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REVIEW
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RAS: Striking at the Core of the Oncogenic Circuitry

Ryan C. Gimple[1,2] *and Xiuxing Wang*[3]*

[1] Division of Regenerative Medicine, Department of Medicine, University of California, San Diego, San Diego, CA, United States, [2] Department of Pathology, Case Western University, Cleveland, OH, United States, [3] Key Laboratory of Antibody Technique of Ministry of Health, Nanjing Medical University, Nanjing, China

Cancer is a devastating disease process that touches the lives of millions worldwide. Despite advances in our understanding of the genomic architecture of cancers and the mechanisms that underlie cancer development, a great therapeutic challenge remains. Here, we revisit the birthplace of cancer biology and review how one of the first discovered oncogenes, RAS, drives cancers in new and unexpected ways. As our understanding of oncogenic signaling has evolved, it is clear that RAS signaling is not homogenous, but activates distinct downstream effectors in different cancer types and grades. RAS signaling is tightly controlled through a series of post-transcriptional mechanisms, which are frequently distorted in the context of cancer, and establish key metabolic and immunologic states that support cancer growth, migration, survival, metastasis, and plasticity. While targeting RAS has been fiercely pursued for decades, new strategies have recently emerged with the potential for therapeutic efficacy. Thus, understanding the complexities of RAS biology may translate into improved therapies for patients with RAS-driven cancers.

Keywords: RAS, cancer, metabolism, immunology, mitogen activated kinase, cancer therapy

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***Correspondence:**
Xiuxing Wang
xiuxingwang81@163.com

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INTRODUCTION

The RAS family represents some of the earliest described oncogenes and its discovery fundamentally transformed our understanding of cancer biology. Originally identified in the 1960s as a viral component that induced formation of sarcomas in rats (1, 2), the RAS oncogenes were later found to be normal components of the human genome (3, 4) that were capable of transforming normal human cells (5, 6). Since these early studies, additional work has highlighted the importance of RAS as a contributor to many human cancers and has more fully elucidated its signaling axis and molecular regulators. As a small membrane-localized GTPase, RAS proteins integrate a number of proliferative signals to establish a tumorigenic cellular circuit when aberrantly activated. Encoded by the *KRAS4A*, *KRAS4B*, *HRAS*, and *NRAS* genes, RAS family members are among the most frequently altered oncogenes in human cancers. In this review, we dissect the oncogenic circuitry established by RAS and discuss its numerous roles in supporting proliferative signaling, survival pathways, metabolic and immunologic functions, and its potential vulnerabilities as a therapeutic target.

RAS SIGNALING CASCADE AND REGULATION

RAS signaling can be activated by a number of cellular receptors including receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and integrin family members. These signaling

cascades initiate RAS activation through assembly of several scaffolding proteins that mediate conversion of RAS from an inactive GDP-bound form to an active GTP-bound state. Epidermal growth factor receptor (EGFR) is a member of the RTK family and one of the best characterized activators of RAS signaling through recruitment of the molecular scaffolding protein growth factor receptor bound protein 2 (GRB2) (7). GRB2 recruits the RAS-guanine exchange factor (RAS-GEF) SOS1, which activates the RAS protein through a conformational change induced by exchanging GDP for GTP. Similarly, other RTK family members including platelet derived growth factor receptor beta (PDGFR-β) can initiate RAS activation through recruitment of GRB2 (8), and colony stimulating factor 1 receptor (CSF-1R) signaling functions through activation of RAS (9). Several GPCRs also function in a RAS-dependent manner with the beta-gamma subunit of GPCRs activating RAS signaling (10). GPCRs activate RAS through stimulation of both non-RTKs (11) (including src, Lyn, and Syk) and RTKs as described above. Certain downstream signaling functions of integrin proteins are also RAS dependent (12).

RAS can be further activated by additional RAS-GEFs including the RAS-GRF and RAS-GRP family members or negatively modulated by a series of RAS-GTPase activating enzymes (RAS-GAPs), including neurofibromin 1 (NF1) (13). These RAS activity regulators are also frequently altered across a number of cancer types. Post-translational modifications are also critical to the functions of the RAS protein. The addition of an isoprenyl group (farnesylation) by farnesyl transferase is essential for RAS localization to the plasma membrane and downstream signaling roles (14). Further, palmitoylation of the NRAS and HRAS proteins by the enzymes DHHC9 and GCP16 promotes membrane localization and efficient signaling (15). Continuous cycles of NRAS and HRAS palmitoylation ensure that these proteins are selectively localized to the Golgi or plasma membrane and not in other intracellular membranes (16, 17). KRAS, however, can localize to the plasma membrane without the requirement of palmitoylation (18). The post-translational membrane anchor that fastens KRAS to the plasma membrane contains unique sequences and electrostatic properties that determine the specific localization of RAS nanoclustering within anionic phospholipids (19). KRAS dimerization is also critical for oncogenic signaling (20).

Further post-translational modifications including mono-ubiquitination favor the active form of RAS (21, 22), while di-ubiquitination decreases downstream signaling output through ERK (23). RAS signaling can be abrogated through ubiquitination by an LZTR1-CUL3 complex, which inhibits its membrane localization (24, 25). RAS acetylation has also been shown to reduce signaling activity, with cells dependent on the protein deacetylases HDAC6 and SIRT2 to maintain RAS signaling (26, 27). Additionally, acylpeptide hydrolase (APEH) contributes to the appropriate localization of RAS to the plasma membrane by regulating phosphatidylserines in the plasma membrane (28) (**Figure 1**).

Following activation, RAS can execute a variety of functions that promote cancer development including oncogenic transcription, cell cycle progression, cellular survival, cell growth and metabolism, and cell motility and migration. First, RAS activates the mitogen-activated protein kinase (MAPK) pathway defined by a RAF-MEK-ERK signaling axis. This pathway activates transcription of a number of proliferative signaling networks driven by FOS, JUN, and ETS family transcription factors, as well as MYC. These factors support cancer cell proliferation through promoting cell cycle entry, angiogenesis, and survival. Second, RAS plays an important role in the activation of the PI3K-AKT signaling network, which supports oncogenic transcription through NF-κB signaling, evasion of apoptosis through inhibition of the pro-apoptotic enzyme BAD, and cell growth and metabolism through mTOR. Third, activation of TIAM1 drives cancer cell motility and migration through a Rac-Rho and Rac-PAX dependent network. Other RAS effectors have been studied extensively (29) (**Figure 1**).

KRAS can also mediate activation of canonical Wnt signaling while suppressing non-canonical Wnt pathways to promote tumor growth. In APC-deficient colon cancers, KRAS-dependent cells specifically upregulate BMP signaling, which activates expression of TAK1/MAP3K7 and downstream transcriptional upregulation of canonical Wnt target genes. This pathway can be targeted with TAK1 kinase inhibitors, which selectively ablate KRAS-mutant colon cancer xenografts (30). KRAS has also been shown to inhibit non-canonical Wnt signaling through sequestering calmodulin and blocking transcription of the Frizzled 8 receptor, a G protein-coupled receptor activator of non-canonical Wnt signaling (31). This represents one distinguishing feature between RAS family proteins, as HRAS is unable to similarly affect this pathway (31). Because non-canonical Wnt signaling reduces activation of canonical Wnt signaling pathways, these studies consistently show that KRAS activates canonical Wnt signaling to support stem-like properties of cancer cells and tumor growth and that this node may be targeted for cancer therapy.

ROLE OF RAS MUTATIONS IN DIFFERENT CANCER TYPES

The Cancer Genome Atlas (TCGA) project identified the RTK-RAS signaling pathway as the most frequently altered oncogenic network in cancer, with 46% of all samples displaying alterations (32). RAS alterations contribute to 20–30% of all human cancers. KRAS mutations are exceedingly common in pancreatic adenocarcinomas and colorectal cancers, while NRAS mutations are more common in melanomas, thyroid cancers, and leukemias (33, 34) (**Figures 2A–C**). Although KRAS, HRAS, and NRAS share functional similarities, KRAS missense gain-of-function mutations tend to occur on the 12th codon, while those in HRAS and NRAS occur on the 61st codon and are differentially utilized across cancer types (33–35) (**Figure 2D**). These mutations act by creating enhanced RAS activity, effectively uncoupling pro-proliferative downstream signaling from growth factor receptors. Alterations in any of these RAS family genes is associated with poor patient prognosis in pan-cancer analyses (33, 34) (**Figure 2E**), and RAS pathway gene alterations frequently

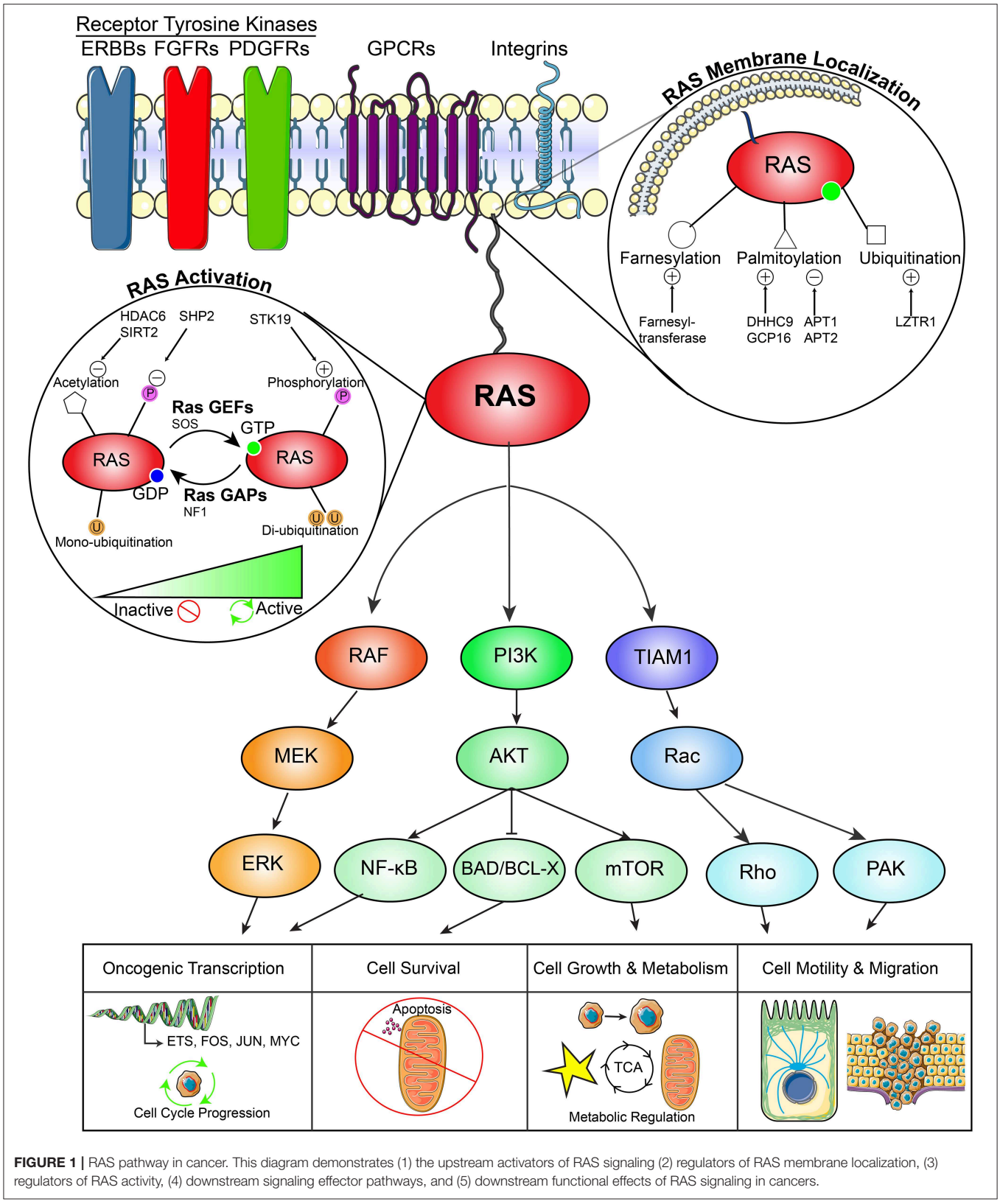

FIGURE 1 | RAS pathway in cancer. This diagram demonstrates (1) the upstream activators of RAS signaling (2) regulators of RAS membrane localization, (3) regulators of RAS activity, (4) downstream signaling effector pathways, and (5) downstream functional effects of RAS signaling in cancers.

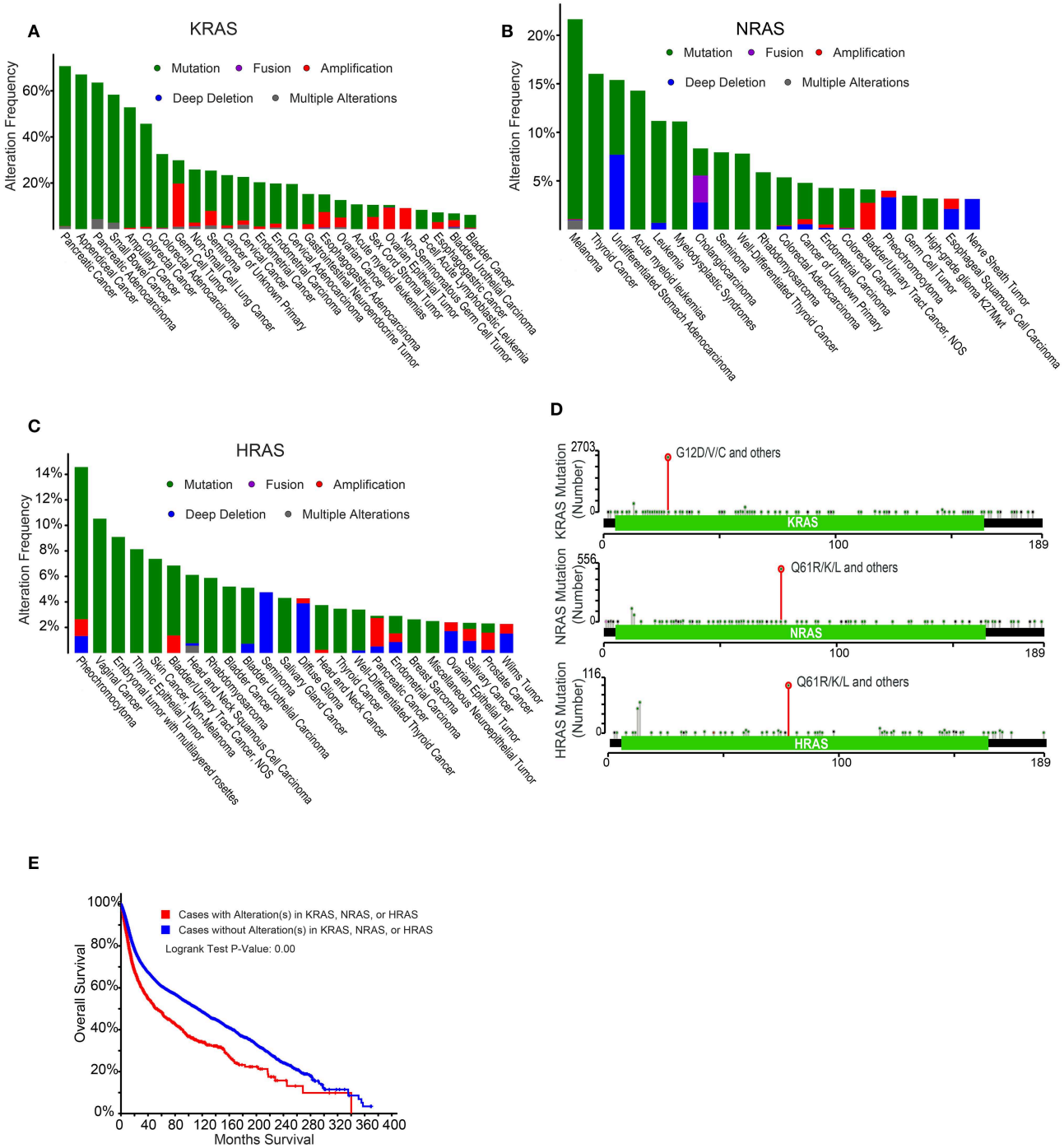

FIGURE 2 | Epidemiology of RAS alterations in cancer. **(A)** Frequency of KRAS alterations across a number of cancer types. Data were derived from Cerami et al. (33) and Gao et al. (34). **(B)** Frequency of NRAS alterations across a number of cancer types. Data were derived from Cerami et al. (33) and Gao et al. (34). **(C)** Frequency of HRAS alterations across a number of cancer types. Data were derived from Cerami et al. (33) and Gao et al. (34). **(D)** Localization of RAS gene mutations across the gene body. Data were derived from Cerami et al. (33) and Gao et al. (34). **(E)** Prognosis of cancer patients with or without alterations in KRAS, NRAS, or HRAS. Data were derived from Cerami et al. (33) and Gao et al. (34).

co-occur with the exception of KRAS-BRAF and KRAS-NRAS gene pairs, which are mutually exclusive (33, 34) (**Figure 3**).

Pancreatic ductal adenocarcinomas (PDACs) are highly lethal and display exceptionally high frequency of KRAS mutations (94% mutant). RAS mutations in PDAC commonly co-occur with CDKN2A mutations and deletions, TP53 mutations, and SMAD4 mutations (36–38). Colorectal cancers are largely initiated by mutations in APC, which lead to uncontrolled Wnt

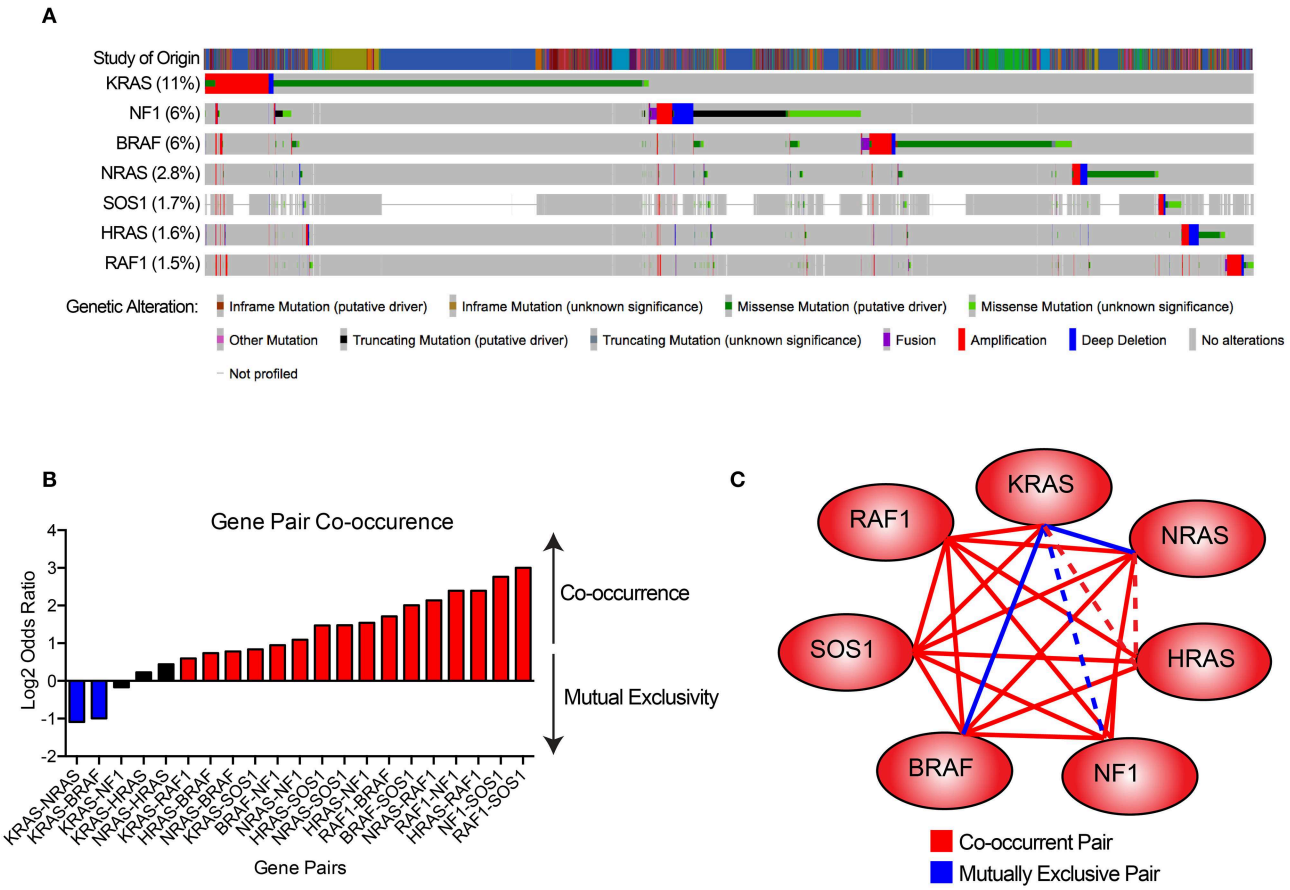

FIGURE 3 | Gene pair co-occurrence among RAS pathway genes. **(A)** Co-occurrence plot RAS pathway genes across a number of cancer types. Data were derived from Cerami et al. (33) and Gao et al. (34). **(B)** Gene pair co-occurrence plot of RAS pathway genes. Blue bars indicate gene pairs that are significantly mutually exclusive, red bars indicate gene pairs that are significantly co-occurrent, and black bars indicate gene pairs without significant co-occurrence. **(C)** Gene pair co-occurrence network. Solid blue lines indicate gene pairs that are significantly mutually exclusive, solid red lines indicate gene pairs that are significantly co-occurrent, and dotted lines indicate gene pairs without significant co-occurrence.

signaling, followed by loss of function of TP53, inactivation of TGF-β signaling, and mutations in KRAS in ∼37% of cases (39). KRAS is the most commonly mutated oncogene in lung adenocarcinoma, occurring in 33% of cases, along with EGFR, BRAF, and TP53 mutations (40). Despite the high prevalence of KRAS mutations and RTK activation in lung adenocarcinomas (and other forms of non-small cell lung cancers), small cell lung carcinomas are characterized by nearly universal inactivation of TP53 and RB1 through mutation or deletion, without alterations in RAS (41). In contrast to pancreatic, lung, and colon cancers, melanomas contain NRAS mutations in 20–30% of cases (42) NRAS is also commonly mutated in acute myeloid leukemias in 15% of cases (43, 44).

The differential mutation rate across cancers suggests that each mutational event may activate distinct signaling events and that each tissue type may be differentially poised to transform following RAS mutation. For example, HRAS displayed a greater capacity to transform fibroblasts than the other RAS family members (45), while in hematopoietic cell models, NRAS demonstrated a stronger transforming potential (46). RAS family members display distinct post-translational modifications, which regulate their subcellular localization and differential signaling preferences, which have been extensively reviewed elsewhere (47–49).

RAS AND METABOLISM

Dysregulated metabolism is a key hallmark of cancer, and activation of RAS signaling supports cancer initiation, maintenance, and progression through driving altered metabolic networks. RAS signaling promotes oncogenic metabolism by coordinating numerous metabolic processes including lipid, nucleotide, and glycolytic pathways. Specifically, RAS signaling supports cellular bioenergetic needs and enhances glucose uptake through induction of the GLUT1 glucose transporter promoting survival in low-nutrient conditions and increased glycolytic metabolism (50). This glucose is shunted away from the tricarboxylic acid (TCA) cycle to support glycolytic metabolism,

protein glycosylation, and nucleotide metabolism through the pentose phosphate pathway (51, 52). Cells also upregulate glutamine metabolism and the phosphoserine biosynthetic pathway through upregulation of biosynthetic enzymes in these pathways (53). KRAS redirects glutamine utilization to support cellular redox balance through transcriptional regulation of the GOT1 (glutamic-oxaloacetic transaminase 1) enzyme and creates a dependency on glutamine metabolism (54). Co-mutation of KRAS with loss of KEAP1 (kelch like ECH associated protein 1) further extended the glycolytic phenotype, dependence on glutamine, and sensitivity to glutaminase inhibitors in lung adenocarcinoma models (55). RAS signaling also acts to support nucleotide biosynthesis via MYC activation. RAS upregulates MAPK signaling, which induces MYC and drives nucleotide metabolism through the pentose phosphate pathway (56).

Increased copy number of mutant oncogenic KRAS that typically occurs later in the process of tumorigenesis further activates glycolytic metabolism and supports glutathione synthesis, but can also direct metabolites into the TCA cycle in lung cancer cells to support tumor progression (57). This mitochondrial metabolism has been shown to be essential for anchorage-independent cell growth in KRAS-driven cancers by promoting generation of reactive oxygen species, which modulate ERK signaling (58). This suggests that differential dosage of KRAS expression can have contrasting effects on cellular metabolism and highlights the evolution of metabolic states throughout tumor development. RAS allelic imbalance and loss of wild-type KRAS alleles can further extend the oncogenic properties of cancer cells and mark the most aggressive undifferentiated cells (59), but also create a dependency on the MAPK signaling pathway with unique sensitivities to pharmacologic MEK inhibition (60).

While cancers rely heavily on endogenous synthesis of substrates for anabolic needs, RAS-driven cancers also utilize mechanisms to recover materials from their extracellular environments in the form of micropinocytosis (61, 62). This process supports cancer cell growth through scavenging extracellular amino acids for use in protein synthesis, and glutamine for a variety of metabolic processes (63). RAS activation can also support cell membrane biosynthesis through fatty acid uptake from lysophospholipids in the surrounding microenvironment, reducing dependence on endogenous lipid synthesis (64). KRAS signaling sustains cancer cells under conditions of nutrient stress by activating an NRF2-ATF4 axis to increase amino acid transport and protein biosynthesis, preventing apoptotic cell death through increased asparagine synthase activity (65).

Despite this metabolic resiliency through increased nutrient scavenging capacity, RAS driven cancers are dependent on autophagy, which is essential for mitochondrial recycling and oxidative capacity (66). Autophagy is essential for proper mitochondrial function and nucleotide synthesis in KRAS-driven tumors (67), as well as for efficient catabolism of fatty acids (68). In RAS driven pancreatic cancers, autophagy is supported by the MiT/TFE family of transcription factors, including MITF, TFE3, and TFEB, which activate genes that promote autophagy and lysosomal pathways to maintain intracellular amino acid pools (69). The acyl-CoA synthetase family member, ACSL3, whose expression is driven by mTOR signaling downstream of RAS, specifically regulates intracellular fatty acid metabolism and utilization in RAS-dependent cancers by supporting fatty acid uptake, accumulation, and β-oxidation (70). Interestingly, RAS-driven metabolic dependencies can also be tissue- and context-dependent. Branched-chain amino acid metabolism is a key dependency in KRAS-driven non-small-cell lung carcinoma (NSCLC) cells in which they are essential for non-essential amino acid and DNA synthesis. However, these metabolic circuits are dispensable in KRAS-driven pancreatic ductal adenocarcinoma (PDAC) cells (71) (**Figure 4**).

RAS IN CANCER METASTASIS

In addition to driving processes essential for early phases of tumorigenesis, RAS activity is important for the acquisition of more malignant features, including supporting metastasis. In mouse models of colorectal cancer, while primary tumors were characterized by a heterogeneous population of cells bearing both oncogenic KRAS mutations and wild-type KRAS, metastatic sites were largely comprised of more uniform cell populations harboring oncogenic KRAS (72). This metastatic phenotype was promoted by transforming growth factor beta (TGF-β) signaling (72). Distinct from heterogeneity in cellular populations with respect to KRAS mutation status, acquisition of multiple oncogenic KRAS mutations within single cells through focal amplifications and loss of the wild-type allele (loss of heterozygousity) can promote tumor metastasis and aggressive properties (59). KRAS also supports metastatic dissemination through repression of Raf Kinase Inhibitory Protein (RKIP), a putative tumor suppressor with roles in cell migration, motility, and epithelial-to-mesenchymal transition (73). Activation of KRAS signaling along with homozygous deletion of LKB1 (also known as STK11 or serine/threonine kinase 11) promoted cancer progression and metastasis in non-small cell lung cancer models (74). In KRAS-driven pancreatic cancer models, deletion of LKB1 enhanced the tumorigenicity and proliferation rate of cancer cells through enhanced serine biosynthesis and S-adenosyl-methionine (SAM), which supports DNA methylation (75).

RAS AND THE IMMUNE SYSTEM

Interactions between cancer cells and the immune system are essential features of cancer biology. In order to survive and thrive, cancer cells must avoid immunoediting by immune effector cells; however, cancer cells also frequently gain proliferative advantage from the surrounding immune microenvironment (76). RAS signaling reduces expression of MHC class I molecules on the surface of cancer cells, rendering them less vulnerable to immune-mediated cell death by cytotoxic T-cells (77, 78). Immune checkpoints such as PD-L1 (CD274) serve to dampen the reactivity of the immune system and to prevent autoimmunity. Cancers frequently subvert this mechanism to avoid being targeted by the immune system. RAS signaling can promote this effect in an MEK-dependent

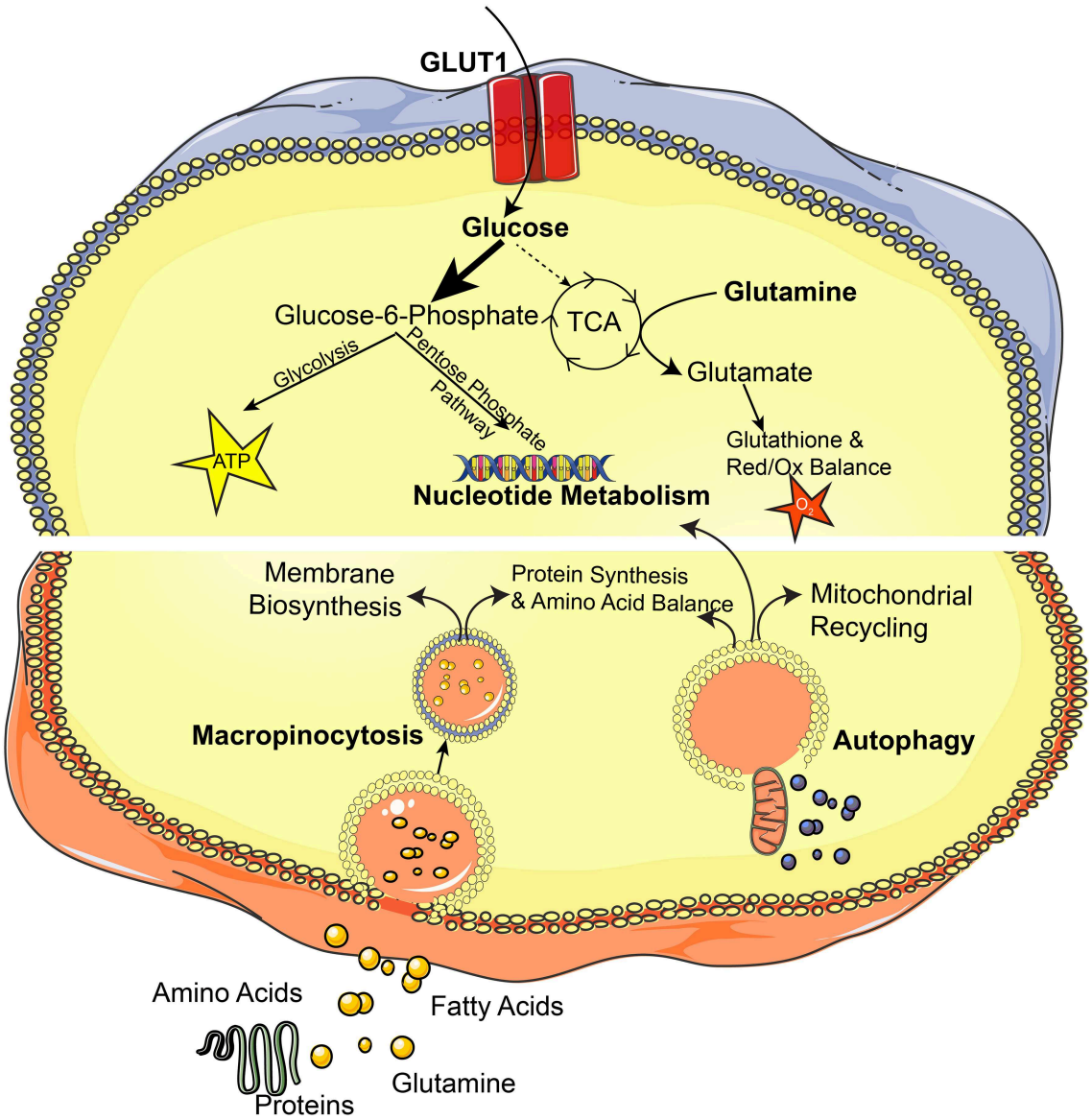

FIGURE 4 | The RAS pathway orchestrates cellular metabolism. This diagram depicts metabolic pathways that are altered in RAS-driven cancers.

manner by stabilizing PD-L1 mRNA through downregulation of tristetraprolin (TTP/ZFP36), an RNA binding protein which typically degrades mRNAs (79). These findings may partially explain the observation that KRAS mutant non-small cell lung cancer patients display better responses to PD-1 inhibition with nivolumab than KRAS wild-type patients (80, 81). In hepatocellular carcinoma models, dual KRAS and MYC signaling can translationally enhance PD-L1 levels by bypassing upstream open reading frames, which typically serve a repressive role (82). This contributes to a more aggressive and metastatic phenotype with the capacity to evade the immune system. KRAS and MYC signaling further cooperate to promote the development of aggressive and invasive adenocarcinomas by recruiting immunosuppressive macrophages via the chemokine CCL9 and excluding T-cells and NK cells via interleukin-23 (IL-23) (83). These alterations allow developing tumors to evade immune-mediated attack. In lung cancer models, KRAS supports expression of IL6-mediated chronic inflammation, which reorganizes the tumor microenvironment by recruiting myeloid derived suppressor cells (84, 85). Targeting MAPK and CDK4/6 pathways in RAS mutant lung cancer cells leads to natural-killer (NK) cell-mediated attack of tumor cells through induction of senescence pathways (86). Activation of the MEK/ERK signaling pathway by the oncogenic KRAS G12D mutation increases secretion of IL-10 and TGF-β from pancreatic cancer cells, which promotes conversion of T-cells to an immunosuppressive regulatory T-cell (Treg) state (87). Additionally, co-mutation with STK11 is associated with a

reduction in NF-κB signaling in RAS mutant tumors and suppression of tumor immunosurveillance while co-mutation with TP53 is associated with increased immune responses (88). This suggests that mutations commonly co-occurring with RAS impinge upon the immune reactivity of RAS driven cancers.

Besides avoiding immune-mediated destruction, cancer cells frequently benefit from a proinflammatory microenvironment that sustain oncogenic processes. In pancreatic intraepithelial neoplasia models of pancreatic cancer precursor lesions, KRAS signaling induced expression of IL-17 receptors on preneoplastic cells and infiltration by IL-17 secreting T-cells, both of which accelerated progression to a neoplastic state (89). RAS signaling also promotes tumor vascularization and inflammation by inducing secretion of IL-8 from cancer cells through MAPK and PI3K pathways (90). Tumor vascularization is further driven by KRAS-mediated induction of hypoxic HIF signaling, which drives expression of vascular endothelial growth factor (VEGF) (91). KRAS activation can activate inflammatory processes in lung cancer models by stimulating accumulation of macrophages and neutrophils through production of inflammatory chemokines (92) (**Figure 5**).

THERAPEUTIC TARGETING OF RAS

Because of the numerous ways in which RAS activity supports tumor cell proliferation, survival, metabolism, microenvironmental interactions, and immune evasion, efficient therapeutic targeting of RAS has been the focus of a large body of research. While it was previously believed that RAS is an undruggable target due to its molecular structure, new insights into its biological functions and molecular regulators may allow for efficient pharmacological inhibition of RAS effectors and discoveries of synthetic lethality.

Direct RAS Inhibitors

Direct inhibition of oncogenic RAS could be a powerful therapeutic approach to ablate RAS-driven tumors. Studies of the molecular structure of the common KRAS G12C variant have informed the development of specific inhibitors that selectively target the mutant form of KRAS and both limit its activation by favoring binding to GDP as well as blocking its downstream signaling through RAF (93). Another compound targeting the KRAS G12C variant, ARS-853, selectively reduced the frequency of the active, GTP-bound KRAS, and inhibited cell proliferation in lung cancer models and suggests that nucleotide cycling between GDP and GTP bound forms are essential for its molecular functions (94, 95). Next-generation forms of KRAS G12C targeting agents, including ARS-1620, demonstrated improved potency compared to earlier generation agents and block oncogenic RAS signaling and tumor growth *in vivo* in a target-specific manner in non-small cell lung cancer models (96). These agents have been extensively reviewed elsewhere (97, 98). In addition to mutation-specific RAS inhibitors, pan-RAS inhibitors that target HRAS and NRAS as well as KRAS have been developed. One of these pan-RAS inhibitors, compound 3,144, efficiently silenced PI3K-AKT and MEK-ERK signaling downstream of RAS and prevented growth of RAS-driven xenograft cancer models. However, some off-target effects and toxicities apparent in this first-generation compound have prevented wide-spread clinical adoption at this time (99). To advance rational design of compounds with RAS targeting potential, computational modeling of RAS three-dimensional structure revealed conformational changes that occur during RAS deactivation, suggesting that stabilizing these inactive forms may reduce RAS signaling efficacy (100). Similar efforts identified a high-affinity allosteric KRAS inhibitor that impairs KRAS signaling and cancer cell growth in cells bearing several distinct types of KRAS activating mutation (101). Detailed conformational dynamics analyses and structural biology approaches uncovered numerous vulnerabilities and co-dependencies of the RAS enzyme, which may be exploited for therapeutic targeting and which have been detailed extensively elsewhere (102, 103).

In addition to small molecule inhibitors, other therapeutic approaches have investigated methods to deliver nucleic acid-based delivery of therapeutic compounds to cancer cells *in vivo*. Using nanoliposomal delivery of KRAS-targeting siRNAs, KRAS mRNA expression could be dramatically reduced with subsequent decrease in tumor growth and metastatic potential in colon and lung cancer models (104). Nanoliposomes can also be used to deliver miRNAs that specifically target KRAS and impair tumor growth and metastasis in lung cancer models (105). Cyclodextrin polymer nanoparticles can also be used to deliver siRNAs to cancer cells *in vivo*. Optimized siRNAs targeting KRAS impaired colon cancer growth *in vivo* while combinatorial inhibition of KRAS and PIK3CA/PIK3CB significantly improved tumor control compared to single agents alone, demonstrating that targets can be effectively multiplexed (106). In contrast to liposomal or other nanoparticle technologies, exosome-mediated delivery of siRNAs have greater efficiency due to longer persistence in the circulation and take advantage of RAS-mediated upregulation of micropinocytosis for greater uptake by RAS-driven cells. Exosomal delivery of siRNAs targeting KRAS reduced expression of KRAS, suppressed tumor formation, and inhibited metastatic progression in mouse pancreatic cancer models (107).

Inhibitors of RAS Modulators

Besides directly targeting the enzymatic domain of RAS, many studies have investigated targeting its subcellular localization. As described previously, RAS relies on a number of factors for post-translational modifications and localization to the cell membrane. The phosphodiesterase PDE-delta binds to farnesylated RAS and promotes its efficient signaling by selectively localizing RAS to the plasma membrane as opposed to intracellular membranes (108). Inhibition of the interaction between PDE-delta and KRAS disrupted RAS localization and signaling and impaired cell proliferation in pancreatic cancer models (109). Additionally, inhibition of the lysophospholipase APT1 with palmostatin B blocked RAS depalmitoylation and impaired RAS localization and signaling efficacy and contributed to re-acquisition of contact inhibition in HRAS-transformed fibroblasts (110). This inhibitor demonstrated similar effects in NRAS-driven hematologic cancer models

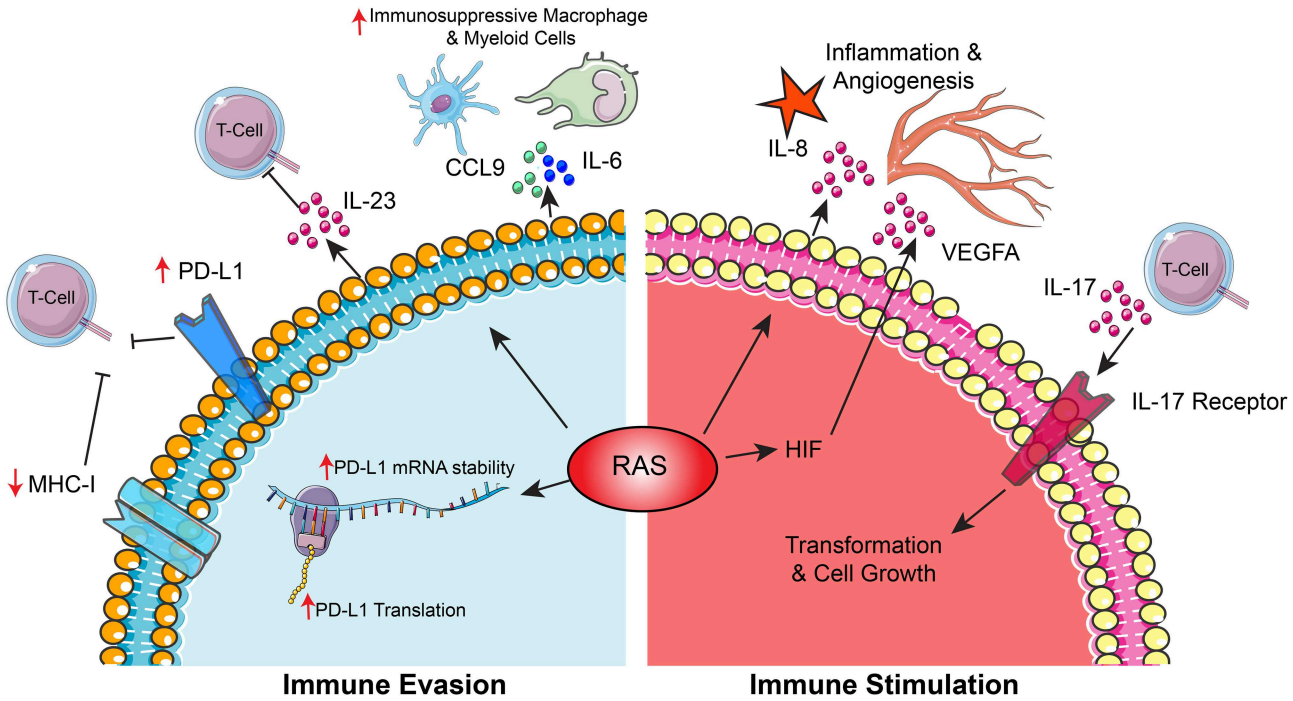

FIGURE 5 | The RAS pathway shapes interactions between cancer cells and the immune microenvironment. This diagram depicts mechanisms by which RAS signaling promotes cancer through (1) supporting cancer cell immune evasion and (2) driving immune-mediated stimulation of cancer cell growth.

(111). Farnesyltransferases are also essential for RAS membrane localization and represent therapeutic targets. Several of these agents have shown promise in clinical trials by disrupting RAS signaling in combination with other therapeutic agents (112–114), although these effects may be based on inhibition of other farnesylation-dependent enzymes beyond RAS. RAS geranylgeranylation following inhibition of farnesyltransferases reactivates RAS signaling and serves as a common resistance mechanism (115). Combinatorial targeting of farnesyl and geranylgeranyltransferases may overcome this resistance (116).

SOS is a RAS-specific guanine exchange factor (GEF) that mediates the conversion of RAS from an inactive GDP-bound state to an active GTP-bound state. Because of this important role in regulating RAS activity, SOS is a natural target for RAS driven cancers. Helical proteins that interrupt the RAS-SOS interaction blocked RAS activation and downstream ERK activity following EGFR stimulation (117). Additional studies have identified small molecules that can interrupt the RAS-SOS interaction and disrupt RAS activation and downstream MAPK and PI3K signaling (118, 119). In order to mediate its downstream effects, RAS binds to a series of effector molecules through a RAS binding domain. Inhibition of this RAS binding domain with the small molecule agent rigosertib impairs the interaction between RAS and RAF, as well as Ral and PI3K, simultaneously incapacitating several downstream RAS effectors and impairing tumor growth *in vitro* and *in vivo* (120). RAS also relies on kinase suppressor of ras (KSR), which serves as a scaffolding factor that links RAS to RAF and allows for MEK activation (121–124). Stabilization of the inactive form of KSR with small molecule compounds blocked this signal transduction from RAS to RAF and enhanced efficacy of MEK inhibitors (125). STK19 activates oncogenic signaling in melanoma cells through selective phosphorylation of mutant NRAS, which supports its interaction with downstream effectors through the RAS binding domain. Pharmacologic inhibitors of STK19 blocked NRAS phosphorylation and impaired melanoma cell growth and tumor formation capacity, and extended survival of tumor-bearing mice (126).

RAS can also be activated by the protein tyrosine phosphatase SHP2 (encoded by the PTPN11 gene). SHP2 binds to receptor tyrosine kinase growth factor receptors through its SH2 domain and mediates activation of RAS through dephosphorylation of RAS, increasing its association with RAF (127, 128). Inhibition of the SHP2 phosphatase domain with a small molecule inhibitor suppressed RAS signaling and impaired proliferation of receptor tyrosine kinase-driven cancer cells *in vitro* and *in vivo,* although RAS-mutant cells were not sensitive to this drug *in vitro* (129). Targeting SHP2 further sensitized pancreatic cancer cells to MEK inhibition and promoted a senescence response in KRAS-mutant non-small cell lung cancer models under nutrient-restricted conditions (130, 131). These findings suggest that combinatorial targeting of signaling elements upstream and downstream of RAS may be a useful therapeutic approach.

Inhibition of Downstream Signaling and Resistance Mechanisms

Aberrant RAS activation can also be targeted through inhibition of downstream signaling elements, such as MEK. Despite these

efforts, targeted therapies are frequently plagued by the robust emergence of resistance. In KRAS mutant cancers, targeting of MEK with trametinib led to compensatory signaling through fibroblast growth factor receptor 1 (FGFR1). Combinatorial therapy using trametinib and FGFR1 inhibition effectively abolished this resistance mechanism and served as a useful combinatorial strategy (132). RAS-driven cancer cells could further overcome MEK inhibition through overexpression of ERBB3. Targeting the related RTKs EGFR and ERBB2 reversed this effect and sensitized to MEK inhibitors (133). Targeting RAF kinases can also reverse resistance to MEK inhibitors through downregulation of MAPK signaling (134). MEK inhibitors further drive compensatory activating phosphorylation of the KSR-1 scaffolding protein, which promotes PI3K-AKT signaling that circumvents inhibition of RAS signaling effectors (135). In the context of RAF or MEK inhibition, YAP1, a component of the Hippo pathway, promoted survival of RAS-mutant cells, with combinatorial inhibition of MEK and YAP1 yielding improved therapeutic efficacy (136). Thus, development of therapeutic resistance following RAS inhibition is exceedingly common. Greater understanding of these resistance mechanisms may allow researchers to collapse the great degree of cellular plasticity in these signaling networks through combinatorial inhibition of survival and escape pathways.

Despite our detailed understanding of the major RAS downstream signaling elements in cancers, recent evidence revealed that the temporal dynamics of signal transduction, and not just the pathway constituents themselves, are critical to the resulting biological effects. Because of this phenomenon, treatment with BRAF inhibitors may have counterproductive effects on RAS signaling by prolonging the typically short pulses of RAS activity into long periods of downstream ERK activation (137). Furthermore, while BRAF inhibitors are effective in blocking growth of cancer cells driven by the BRAF-V600E mutation, BRAF inhibition paradoxically activates MAPK signaling in KRAS mutant tumors through inducing increased dimerization of BRAF with RAS (138). Because the complexity of these signaling pathways has not been completely elucidated, caution must be used when developing therapeutic agents and their downstream effects must be empirically determined.

Identification of RAS-Specific Synthetic Lethality

In addition to targeting RAS signaling directly through its enzymatic activity or indirectly through its regulators or downstream signaling effectors, therapeutic targeting of dependencies established by oncogenic RAS is a promising approach. The unique cellular states established by RAS activation create new nodes of fragility that may be amenable to anti-cancer therapies. Increased RAS copy number engages a glycolytic switch which increases glycolysis and shifts glucose utilization toward the TCA cycle and glutathione synthesis. These metabolic changes create sensitivity to glutathione synthesis inhibitors (57). Loss of wild-type RAS further sensitized cells to MEK inhibition, suggesting that allelic imbalance at the KRAS locus can impact dependency on downstream signaling elements (60). Additionally, increased levels of the GLUT1 glucose transporter facilitates selective sensitivity of RAS driven cancers to vitamin C, the oxidized version of which is preferentially imported, depleting intracellular glutathione, and generating oxidative stress (139). Other targeting approaches have leveraged oxidative stress to selectively ablate NF1- or KRAS- mutant tumors through combinatorial therapy with HDAC and mTOR inhibitors, which suppress glutathione synthesis and the thioredoxin antioxidant pathway (140). These findings suggest that RAS driven cancers are particularly vulnerable to oxidative damage and are unable to efficiently cope with oxidative stress. KRAS-driven cancers employ micropinocytosis to scavenge nutrients from the extracellular environment. Through interacting with cell surface integrins, the carbohydrate binding protein galectin-3 mediates formation of macropinosomes and reduces reactive oxygen species by recruiting KRAS clusters on the cell membrane to promote RAS signaling. This event can be effectively targeted with galectin-3 inhibitors (141).

Whole genome shRNA and CRISPR screening strategies have identified RAS-specific synthetic lethalities, elucidating potential novel therapeutic targets. Cells with oncogenic RAS rely on TBK1, an IκB kinase, to activate NF-κB signaling to prevent apoptosis (142). KRAS-driven non-small cell lung cancers also rely on the nuclear export receptor XPO1, which clears nuclear IκBα and supports NF-κB activity. KRAS-mutant models are selectively sensitive to small molecule inhibition of XPO1 (143). RAS mutant non-small cell lung cancers are specifically dependent on GATA2, a transcription factor that regulates the proteasome, Rho signaling pathways, and maintenance of NF-κB signaling via the IL-1 pathway (144). Collectively, these results point toward NF-κB signaling as an essential pro-survival signal selectively utilized by RAS driven cancers. Furthermore, the protein kinase STK33 is a RAS-dependent essential factor that inhibits mitochondrial apoptosis downstream of S6-kinase (S6K1) signaling (145). In the context of MEK inhibition, the mitochondrial anti-apoptotic gene BCL-XL is essential in RAS-driven cancers. Combinatorial inhibition of BCL-XL with MEK signaling enhanced cell death in colorectal, lung, and pancreatic cancers bearing RAS mutations, suggesting that BCL-XL displays a synthetic lethal interaction with RAS in a context-specific manner (146).

Other screens have demonstrated increased dependence on ribosomal biogenesis and translational control, protein neddylation, protein sumoylation, RNA splicing pathways, and mitotic control in RAS mutant cancer models (147). PLK1, a kinase involved in centrosome maturation and spindle assembly during mitotic progression, was specifically essential and targeting this kinase with a small molecule inhibitor selectively targeted RAS mutant cells. This dependence on mitotic machinery and sensitivity to mitotic stress was specific to RAS-mutant cells when compared to PIK3CA driven cells (147). The cell cycle regulator CDK4 also displays a synthetic lethal relationship with KRAS in non-small cell

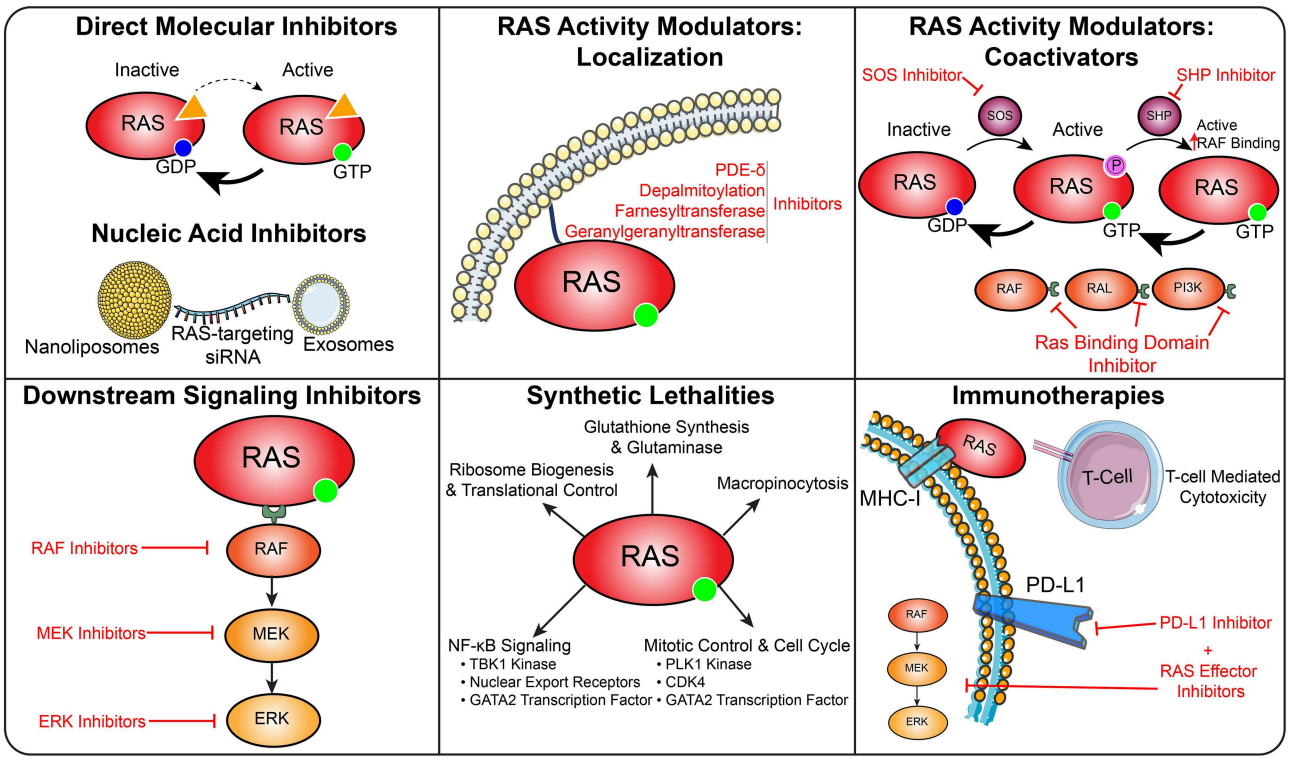

FIGURE 6 | Therapeutic targeting of RAS in cancer. This diagram depicts several strategies to therapeutically target RAS driven cancers.

lung cancers (148). A guanine nucleotide exchange factor for Rac family GTPases, PREX1, is essential for MAPK activation in RAS mutant acute myeloid leukemias, and cells driven by oncogenic RAS were sensitized to Rac/PAK family inhibitors (149).

Immunotherapies

Therapeutic approaches that harness the immune system to target cancers have emerged as an effective strategy. Recently, CD8+ T-cells have been isolated from a patient with metastatic colorectal cancer that specifically recognize mutant KRAS. *Ex vivo* expansion of this population followed by reinfusion into the patient led to reduction in metastatic burden, suggesting that immunotherapeutic approaches may be effective in targeting RAS (150, 151). Further immunotherapeutic efforts have utilized T-cell receptors engineered to specifically target oncogenic forms of KRAS to control tumor growth in pancreatic cancer models (152) In addition to direct targeting of RAS antigens, immunotherapeutic approaches have been explored in combination with inhibition of downstream RAS signaling elements. In BRAF-driven melanomas, combination of BRAF, MEK, and immune checkpoint inhibition through PD-L1 inhibitors enhanced cancer cell death and displayed efficacy in early clinical trials for metastatic melanoma (153, 154). Combinations of MEK and BRAF inhibitors with PD-L1 inhibitors demonstrated some promise in metastatic colorectal cancers and melanomas in early clinical trials (155, 156). PI3K signaling downstream of RAS controls interactions between cancer cells and the immune microenvironment. While overactive PI3K signaling driven by PTEN mutations reduced T-cell-mediated cytotoxicity, treatment with a PI3Kβ inhibitor enhanced the efficacy of anti-PD1 antibodies in melanoma models (157) (**Figure 6**).

CONCLUSIONS

RAS family members are some of the most commonly altered genes in cancer. Perturbations of RAS signaling establish robust oncogenic circuits that drive tumor initiation, progression, growth, and survival. Despite our deep knowledge of the direct downstream signaling effectors of the RAS pathway, continued exploration has revealed new insights into the similarities and differences between RAS family members and their preference for particular cancer types. These efforts have also uncovered the more distal downstream consequences of RAS signaling across cancers, including its rewiring of cellular metabolism and capacity to unlock nutrient scavenging pathways, its role in metastasis, and its dual role in regulating the immune microenvironment. These processes endow cancer cells with the plasticity required for survival in dynamic conditions, but also create key vulnerabilities, which can be therapeutically targeted through a number of avenues. Taken together, a deeper understanding of RAS biology will critically inform clinical care

and serves as a model for interrogation of other driver alterations in cancer.

AUTHOR CONTRIBUTIONS

RG and XW contributed to the conception, research, writing, editing, and design of figures for this manuscript.

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REVIEW
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New Horizons in *KRAS*-Mutant Lung Cancer: Dawn After Darkness

Haitang Yang[1], *Shun-Qing Liang*[1,2], *Ralph A. Schmid*[1] *and Ren-Wang Peng*[1]*

[1] Department of General Thoracic Surgery, Department of BioMedical Research, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland, [2] University of Massachusetts Medical School, Worcester, MA, United States

In non-small cell lung cancer (NSCLC), the most frequent oncogenic mutation in western countries is *KRAS*, for which, however, there remains no clinically approved targeted therapies. Recent progress on high biological heterogeneity including diverse *KRAS* point mutations, varying dependence on mutant *KRAS*, wide spectrum of other co-occurring genetic alterations, as well as distinct cellular status across the epithelial-to-mesenchymal transition (EMT), has not only deepened our understanding about the pathobiology of *KRAS*-mutant NSCLC but also brought about unprecedented new hopes for precision treatment of patients. In this review, we provide an update on the most recent advances in *KRAS*-mutant lung cancer, with a focus on mechanistic insights into tumor heterogeneity, the potential clinic implications and new therapies on horizons tailored for *KRAS*-mutant lung cancer.

Keywords: lung cancer, *KRAS*, mitogen-activated protein kinases, heterogeneity, targeted therapy, immunotherapy

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***Correspondence:**
Ren-Wang Peng
renwang.peng@insel.ch

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INTRODUCTION

Lung cancer is the most common cancer with high lethality (1). Carcinogenic Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation is the most common gain-of-function alteration, accounting for ∼30% of lung adenocarcinomas in western countries and about 10% of Asian lung adenocarcinomas (2).

As a membrane-bound small GTPase, KRAS switches between the active GTP-bound and inactive GDP-bound status, which is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (3). The intrinsic GTPase activity of RAS is rather low, but in the presence of GAPs, such as neurofibromin 1 (NF1), its hydrolytic activity can be increased by several orders of magnitude. Reactivation of GDP-bound RAS is mediated by GEFs, such as son of sevenless homolog 1 (SOS1), which promotes the release of bound GDP, and then cellular GTP will replace GDP to bind to RAS. Carcinogenic mutations impair the ability of KRAS to hydrolyze GTP and are thought to lock the oncoprotein in a constitutively active state by activating KRAS downstream signaling cascades, leading to uncontrolled cell proliferation and survival. In patients with lung cancer harboring *KRAS* mutations, the most mutations occur in codon 12, whereas mutations in codons 13 and 61 are less frequent (4).

In lung cancer, considerable progress in developing molecularly-driven therapeutics has been made in the past decades, mainly including targeted therapies against oncogenic drivers, such as *EGFR*, *HER2*, *EML4-ALK*, *MET*, *ROS1*, and *BRAF* mutations, and immunotherapies in non-oncogene-driven lung cancer, such as PD1 and PDL1 alterations (5, 6). However, for *KRAS*-mutant lung cancer, the treatment options are still limited, and chemotherapies remain the first-line recommendation.

In this review, we update the recent clinically relevant aspects of the pathobiology of *KRAS*-mutant non-small cell lung cancer (NSCLC), mainly focusing on tumor heterogeneity, therapeutic implications, and new treatment opportunities.

HETEROGENEITY IN *KRAS*-MUTANT LUNG CANCER

Diverse Point Mutations in *KRAS*

In lung cancer, *KRAS* mutations occur primarily in adenocarcinoma, whereas they are only rarely seen in squamous cell carcinoma (**Figure 1**). Diverse point mutations exist within *KRAS*, the majority of which affect codon 12 of the protein in NSCLC (**Figure 1**), leading to amino acid substitutions that impair the intrinsic hydrolytic activity and render the KRAS oncoprotein constitutively active.

In lung cancer, the presence of KRAS amino acid substitution influences patients' prognosis and is negatively associated with patient response to targeted therapy (7, 8) and chemotherapy (9–11). Molecular modeling studies showed that different conformations imposed by distinct *KRAS* oncogene substitutions could lead to altered association with downstream signaling transducers (12). Specifically, compared to wild-type *KRAS*, the mutant $KRAS^{G12C}$ or $KRAS^{G12V}$ is less dependent on AKT, which, however, is more intimately engaged by other mutant KRAS proteins.

Mutant KRAS with different amino acid substitutions may also associate with distinct biological behavior (13) and can lead to different clinical outcomes (14–16). In *KRAS*-mutant lung cancer, tumors carrying $KRAS^{G12C}$ exhibited higher ERK1/2 phosphorylation than those with $KRAS^{G12D}$ (17). In supporting this observation, studies with genetically engineered mouse model showed that $Kras^{G12C}$ tumors were significantly more sensitive to MEK inhibitor than the $Kras^{G12D}$ ones and that MEK inhibition significantly increased chemotherapeutic efficacy and progression-free survival (PFS) of $KRAS^{G12C}$ mice.

Taken together, different amino acid substitutions in oncogenic KRAS lead to heterogeneity in biological behaviors of the mutant protein, implying the need of genotype-specific analysis to identify clinically relevant subgroups of patients that may ultimately influence treatment decisions. It also should be taken into account for different downstream signaling pathways to be inhibited for patients with tumors carrying different KRAS amino acid substitutions.

KRAS Dependence Score and EMT

The concept of *KRAS* dependence or independence was proposed based on the observations that in both patients and cell lines, tumors frequently exhibit unexplained intrinsic resistance to *KRAS*-targeted therapy, by either inhibitors or genetic ablations. Mutant *KRAS* has been considered as an oncogenic driver. However, whether it is indispensable in each tumor carrying this oncogene is not clear. Early evidence suggested that not all *KRAS*-mutant tumor cells are dependent on KRAS (18, 19), and that some *KRAS*-mutant cancer cells, including lung (20) and pancreatic cancer cells (21, 22), can survive in the absence of the *KRAS* oncogene. These observations provide additional layers of evidence that make targeting *KRAS*-mutant tumors more complex.

Oncogenic KRAS can activate various downstream effector pathways, and the best characterized are phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) (23–25). Oncogenic *KRAS* signaling proceeded by different downstream effectors may lead to phenotypic variance in cancer, but to what extent the downstream effectors contribute to the oncogenic phenotype is not fully understood. Recently, Yuan et al. designed a combinatorial siRNA-based approach to functionally discern the link between KRAS downstream effectors and phenotypic variation in a large panel of cancer cell lines, and identified two major subtypes within *KRAS*-mutant cancers based on the dependence on KRAS or RSK (Ribosomal Protein S6 Kinase A1) (25). Interestingly, besides the distinct morphologies and effector landscapes, the two subtypes also differ in metabolic status with therapeutically tractable vulnerabilities. The heterogeneity in effector signaling pathways across *KRAS*-mutant cells presents a significant challenge to identify universal synthetic partners lethal to mutant *KRAS*.

It is well-documented that the epithelial-to-mesenchymal transition (EMT) process is closely related to therapy resistance. Interestingly, *KRAS*-mutant cancer cells dependent on or addicted to *KRAS* oncogene are more associated with an epithelial phenotype, whereas those independent of *KRAS* adopt a mesenchymal phenotype (18). Importantly, *KRAS*-mutant cancer cells differing in EMT status vary in their responses to MEK inhibitors (26), as EMT rewires the expression of receptor tyrosine kinases (RTKs), a consequence of differential feedback activation of the MAPK pathway following MEK inhibition. In epithelial-like cancer cells, ERBB3 is preferentially activated by feedback signaling, which reactivates MEK and AKT signaling. In mesenchymal-like *KRAS*-mutant cancer cells, reactivation of MEK and AKT was dominantly driven by FGFR1. Signaling transduced by FGFR is normally suppressed by the sprouty proteins (SPRY4), but MEK inhibition represses the negative regulation of SPRY4. In line with this, another independent study using short hairpin (sh) RNA screen had similar findings in *KRAS*-mutant lung and pancreatic cancer cells (27). These findings provide a strong therapeutic rationale to treat epithelial *KRAS*-mutant lung cancer (high epithelial markers) with clinically available ERBB and MEK inhibitors, and mesenchymal-like *KRAS*-mutant lung cancers (high FGFR1) by combined therapy with FGFR and MEK inhibitors.

The association of tumor response to MEK inhibitor therapy to EMT status of cancer cells was further investigated by a more recent study (28). Peng et al. identified an inverse correlation between MAPK signaling dependency and a zinc finger E-box binding homeobox 1 (ZEB1)–mediated EMT in patient samples harboring *KRAS*, *BRAF,* or *NRAS* mutations. Mechanistic results indicated that MAPK dependency is dictated by the functional interplay between scaffold protein interleukin-17 receptor D (IL17RD) and ZEB1. Mechanistically, in mesenchymal-like *KRAS*-mutant lung cancer cells, ZEB1 directly represses IL17RD to mediate the resistance to MEK inhibitors. Based on this, ZEB1 suppression by miR-200 expression or histone deacetylase

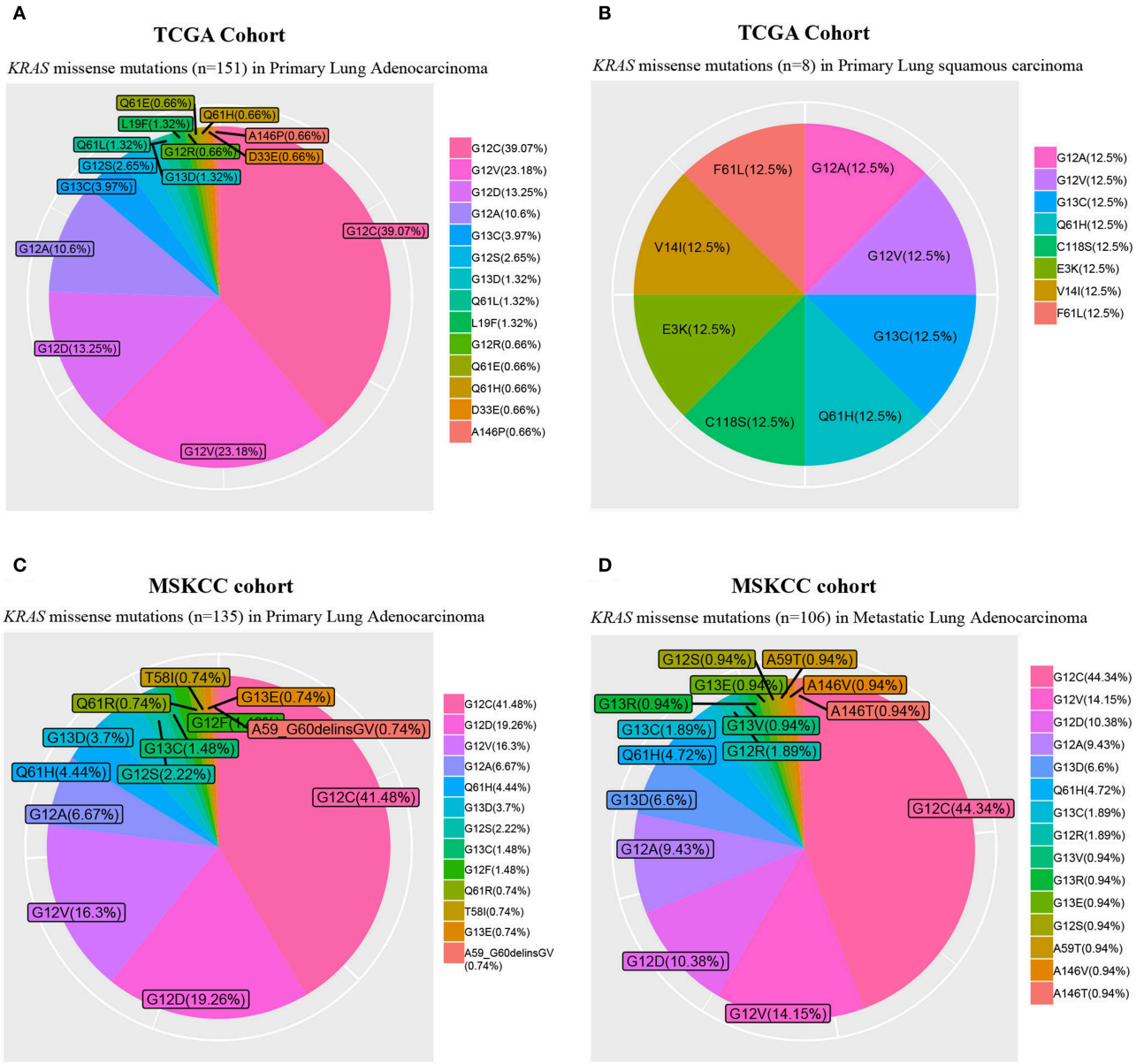

FIGURE 1 | Frequency of *KRAS* missense mutations. The Cancer Genome Atlas (TCGA) primary lung adenocarcinoma ($n = 489$; **A**) and squamous cell carcinoma ($n = 492$; **B**) cohorts, and Memorial Sloan Kettering Cancer Center (MSKCC) primary ($n = 471$; **C**) and metastatic lung adenocarcinoma ($n = 444$; **D**) cohorts.

inhibitor (mocetinostat) re-sensitized mesenchymal cells to MEK inhibition and markedly reduced *in vivo* tumor growth. This study provided the mechanistic support for combinatorial treatment (MEK plus histone deacetylase inhibitors) for *KRAS*-mutant lung cancer, and, again, highlighted the importance of stratification of epithelial and mesenchymal subsets in decision-making for treating *KRAS*-mutant lung cancer.

Genetic Alterations Co-occurring With KRAS Mutations

Heterogeneity in *KRAS*-mutant tumors also arises from co-occurring alterations of other genes, e.g., *TP53*, *CDKN2A/2B*, *STK11*, and *KEAP1* (**Figure 2**). Compelling evidence showed that co-occurring genomic changes could profoundly affect biological behaviors (29–31), clinical outcomes (32), and therapeutic vulnerabilities of *KRAS*-mutant cancers (33, 34).

An integrative study of genomics, transcriptomics, and proteomics in early-stage and chemo-refractory *KRAS*-mutant lung adenocarcinomas identified three major subsets defined by co-occurring genetic alterations in *STK11/LKB1* (KL subgroup), *TP53* (KP subgroup), and *CDKN2A/B* (KC subgroup) (29). The three subgroups differ in biological properties and therapeutic vulnerabilities, with KC tumors associated with suppressed mTORC1 signaling and KL tumors with lower expression of

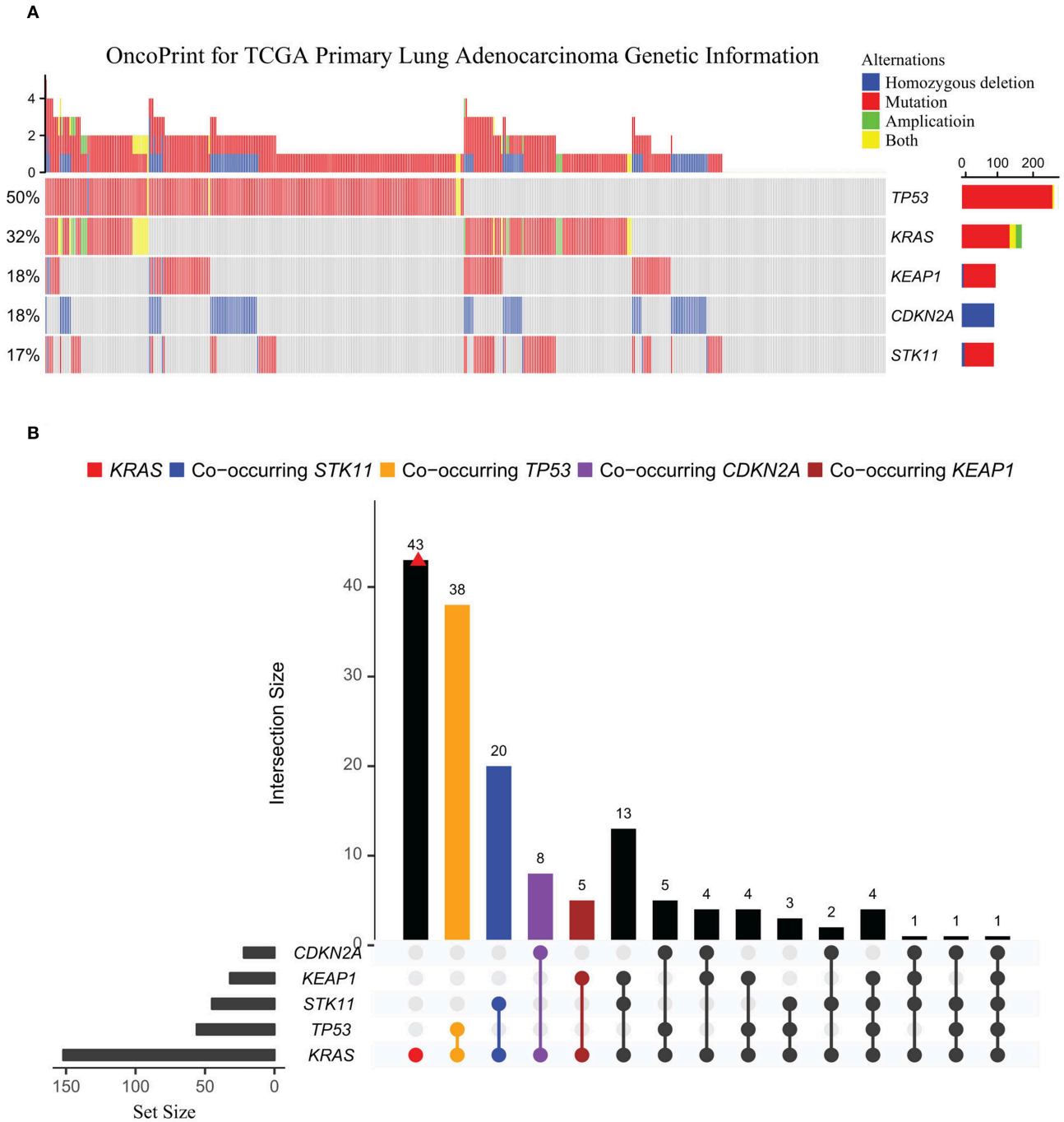

FIGURE 2 | Oncoprint for common genetic alterations co-occurring with *KRAS* in TCGA lung adenocarcinoma cohort. **(A)** The genetic landscape of *KRAS, TP53, KEAP1, CDKN2A,* and *STK11* in TCGA primary lung adenocarcinoma cohort ($n = 489$). **(B)** UpSetR plot visualizing the intersections of other mutations co-occurring with *KRAS*-mutant across TCGA lung adenocarcinomas. The intersection points are indicated by different colors: *CDKN2A* (purple), *KEAP1* (brown), *STK11* (blue), and *TP53* (orange). Intersections among the co-occurring genes were connected with a line, with frequency of each co-occurring mutation type shown on the bar plot. Set metadata is shown to the left (charts).

immune markers (e.g., PD-L1) if *KEAP1* co-mutated, while higher levels of somatic mutations, inflammatory and immune checkpoint markers, and prolonged relapse-free survival were observed in KP tumors. Further, KL cells exhibited heightened vulnerability to HSP90 inhibition. This work argued that genomic alterations co-occurring with mutant KRAS stratify lung adenocarcinomas and define pathobiological properties and therapeutic vulnerabilities.

A more recent study of a large patient cohort ($n = 330$) with advanced *KRAS*-mutant lung cancer identified co-mutated *KEAP1* as an independent prognostic marker for poorer survival [HR = 1.96; $P < 0.001$] and as being associated with less response to chemotherapy [HR = 1.64; $P = 0.03$] and immune therapy [HR = 3.54; $P = 0.003$] (30). Another study showed that presence of co-mutated *Trp53* reduces sensitivity to combined treatment with MEK inhibitor and chemotherapy in $Kras^{G12C}$-driven murine lung cancer, which supports further clinical investigations of the combination therapy for patients with lung cancer harboring $KRAS^{G12C}$ and wild-type p53 (17). Finally, yet importantly, *STK11/LKB1* alterations have been described as a major driver of primary resistance to PD-1 blockade in *KRAS*-mutant lung adenocarcinoma (31, 35).

Supporting this, a recent study (36) showed that among 377 non-squamous NSCLC patients treated with platinum-doublet chemotherapy (carboplatin or cisplatin and pemetrexed) plus pembrolizumab (anti-PD1), the therapy response was significantly associated with the genetic status of *STK11*. Specifically, patients with genomic alterations of *STK11* ($N = 102$) were associated with significantly shorter PFS (4.8 vs. 7.2 months, HR 1.5, 95% CI 1.1–2.0; $P = 0.0063$) and shorter overall survival (10.6 vs. 16.7 months, HR 1.58, 95% CI 1.09–2.27; $P = 0.0083$) compared with patients without *STK11* alteration ($N = 275$). Also, the objective response rate (RR) was significantly different between the two groups (32.6 vs. 44.7%, $P = 0.049$). More importantly, the addition of pembrolizumab to platinum-doublet chemotherapy did not significantly improve PFS (4.8 vs. 4.3 months, HR 1.13, 95% CI 0.83–1.54, $P = 0.75$) or overall survival (10.6 vs. 10.3 months, HR 1.03, 95% CI 0.71–1.49, $P = 0.79$) compared to the chemotherapy alone (36). This study defines a subgroup of patients with *STK11* alterations who do not benefit from immunotherapy, indicating the importance of cancer genetic information for stratification of patients who would benefit from immune checkpoint blockade. Apparently, co-occurring alterations further increase the heterogeneous complexity, which may explain inconsistent outcomes of clinical trials with *KRAS*-mutant lung cancers.

NEW HORIZONS FOR TREATING *KRAS*-MUTANT LUNG CANCER

Refocusing on Direct Targeting of KRAS

For decades, KRAS was considered undruggable due to its high affinity for GTP and the lack of a clear binding pocket. Enormous attempts and efforts had been made, but all failed to identify compounds that could effectively and directly target mutant RAS. Since then, there has been little advance. However, with new technologies in drug development and novel mechanistic insights into RAS biology, attention has been refocused on the approach that directly interferes with the function of RAS oncoproteins, with more effort given to find the way to target mutant alleles specifically.

Earlier studies have identified small molecules selectively recognizing and irreversibly inactivating one specific *KRAS*-mutant allele harboring a G12C amino acid substitution (37, 38). A breakthrough of direct RAS targeting was finally made by Ostrem et al., who, by using a novel screening technology called tethering, developed a new strategy to target mutant $KRAS^{G12C}$ specifically without affecting the wild-type protein (37). This work also suggested that the previous perception of mutant KRAS was persistently locked in its active GTP-bound state might not be true.

Later on, Lim et al. reported the synthesis of a GDP analog, SML-8-73-1, which contains an electrophilic chloroacetamide attached to the β-phosphate. This analog can covalently modify cysteine 12 of $KRas^{G12C}$ and, as a result, it competes with GTP and GDP for active site binding in a cellular context (38). Despite the pioneering development of the $KRAS^{G12C}$-specific inhibitors, follow-up studies indicated that these initial compounds showed only limited potency (39, 40). In a search for more effective compounds or analogs, ARS853 was developed (40), which selectively reduced KRAS-GTP levels by more than 90% and increased the *in vitro* hydrolytic reaction rate by 600-fold compared to the initial compound used in Ostrem et al. (37). At the micromolar range, ARS853 potently suppressed MAPK and PI3K-AKT signaling. Thus, $KRAS^{G12C}$ mutant protein is in a dynamically rather than a statically active state and targeting the inactive, GDP-bound form of KRAS is a realistic and promising anti-RAS therapeutic. These striking findings were recently translated into *in vivo* studies, in which a new covalent $KRAS^{G12C}$-specific inhibitor, ARS-1620, showed rapid and durable tumor regression in mice (41).

These studies prompt a revisit to target KRAS oncoproteins directly. Recent discoveries have enabled further development and investigation of more compounds of this family in clinical trials (**Table 1**). Encouraging phase I clinical trial data of AMG510 (Amgen, clinical trial information: NCT03600883) in 32 patients with $KRAS^{G12C}$ mutation (14 with NSCLC, 19 with colorectal cancer, and 2 with appendix cancer) were just released in ASCO 2019. Five of 10 evaluable patients with NSCLC had a partial response, and four had stable disease, in total achieving a disease control rate of 90% (9/10). Additionally, 13 of 18 evaluable patients with colorectal cancer experienced stable disease. Twenty-six patients were still under study, and nine discontinued. Importantly, the treatment was well-tolerated, with primarily grade 1 events (68%). Two grade

TABLE 1 | Ongoing clinical trials involving direct targeting of KRAS.

Compounds	Company	Mechanism	Clinical trial
AMG 510	Amgen/Carmot Therapeutics	$KRAS^{G12C}$ inhibitor	NCT03600883
MRTX849	Mirati (ex Array)	$KRAS^{G12C}$ inhibitor	NCT03785249
KRAS TCR	Gilead (ex Kite/NCI)	Anti-$KRAS^{G12D}$ engineered T-cell receptor	NCT03745326
AZD4785	AstraZeneca/Ionis	KRAS antisense oligonucleotide	NCT03101839

3 treatment-related AEs were reported (anemia and diarrhea). No grade 4 or more severe treatment-related adverse effects were reported. MRTX849 is another potent, highly selective, and orally available small-molecule inhibitor of $KRAS^{G12C}$ (42). MRTX849 shows broad-spectrum anti-tumor activity in a panel of patient- and cell-derived *in vivo* tumor models with KRAS G12C-substitution, with complete tumor regression observed in a subset of these models.

Different from the inhibitors that directly target mutant KRAS, AZD4785 is a *KRAS* antisense oligonucleotide that targets the *KRAS* gene irrespective of its mutation status (43). Despite AZD4785 being safe and well-tolerated, the first phase I trial failed, which might be due to the fact that AZD4785 targets both mutant and wild-type *KRAS* mRNA for degradation.

Tran and colleagues described a case of a patient with metastatic colorectal cancer treated with autologous T cells specific for mutant $KRAS^{G12D}$, which was restricted to the major histocompatibility complex class I allele HLA-C*08:02 (44). Despite the rarity of HLA-C*08:02, this study demonstrated the promise of T-cell–based immunotherapy for targeting $KRAS^{G12D}$ and HLA-C*08:02. Further evaluation in more patients is warranted.

Whether direct inhibition of KRAS with these new compounds is sufficient remains a question, given the presence of KRAS independence in tumor cells harboring *KRAS* mutations. Concurrent inhibition of collateral dependencies may be required to potentiate the effectiveness of those compounds.

Reinforcing MEK Inhibitors

To date, most efforts to treat cancers with *RAS* mutations have focused on targeting downstream effectors of mutant RAS, such as RAF, MEK, or PI3K, each of which is druggable. Although, as described above, different KRAS mutations show a preference for activating different downstream signaling, hyperactivation of the mitogen-activated protein kinase (MAPK) pathway is generally recognized as a key feature in *KRAS*-driven lung cancer cells. One reason is that the G12C substitution (44%), the most common subtype in *KRAS*-mutant lung cancer, shows more prominent engagement with MAPK signaling. Supporting these findings, we performed pooled drug sensitivity analysis based on publicly available dataset in Genomics of Drug Sensitivity in Cancer, which revealed that, compared with *KRAS*-wild-type lung cancer cells, *KRAS*-mutant lung cancer cells are exclusively more sensitive to various MEK inhibitors rather than those targeting other oncogenic pathways (**Figure 3**). This explains why MEK inhibitors have been the most widely investigated, typically as a combination therapy, despite the presence of multiple inhibitors that are being explored to target different KRAS-activated pathways.

Rethinking Combination Treatment With Chemotherapy

In the clinic, conventional chemotherapy is widely used to treat patients with *KRAS*-mutant NSCLC, although chemotherapy plus immune checkpoint blockade has been recently approved as the first-line regimen for NSCLC, including patients with *KRAS* mutations (45, 46). Clinical trials evaluating the efficacy of selumetinib, a potent MEK inhibitor, to potentiate chemotherapy, have recently been conducted.

A survival benefit of selumetinib plus docetaxel in comparison with docetaxel alone was demonstrated in a phase II clinical trial (47). Specifically, the primary endpoint of the study—median overall survival—was 9.4 months for the combination (selumetinib plus docetaxel) compared with 5.2 months for the control (placebo and docetaxel), although this difference was not statistically significant [hazard ratio (HR) for death 0·80, 80% CI 0·56–1·14; one-sided $p = 0·21$]. The median PFS was significantly improved in patients receiving selumetinib (5.3 vs. 2.1 months; HR for progression 0·58, 80% CI 0·42–0·79; one-sided $p = 0·014$) as was the response rate (37% vs. 0%; $p < 0·0001$). Subsequently, a subgroup analysis demonstrated that patients harboring G12V and G12C *KRAS* mutations appeared to experience higher RR and longer PFS for the combination arm, which was recently confirmed in preclinical mouse model (17).

Following this encouraging result, a further phase III clinical trial in *KRAS*-mutant lung cancer was conducted, which, however, failed to reproduce the significant benefit in patients treated with the combination compared with docetaxel alone (48). In this larger cohort trial, median PFS was 3.9 months with the combination group and 2.8 months with the control group (difference, 1.1 months; HR, 0.93 [95% CI, 0.77–1.12]; $P = 0.44$). Median overall survival was 8.7 and 7.9 months, respectively (difference, 0.9 months; HR, 1.05 [95% CI, 0.85–1.30]; $P = 0.64$). There is a marginally significant objective RR (20.1% in combination group vs. 13.7% in the control group; odds ratio, 1.61 [95% CI, 1.00–2.62]; $P = 0.05$). Whereas, the inconsistency with previous phase II trial results was unclear, a multitude of possible mechanisms, such as the aforementioned genomic (diverse point mutations and co-occurring alterations) and phenotypic (different EMT status) heterogeneity within the recruited patients, might be anticipated. In addition, chemotherapeutics used for combination treatment may need to be reconsidered in future studies, given that distinct amino acid substitutions of KRAS oncoproteins differed in their response to the commonly-used chemotherapy agents (9, 11). Nonetheless, these results did not rule out the effectiveness of this combination therapy in a subgroup of patients with *KRAS* mutations and deserved more detailed analysis, which might provide mechanistic information that facilitates patient stratification and prediction of potential responders.

Trametinib is another selective and potent MEK inhibitor that has been clinically approved for *BRAF* mutant cancers (mainly melanoma). Like selumetinib, the efficacy of trametinib, alone or in combination with docetaxel, has been evaluated in *KRAS*-mutant NSCLC. In a phase II trial, trametinib as a monotherapy showed RR and PFS similar to docetaxel in previously treated *KRAS*-mutant NSCLC (49). Another phase II study with *KRAS*-mutant NSCLC (n=54, including 19 with G12C, 9 with G12D, 9 with G12A) documented a trend toward worse PFS (HR = 1.86, $p = 0.06$) and survival (HR=1.80, $p = 0.14$) in G12C patients compared to non-G12C patients (50). Trametinib plus docetaxel had a RR of 33% and median

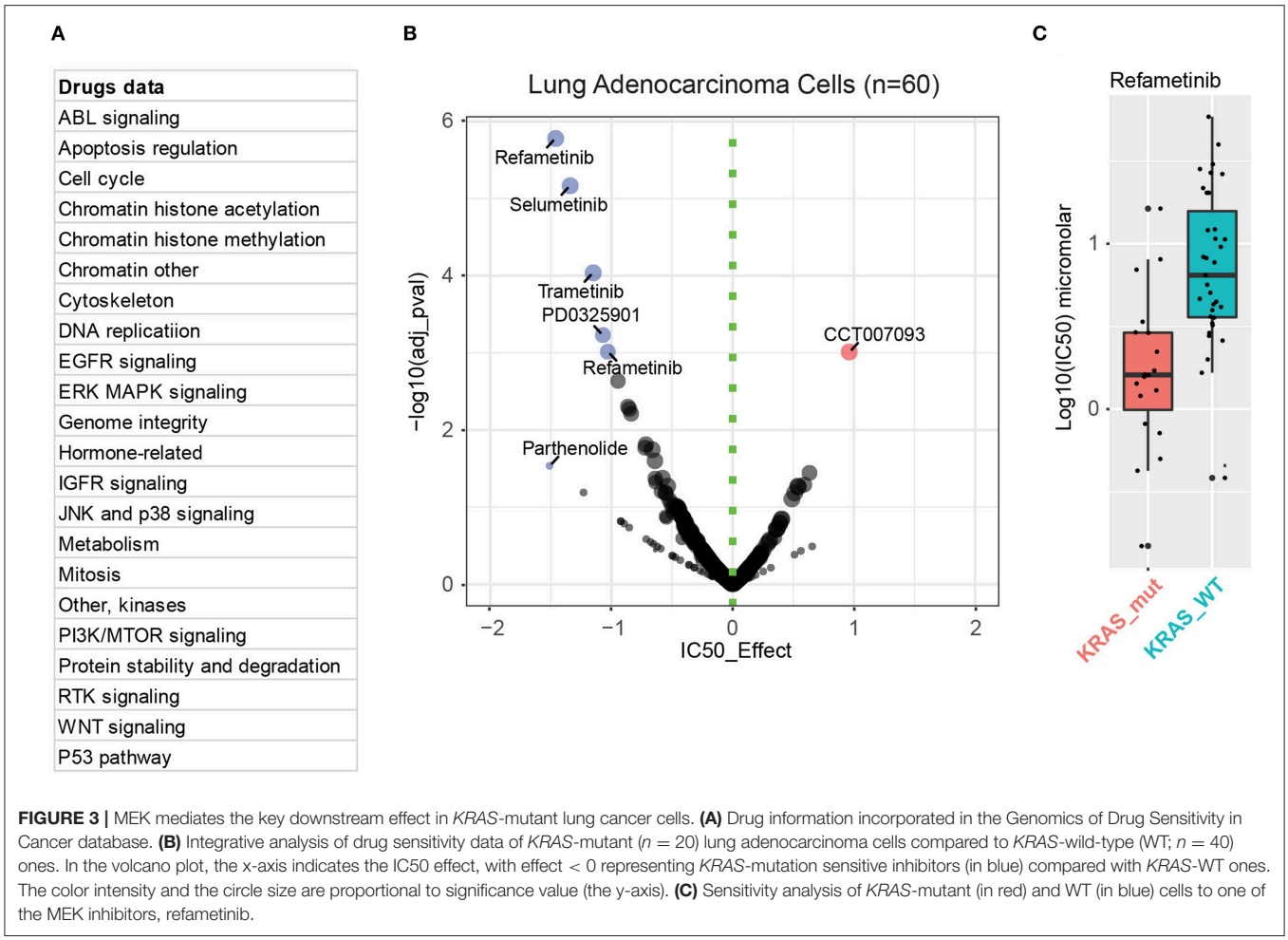

FIGURE 3 | MEK mediates the key downstream effect in *KRAS*-mutant lung cancer cells. **(A)** Drug information incorporated in the Genomics of Drug Sensitivity in Cancer database. **(B)** Integrative analysis of drug sensitivity data of *KRAS*-mutant ($n = 20$) lung adenocarcinoma cells compared to *KRAS*-wild-type (WT; $n = 40$) ones. In the volcano plot, the x-axis indicates the IC50 effect, with effect < 0 representing *KRAS*-mutation sensitive inhibitors (in blue) compared with *KRAS*-WT ones. The color intensity and the circle size are proportional to significance value (the y-axis). **(C)** Sensitivity analysis of *KRAS*-mutant (in red) and WT (in blue) cells to one of the MEK inhibitors, refametinib.

survival of 11.1 months in patients with recurrent *KRAS*-mutant NSCLC. These results suggest that clinical responses to combined trametinib and docetaxel may differ between G12C and non-G12C patients.

Rewiring SHP2 Activities

SHP2 is a non-receptor protein tyrosine phosphatase, encoded by the *PTPN11* gene that is ubiquitously expressed. SHP2 is involved in signal transduction downstream of multiple growth factors, cytokine, and integrin receptors and, not surprisingly, functions as an essential player in oncogenesis (51, 52). Upon the activation of RTKs, the SH2 domain of SHP2 binds to the phosphorylated tyrosine residues and various substrates, such as RTKs, scaffolds and adaptor proteins, which enables SHP2 in its active state for enzymatical removal of phosphates (dephosphorylation) from the substrates.

Previous studies demonstrated that the adaptive reactivation of MAPK signaling in the presence of a MEK inhibitor was mediated by the loss of MAPK-dependent negative feedback loops and the consequent induction of RTKs signaling (26, 53). Recent studies in *KRAS*-mutant lung cancer (54, 55) and pancreatic adenocarcinoma (55), *KRAS*-amplified gastric carcinoma (56), and multiple other cancer models expressing mutant or wild-type *KRAS* (57, 58) revealed that the anti-tumor effect of MEK inhibitor treatment could be dramatically potentiated by concurrent SHP2 inhibition.

Specifically, targeting of MEK alone is frequently hampered by adaptive resistance, which is complex and context-dependent, and can involve activation of various RTKs, including ERBB family, AXL, PDGFR-α, or FGFR1 (26, 53, 59–62). Strikingly, SHP2, the key integrator of RTK-RAS signaling, was necessary for various contexts upon MEK blockade and required to re-establish MAPK signaling. Strong synergy was observed when SHP2 and MEK were simultaneously targeted, resulting in sustained inhibition of tumor growth in different cancer models.

These studies provided compelling evidence supporting further investigations of combining SHP2 and MEK inhibitors for patients with *KRAS*-mutant cancer. Fitting this tendency, the recent development of potent allosteric SHP2 inhibitors strengthens the interest in targeting SHP2 in cancer (63). A clinical trial (NCT03114319) investigating TNO155, an SHP2 inhibitor, in patients with *K-/N-/H-RAS*, *BRAF*, or *PTPN11* mutant tumors is ongoing.

Resurging Autophagy Inhibition

Potential therapeutic interventions to inhibit autophagy have been extensively studied in cancer. Tumor cells depend on

macroautophagy to cope with oncogene-induced metabolic stress. Notably, in human cancer cell lines or tumors bearing *KRAS* mutations, high levels of basal autophagy were observed, making inhibition of autophagy therapeutically actionable in *KRAS*-driven tumors (64, 65).

Three simultaneously published studies signal a resurgence of interest to inhibit autophagy in *KRAS*-driven cancer (66–68). These studies indicated that upon the inhibition of the MAPK pathway, *KRAS*-mutant tumors depend on autophagy for survival and that, as a result, blocking this protective mechanism by concomitant inhibition of autophagy and MEK or ERK kinases is likely to be therapeutically beneficial in patients with *KRAS*-mutant pancreatic ductal adenocarcinoma, *NRAS*-mutant melanoma, and *BRAF*-mutant colorectal cancer (66, 67). More importantly, Kinsey et al. initiated off-label treatment for a patient with metastatic PDAC with trametinib and hydroxychloroquine, both of which have been clinically approved for other indications. They observed a striking disease response with a 50% reduction in tumor burden without toxicity (67).

Based on the intriguing findings, further clinical investigations are required to determine the benefits of combined MEK and autophagy for patients with activating mutations in the RAS–RAF–MEK–ERK pathway.

Rewiring KRAS Activation

In normal cases, KRAS is activated in response to signaling from upstream RTKs. However, oncogenic *KRAS* mutations "lock" the protein in a constitutively active state, activating KRAS-dependent signaling in a RTKs-independent pattern. In line with this, clinical trials confirmed that patients with *KRAS*-mutant cancers generally have a poor response to the first generation of EGFR inhibitors, such as erlotinib and gefitinib, and the presence of *KRAS* mutations is used as a biomarker to exclude patients for EGFR inhibitor therapy (7, 8, 69).

However, recent studies have challenged this paradigm, which instead demonstrated that the activation of ERBB signaling was required for $KRAS^{G12D}$-driven lung tumorigenesis in preclinical mice models and that pan-ERBB inhibition other than EGFR inhibition alone was strikingly effective to inhibit *KRAS*-mutant tumor growth and progression (61, 62).

In humans, the ERBB family contains HER1 (EGFR, ERBB1), HER2 (NEU, ERBB2), HER3 (ERBB3), and HER (ERBB4). Previous studies showed that ERBB3 activation was associated with resistance to MEK inhibition in *KRAS*-mutant NSCLC cells (26, 59). In a recent study by Kruspig et al. (61), the authors showed that multiple ERBB ligands (e.g., Areg, Ereg, Nrg3, Nrg4, and Hbegf) and receptors (for example, Erbb2 and Erbb3) were highly expressed in a $KRAS^{G12D}$ mouse model. Neratinib, a multi-ERBB inhibitor (70, 71), almost completely suppressed the emergence of tumors. In sharp contrast, erlotinib failed to reproduce the same effect. Further mechanistic analyses of seemingly contradictory results revealed that ERBB activity establishes a feed-forward loop to amplify signaling through the core RAS-ERK cascade to sustain survival and proliferation in *KRAS*-mutant NSCLC. Indeed, pan-ERBB inhibition enhanced the potency of MEK inhibition *in vitro* and *in vivo*.

Similarly, an independent study by Moll et al. demonstrated a requirement for ERBB signaling to support the progression of $KRAS^{G12D}$-driven lung cancer (62). In this study, an independent pan-ERBB inhibitor, afatinib, was used. Genetic mouse models revealed that EGFR deletion attenuates mutant KRAS activity and transiently reduces tumor growth. However, EGFR inhibition initiated a rapid resistance mechanism involving non-EGFR ERBB family members, which triggered a tumor escape mechanism. This provided an explanation, at least to some extent, for the poor unresponsiveness of *KRAS*-mutant lung cancer patients to the first-generation TKIs targeting EGFR alone. More importantly, afatinib blocked compensatory ERBB2 and ERBB3 activation, whereas erlotinib and gefitinib did not. Together, these studies suggested the therapeutic potential of pan-ERBB inhibitors in *KRAS*-driven tumors.

Notably, both studies revealed a requirement for simultaneous inhibition of multiple ERBB while targeting a single member of the family was not effective. These preclinical studies provide new insights into *KRAS*-driven tumorigenesis and bring new hope for *KRAS*-mutant lung cancer patients. Further trials, such as combined MEK with pan-ERBB inhibitors, are highly needed to determine the translational significance of the pan-ERBB inhibition strategy for patients, which can be easily and quickly conducted given that both inhibitors have been clinically approved (72, 73).

Re-examining Downstream Partnership: CRAF (RAF1) but Not A-RAF or B-RAF

KRAS oncogenes signal through a cascade of downstream effectors, among which the most important one is the RAF/MEK/ERK cascade. The direct RAS downstream effectors within the RAF/MEK/ERK pathway are the RAF kinases, including A-, B-, and C-RAF. However, it is not well-known how these individual RAF kinases contribute to *KRAS*-mutant tumor initiation and development.

Recent studies showed that C-RAF rather than B-RAF plays a crucial role in mediating KRAS oncogenic signaling (74, 75). Targeting of C-Raf rather than of B-Raf kinase could recapitulate the effect of *Kras* ablation and effectively inhibit tumor development without inducing significant toxicities in mouse models of *Kras*/*Trp53*-mutant lung adenocarcinoma. This work suggested that distinct RAF kinases likely play different roles in mediating KRAS oncogenic signaling.

In a more recent study, ablation of *B-* or *C-RAF* was concomitant with $Kras^{G12V}$ induction (76). C-Raf ablation completely prevented *Kras*-driven NSCLC without inducing deleterious effects, which, however, was not the case with B-Raf ablation, indicating that B-Raf is dispensable for Kras oncogenic signaling. Moreover, ablation of *C-Raf* did not affect Mek or Erk phosphorylation, suggesting that C-Raf-mediated Kras signaling is independent of the MAPK cascade. Further, the same group showed that combined inhibition of C-Raf and Egfr induced complete regression of pancreatic ductal adenocarcinomas in *Kras*/*Trp53*-driven GEM models and PDXs without apparent toxicity (77). Together, these studies provided compelling evidence that C-Raf, but not B-Raf or A-Raf, may

mediate the oncogenic signaling in *KRAS*-driven cancer. More importantly, the therapeutic effect observed by ablation of *C-Raf* was likely due to disrupting the interaction of the C-Raf protein with other partners, such as BCL2, ASK1, MST2, and ROKα (76), whereas not via modulating MAPK cascade that is also essential for normal homeostasis. This might explain, to some extent, why the elimination of *C-Raf* did not induce systemic toxicity, in contrast to MEK inhibitors.

Notably, the therapeutic effect achieved by *C-Raf* ablation could not be reproduced by three C-Raf inhibitors that are designed to block the kinase activity other than the protein expression, confirming that the non-kinase activity of C-RAF instead of the conventional MAPK cascade is critical for the ability of *KRAS*-dependent oncogenic transformation. The striking finding of these studies, which is at odds with the currently ongoing efforts to develop C-RAF kinase inhibitors, implies, instead, the need for strategies to block C-RAF kinase-independent activities or induce its degradation.

Revitalizing Chemotherapy

Currently, the platinum-based chemotherapy is still widely used for patients with *KRAS*-mutant lung cancer. However, the efficacy of chemotherapy is very limited, and durable response is generally short. Considerable efforts have been made to potentiate the efficacy of chemotherapy in *KRAS*-mutant cancer. Unfortunately, a recent phase III study has once again frustrated this attempt, which showed no additional survival benefit from combined MEK inhibitors compared to docetaxel alone (48).

Oncogenic KRAS signaling also involves PI3K-AKT-mTOR, via the interaction with the catalytic subunits of PI3K (78–80). Blocking RAS-mediated PI3K activation has also shown to inhibit the progression of *KRAS*-driven tumors. However, high toxicities of targeting PI3K, AKT or mTOR, in combination with MEK inhibitors, have prevented their approval for use in human patients (81–83).

We recently found that activation of mTOR signaling mediates a key resistance mechanism to chemotherapy in *KRAS*-mutant lung cancer (84). We observed exclusively hyperactivated mTOR signaling in lung cancer patient samples with *KRAS*-mutations but not in those carrying wild-type *KRAS*. Combined clinically approved mTOR inhibitor and chemotherapy showed a strong synergism in inhibiting proliferation of cancer cells harboring *KRAS*-mutation specifically. Additionally, the efficacy of this combination treatment correlates with the magnitude of mTOR activity induced by chemotherapy alone. Our results pinpoint a rational and readily translatable strategy that combines mTOR inhibitors with standard chemotherapy to treat *KRAS*-mutant lung cancer.

A recently published study provided novel hints to potentiate platinum-based chemotherapy in multiple cancer types (85). Jin et al. used multi-step kinome screens and identified MAST1, an AGC serine/threonine protein, as a key mediator of cisplatin resistance. The mechanistic study showed that MAST1 expression was increased in resistant cells and functioned as a MAP3K (MAPK kinase kinase), thereby activating MEK1 in cisplatin-resistant cells. Knockdown of *MAST1* re-sensitized the resistant cells to cisplatin *in vitro* and *in vivo*. Further investigations showed that cisplatin directly binds to cysteine 142 site of MEK1, restricting its access to C-RAF that typically phosphorylates MEK1. In this case, MAST1 took over C-RAF to re-activate MAPK cascade in cisplatin-resistant cells, and inhibition of MAST1 led to decreased MEK1 phosphorylation, explaining the effectiveness of targeting MAST1 in overcoming cisplatin resistance. More interestingly, MAST1 expression was shown to be specific to cisplatin rather than other similar agents (for instance, 5-fluorouracil) that interfere with DNA replication. This finding may be particularly relevant in the oncogenic *RAS/BRAF* setting, which mainly activates downstream MAPK signaling.

In a recent study (86), we reported that pemetrexed-resistant *KRAS*-mutant lung cancer cells assume a mesenchymal phenotype and cross-resist MEK inhibitors. Mechanistically, acquisition of resistance enables *KRAS*-mutant lung cancer cells to bypass canonical KRAS effectors but entail hyperactive AXL/eIF4E, increased protein turnover in the endoplasmic reticulum (ER), and adaptive activation of an ER stress-relief unfolded protein response survival pathway whose integrity is maintained by HSP90. In line with these mechanistic findings, HSP90 inhibitors synergistically enhance antitumor effects of pemetrexed and MEK inhibitors in multiple *in vitro* and *in vivo* models, validating a rational combination strategy to treat *KRAS*-mutant lung cancer.

Reactivating Anti-tumor Immunity

Immune surveillance is generally dormant in cancer via dysregulation of immune checkpoints, such as the upregulation of the immunosuppressive protein PD-L1 for the evasion of the host immune system. Considerably convincing evidence has shown the importance of immune checkpoint blockade for treating various cancers (87). However, the therapeutic efficacy varies individually due to the high heterogeneity of tumors and the lack of reliable biomarkers to stratify the patients. Currently, limited biomarkers, such as PD-L1 expression (88) and tumor mutation load (TMB) (89–91), are clinically used to predict the immunotherapy benefit.

Interestingly, several preclinical studies suggested that tumors harboring *KRAS* mutations might be associated with a vulnerability to immunotherapies, in particular those with concomitant *TP53* mutations (29, 92, 93). Mechanistic studies indicated that oncogenic KRAS could stabilize PD-L1 mRNA through post-transcriptional modifications of the AU-rich element binding protein tristetraprolin (TTP) (94). Specifically, KRAS-MEK signaling contributes to phosphorylation and inhibition of TTP through the kinase MK2. In the same study, a high correlation between MAPK activation and elevated PD-L1 expression was observed in *KRAS*-mutant human lung and colorectal tumors.

A landmark trial (KEYNOTE-024) demonstrated that pembrolizumab was superior to chemotherapy in advanced NSCLC (potentially including patients with *KRAS*-mutations), among which more than 50% had high PD-L1 expression (95). Follow-up studies of the KEYNOTE-024 cohort revealed continuous survival benefit in patients treated with

pembrolizumab as first-line monotherapy compared to those treated with chemotherapy (96).

Importantly, two remarkable phase III trials (KEYNOTE-189 and KEYNOTE-407) demonstrated that addition of pembrolizumab (Keytruda, anti-PD1) could significantly prolong the survival of NSCLC patients (45, 97). The promising results from these studies have led to the approval of pembrolizumab in combination with chemotherapy (carboplatin-pemetrexed) for metastatic, non-squamous NSCLC, and the approval of pembrolizumab in combination with chemotherapy (carboplatin and Taxol) for patients with squamous lung carcinoma, excluding those carrying *EGFR* or *ALK* mutations. Although patients with *KRAS*-mutant NSCLC were potentially included in the two studies, specific efficacy of the combination therapy on *KRAS*-mutant NSCLC remains to be investigated.

A recent systematic review and meta-analysis study, which integrated multiple randomized clinical trials, showed that immune checkpoint inhibitors significantly prolonged overall survival in the *KRAS*-mutant subgroup (HR, 0.65; 95% CI, 0.44–0.97; $P = 0.03$) but not in the *KRAS* wild-type one (HR, 0.86; 95% CI, 0.67–1.11; $P = 0.24$; interaction, $P = 0.24$) (98). Another meta-analysis study that incorporated 509 patients (138 of *KRAS*-mutant and 371 with *KRAS*-wild-type NSCLC) showed that, compared to docetaxel chemotherapy, immune checkpoint inhibitors improved overall survival in patients with previously-treated *KRAS*-mutant NSCLC (HR = 0.64 [95% confidence interval, 0.43–0.96], $P = 0.03$) (99), but not in patients with wild-type *KRAS* (HR = 0.88 [95% confidence interval, 0.68–1.13], $P = 0.30$). These results indicate that *KRAS* mutational status is a potential biomarker for survival benefits to immune checkpoint inhibitors. However, two other studies with advanced non-squamous NSCLC reported that the efficacy of immune checkpoint inhibitors is independent of *KRAS*-mutant status (100, 101). Thus, further studies with a stratification of *KRAS* genetic status are still needed.

Although, preclinical studies using immune-competent mouse models verified the promising efficacy of checkpoint blockade in the *Kras*-mutant setting (92, 102, 103), a majority of these studies relied on mouse models with a single genetic background, which limits the power to assess the potential influence of other co-occurring mutations (e.g., *STK11* alterations). Co-occurring genetic mutations, which can lead to differential downstream effectors engaged by mutant *KRAS*, have been reported to significantly affect the tumor immune signatures (25, 29, 31, 35) and responses to immunotherapy (30). This might explain why a pooled analysis of patient response data does not consistently support the association between benefits of immunotherapy and *KRAS* mutations (98–101).

CONCLUSIONS

Targeting KRAS has represented a tremendous unmet clinical need. Nevertheless, the challenge of clinical treatment of *KRAS*-mutant cancers seems not to be insurmountable. Now, a new wave of attempts is motivated to target KRAS directly, which has long been considered undruggable. A striking response has been achieved with AMG510 in patients with $KRAS^{G12C}$. New treatment strategies based on a deeper understanding of the pathobiology of oncogenic *KRAS,* such as abolishing C-RAF, blocking the universal rewiring of SHP2, and the protective autophagy in response to MEK inhibitors are highly promising with preliminary success in human patients. Conventional approaches, such as combined chemotherapy and mTOR inhibitors, as well as combined cisplatin and MAST1 inhibitors, are also encouraging but require further investigations in patients. The great success of immunotherapy has been witnessed in the treatment of patients with various tumors, but more evidence is required in cancer patients with *KRAS* mutations.

Given the presence of a variety of potent and specific chemicals, treating *KRAS*-mutant lung cancer remains a significant challenge, implying that the problem might be mechanism-related rather than the efficacy of targeting itself. A critical point is a high heterogeneity within *KRAS*-mutant tumors. To maximize the patient benefit, it cannot be more important than molecularly guided stratification on top of *KRAS* mutations.

AUTHOR CONTRIBUTIONS

HY and S-QL wrote the manuscript. RS reviewed the manuscript. R-WP outlined and revised the manuscript.

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The Importance of microRNAs in RAS Oncogenic Activation in Human Cancer

Roberta Roncarati[1,2], *Laura Lupini*[1], *Ram C. Shankaraiah*[1] *and Massimo Negrini*[1]*

[1] *Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy,* [2] *CNR, Institute of Genetics and Biomedical Research, National Research Council of Italy, Milan, Italy*

microRNAs (miRNAs) regulate gene expression by modulating the translation of protein-coding RNAs. Their aberrant expression is involved in various human diseases, including cancer. Here, we summarize the experimental pieces of evidence that proved how dysregulated miRNA expression can lead to RAS (HRAS, KRAS, or NRAS) activation irrespective of their oncogenic mutations. These findings revealed relevant pathogenic mechanisms as well as mechanisms of resistance to target therapies. Based on this knowledge, potential approaches for the control of RAS oncogenic activation can be envisioned.

Keywords: microRNA, RAS, cancer, MAPK, target therapies

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***Correspondence:**
Massimo Negrini
massimo.negrini@unife.it

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INTRODUCTION

microRNAs (miRNAs) are small (19–24 nucleotides) non-coding RNAs discovered in 1993 in studies related to embryonic development of C. *elegans* (1, 2). Their importance significantly increased following the discovery of their existence in all eukaryotic organisms (3). Currently, 2,654 mature miRNAs, originating from 1917 precursors, are described in humans (http://www.mirbase.org/) (4, 5). Their main function is to negatively regulate gene expression at the post-transcriptional level through the interaction of their "seed" portion by sequence homology typically with the 3′ non-coding regions of messenger RNAs (mRNAs). Through this interaction, miRNAs limit translation, or promote degradation of target mRNAs (6, 7).

The modulation of target mRNAs by miRNAs is complex, considering that each mRNA is generally targeted by multiple miRNAs, and the strength of this interaction is variable (8). Classically, it has been thought that each miRNA can interact with hundreds of target mRNAs. However, recent reports have highlighted RNA transcripts inducing degradation of respective interacting miRNAs through a mechanism known as "target-directed miRNA degradation" (TDMD) (9, 10). Added to the complexity of these direct interactions is the fact that some long non-coding RNA (lncRNA) could function as "sponges," that act as a buffer and prevent the action of miRNAs on target protein-coding mRNAs (11, 12). Lastly, it is also important to consider that cell co-localization of each miRNA with the target mRNAs is necessary and depends on the eventual tissue-specific expression of each of the interacting RNAs.

Thus, miRNAs, taken together, represent an essential phase in the regulation of gene expression by modulating the translation of the entire transcriptome (13, 14). Given their biological importance, their deregulation plays a significant role in pathogenic mechanisms, including the neoplastic transformation (15, 16). The first evidence associating miRNAs with human malignant diseases was the discovery of miR-15 and

miR-16 in the minimal region of deletion at chromosome 13q14 in chronic lymphatic leukemia (17). Since this seminal study, a myriad of other studies has confirmed the role of miRNAs in tumorigenesis and other human diseases as well.

miRNAs AS DIRECT REGULATORS OF RAS

The first functional evidence to establish a molecular link between the deregulation of miRNAs with an explicit oncogenic pathway was published in 2005 when Slack and collaborators reported the importance of the downregulation of members of the *let-7* miRNA family with the activation of oncogenes of the RAS family (18). The study demonstrated that the 3′ UTRs of KRAS, NRAS and HRAS mRNAs comprised multiple complementary *let-7a* binding sites. The enforced expression of let-7 could indeed reduce RAS protein levels (18). Conversely, let-7 downregulation could lead to the loss of its post-transcriptional control, causing RAS over-expression and activation. This study was decisive in proving that aberrant expression of miRNAs could play an important role in tumor initiation and progression, and paved the way for studies that extended miRNA involvement to all phases of neoplastic initiation and progression (19).

The involvement of RAS (KRAS, NRAS, HRAS) in human tumors is mainly associated with the presence of activating mutations at codons 12, 13 and 61, able to activate various molecular pathways, which play a key role in a large number of tumor traits, spanning from cell proliferation, cell survival, cytoskeleton organization, motility, and more (20). The demonstration of the role of miRNAs in the abnormal regulation of RAS thus represented another important mechanism involved in key steps of tumorigenesis.

Since then, quite a few other reports have demonstrated the modulation of RAS by miRNAs. In many cases, the interaction was only predicted by computer algorithms, but several studies have experimentally validated these interactions. **Table 1** lists the microRNAs for which the ability to modulate the expression of KRAS, NRAS, or HRAS has been experimentally confirmed.

As mentioned, let-7 was the first, and probably the most important miRNA implicated in the regulation of genes of the RAS family (18). In the human genome, 12 loci are known to encode for members of the let-7 family: let-7a-1, -2, -3; let-7b; let-7c; let-7d; let-7e; let-7f-1, -2; let-7g; let-7i; miR-98. While it is described that members of the let-7 family are up-regulated in the course of cell differentiation, numerous studies have reported the reduction of let-7 expression in different tumor types (21, 22). Already in 2004, Takamizawa et al. demonstrated the downregulation of let-7 in non-small cell lung carcinoma (NSCLC) (23, 24) and documented its prognostic significance. Furthermore, in line with these observations, they proved that enforced expression of let-7 miRNA could inhibit *in vitro* cell growth of the lung adenocarcinoma A549 cells (23, 25–27). These studies were further confirmed in murine *in vivo* models of NSCLC (28, 29) and revealed that let-7 mimics could represent potential therapeutic molecules.

TABLE 1 | Human microRNAs targeting RAS family members.

miRNA	HRAS	KRAS	NRAS
hsa-let-7a-5p	1	1	1
hsa-let-7b-5p	1	1	1
hsa-let-7c-5p		1	1
hsa-let-7g-5p		1	
hsa-miR-1-3p		1	
hsa-miR-16-5p		1	
hsa-miR-18a-3p		1	
hsa-miR-20a-5p			1
hsa-miR-26a-5p			1
hsa-miR-27a-3p		1	1
hsa-miR-96-5p		1	
hsa-miR-98-3p			1
hsa-miR-98-5p			1
hsa-miR-124-3p			
hsa-miR-126-3p		1	
hsa-miR-134-5p		1	
hsa-miR-139-5p	1		
hsa-miR-143-3p	1	1	
hsa-miR-145-5p			1
hsa-miR-148b-3p			1
hsa-miR-152-3p		1	
hsa-miR-155-5p		1	
hsa-miR-181a-5p	1	1	1
hsa-miR-181c-5p		1	
hsa-miR-181d-5p	1		
hsa-miR-193a-3p		1	
hsa-miR-193b-3p		1	
hsa-miR-199a-5p		1	
hsa-miR-200c-3p		1	
hsa-miR-206		1	
hsa-miR-214-3p			1
hsa-miR-216b-5p		1	
hsa-miR-217		1	
hsa-miR-224-5p		1	
hsa-miR-340-5p		1	
hsa-miR-365a-3p		1	
hsa-miR-384		1	
hsa-miR-433-3p		1	
hsa-miR-452-5p		1	
hsa-miR-487b-3p		1	
hsa-miR-543		1	1
hsa-miR-613		1	
hsa-miR-622		1	
hsa-miR-663a	1		
hsa-miR-4689		1	

Data from miRTarBase (http://mirtarbase.mbc.nctu.edu.tw).

Given the proven interaction of let-7 with members of the RAS family, it is plausible that the observed effects were due to the modulation of RAS. However, let-7 can also regulate additional important oncogenes such as c-MYC, high-mobility group A

(HMGA), Janus protein tyrosine kinase (JAK), signal transducer and activator of transcription 3 (STAT3) (30). Its action as a tumor suppressor gene is therefore achieved through the ability to interact with multiple oncogenes and inhibit the activation of their molecular pathways (18, 28).

Essentially all types of human cancer present a general down-regulation of let-7 (21). Among others, the modulation of RAS by let-7 was demonstrated in colorectal cancer (CRC) where let-7 is strongly down-regulated in tumor tissues compared to adjacent healthy tissues. Similar to the study on NSCLC cells, let-7 was also shown to act as a growth suppressor in human CRC cells (31).

Confirming the importance of RAS regulation by let-7, the discovery of the LCS6 polymorphism (Let-7 Complementary Sites 6, rs61764370) in the KRAS 3′ UTR region further demonstrated let-7 expression altering interaction. This polymorphism has been associated with a greater risk of developing tumors and worse prognosis in lung, oral, and colorectal cancer (32–34).

An understanding of a mechanism leading to let-7 down-regulation in cancer came from studies on LIN28 in mammals. Lin28 and Lin28b act as RNA binding proteins that are able to associate with the terminal loop of the precursors of let-7 family miRNAs and block their processing into mature miRNAs (35–38). Since LIN28 is over-expressed in human cancer, this mechanism causes let-7 down-regulation, which establishes a connection with RAS and other cancer-associated signalings.

Let-7 is not the only miRNA involved in the regulation of RAS (HRAS, KRAS, or NRAS) (39). Among the miRNAs involved in the regulation of members of the RAS family, miR-143 and miR-145, co-expressed in the same primary transcript, can target both KRAS and NRAS, and have been found to be down-regulated in numerous human tumors (40–42). Already in 2003 Michael et al. documented a significant reduction of miR-145 in CRC compared to normal mucosa (43) and in 2014, Pagliuca et al. confirmed that the miR-143/miR-145 cluster, highly expressed in normal colon, was significantly decreased in CRC (44). Their reduced expression has been correlated with p53 mutations capable of reducing the maturation process of these miRNAs (45).

Very similar to let-7, members of the miR-181 family were shown to target all the RAS family members (HRAS, KRAS, and NRAS). They were found downregulated in different types of cancer, such as oral squamous cell carcinoma (46, 47), gastric cancer (48), and gliomas (49). These findings suggest that miR-181 down-regulation is one of the mechanisms leading to oncogenic RAS activation in these tumors.

It is notable that in spite of KRAS activation by gene mutation in 90% of the cases in pancreatic cancer, various miRNAs capable of directly targeting KRAS are simultaneously downregulated. Specifically, miR-96, miR-126, and miR-217 (50–53). Since the reduced expression of these miRNAs correlates with higher KRAS expression, these alterations likely represent a mechanism for strengthening the already activated RAS signaling.

Another noteworthy miRNA capable of targeting KRAS is miR-134. It was found downregulated in glioblastoma and renal cell carcinoma (54, 55). miR-134 downregulation correlated with the activation of the MAPK pathway and its enforced expression in renal cancer cells could inhibit *in vitro* migration and invasive traits.

Oncogenic mutations resulting in RAS activation are prevalent in most human tumors, but there are exceptions. RAS mutations in HCC are rare events but paradoxical wild-type RAS activation is common (56). Dietrich et al. (57) discovered that wild-type KRAS expression was increased in HCC compared to non-tumor liver and revealed an inverse correlation with miR-622 expression.

In addition to the above-mentioned examples, several other miRNAs were proven to target and inhibit the expression of RAS oncoproteins (**Table 1**). These miRNAs are generally downregulated in tumors, thus concurring with reciprocal overexpression and activation of RAS, irrespective of activating gene mutations.

miRNAs AS RAS EFFECTORS

The interplay between miRNAs and RAS is not only represented by miRNAs acting as negative modulators of RAS but also includes downstream miRNA effectors. The most significant is undoubtedly miR-21, which is up-regulated by KRAS oncogenic mutants in non-small-cell lung cancer (58), laryngeal squamous cell carcinoma (59), and pancreatic adenocarcinoma (60) as well as many other human cancers. miR-21 is a known oncomiR capable of blocking the expression of tumor suppressor genes antagonists of the PI3K-AKT pathway, such as PTEN, or of the RAS-MAPK pathway, such as PDCD4 or RASA1 (61–63) (**Figure 1**).

miRNAs AS REGULATORS OF RECEPTOR TYROSINE KINASES (RTKS)

RAS is a crucial node that connects receptor tyrosine kinases (RTKs) with downstream molecular pathways (**Figure 1**). Hence, miRNAs can affect RAS activity by acting on RTKs as well as MAPK, PI3K, or other pathways.

It is a well-known notion that RAS activation is physiologically triggered by RTKs, a category of transmembrane receptors that become activated in response to growth factors. Several miRNAs are known to target RTK mRNAs and their dysregulation can lead to inappropriate activation of the targeted RTK. Just to mention a few examples, miR-7, miR-539 and miR-103-3p can target and modulate the expression of the epidermal growth factor receptor (EGFR) (64–66); miR-26a was shown to target c-MET, the hepatocyte growth factor receptor (67); miR-199-3p can target the vascular endothelial growth factor receptors 1 and 2 and the VEGFA ligand (68); miR-7 and miR-98 can target the insulin growth factor receptor gene (64, 69).

All the above-mentioned miRNAs were found dysregulated in a variety of human cancers. miR-539 is downregulated in breast cancer (BC) tissues and cell lines. miR-539 enforced expression could inhibit BC cells proliferation and tumor growth *in vitro* and *in vivo* (65). miR-7 is downregulated in breast and colorectal cancer (CRC) cells (64, 66) and its reduced expression in BC patients correlated with higher stage, grade,

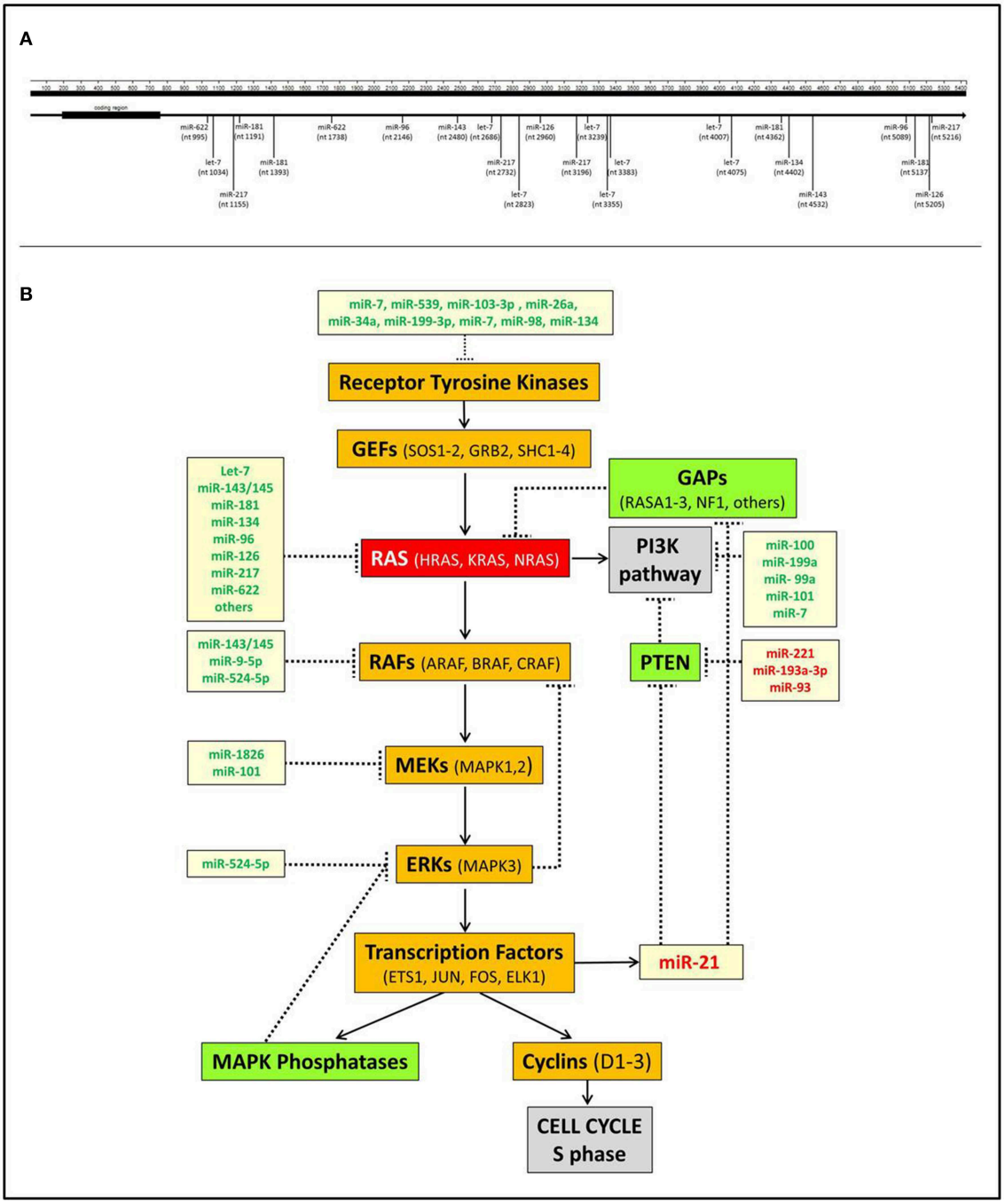

FIGURE 1 | Interactions of miRNAs with RAS. **(A)** Scheme of the direct interactions of miRNAs with the 3′UTR of KRAS. Data were derived from TargetScan v7.2 (http://www.targetscan.org) and from Johnson et al. (18), Chen et al. (40), Jiao et al. (53), Liu et al. (55), and Dietrich et al. (57). **(B)** A simplified scheme of the interplay between miRNAs and RAS pathways. It shows that several miRNAs negatively regulate the MAPK and PI3K RAS-linked pathways at different points. Conversely, miR-21, which is transcriptionally induced by the transcription factor ELK1, inhibits the MAPK and PI3K suppressors GAPs and PTEN, thereby further promoting RAS activation. miRNAs indicated in green are downregulated in tumors, miRNAs indicated in red are upregulated.

and poor prognosis (64). The tumor suppressor activity of miR-103-3p was confirmed by the anti-proliferative effects after its enforced expression in lung cancer cell lines; furthermore, the downregulation of miR-103a-3p in NSCLC was associated with poor prognosis (66). miR-26a reduced levels were associated with poor prognosis in Hepatocellular carcinoma (HCC) (67). MiR-26a can also control the expression of VEGFA in HCC cells and impairs VEGFR2-signaling thereby controlling angiogenesis. miR-199-3p, another miRNA that can target VEGFR1, VEGFR2, and the ligand VEGFA (68), is frequently down-regulated in HCC

and it has been shown to have *in vitro* and *in vivo* anti-tumor activity in HCC models (68, 70). MiR-98 is down-regulated in retinoblastoma, where it also represents a prognostic marker (69).

The above-reported miRNAs are just a few examples to show how their deregulation can lead to RTKs overexpression and consequently activation of RAS and its downstream pathways. The latter are themselves regulated by miRNAs, whose deregulation may directly cause the activation of RAS downstream effectors independently from RAS triggering.

miRNAs AS REGULATORS OF MAPK PATHWAY

The MAPK pathway is a well-studied pathway that promotes cell proliferation and is controlled by RAS activation. It includes several effectors with oncogenic function, widely studied in different types of tumors and whose mutations also represent tumorigenic mechanisms.

BRAF is probably the most studied element of the MAPK pathway. BRAF activation has been associated with a missense mutation V600E, commonly found in melanoma and thyroid cancer, but also present at low frequency in several other types of human cancer (71). As expected, various miRNAs can target and regulate BRAF expression. KRAS targeting miR-143 and miR-145, that we have mentioned above, can also target BRAF, indicating a very important role of these miRNAs in regulating the MAPK pathway at several levels (44). As mentioned earlier, these miRNAs are frequently downregulated in various types of cancer. miR-9-5p is another miRNA targeting BRAF, which was shown to be down-regulated in papillary thyroid carcinoma (65).

Further downstream of MAPK pathway cascade, MEK1/MEK2 (also called (MAP2K1 and MAP2K2) as well as ERK1/ERK2, are also targets of miRNAs. miR-1826 can target MEK1. It is down-regulated in bladder cancer and its reduced expression is associated with more severe pathological traits (pT and grade) (72). miR-101 can also target MEK1. This miRNA exhibits reduced expression in diffuse large B cell lymphoma (DLBCL) and it is associated with a worse prognosis (73). miR-665 has been also shown to indirectly activate MEK in BC cells by targeting the nuclear receptor subfamily 4 group A member 3 (NR4A3) gene. This miRNA is upregulated in breast cancer where its upregulation is associated with metastasis and poor survival (74).

miRNAs THAT ACT ON MULTIPLE TARGETS OF THE RAS PATHWAY

Among the several miRNAs that regulate elements of the RAS-centered pathways, some miRNAs target multiple genes belonging to the pathway thus reinforcing their role in modulating MAPK pathway activation.

In this respect, miR-134 is a typical example, as its target genes not only include KRAS (75), but also EGFR (76), HER2 (77), STAT5B (54), and PIK3CA (78), which are upstream and downstream elements of the RAS-centered pathways. This miRNA is downregulated in numerous types of human cancers, where it affects cell proliferation, survival, invasiveness, metastasis, and apoptosis [reviewed in (79)]. This miRNA exemplifies the deregulatory action of single miRNA and consequent wide effects on tumorigenic signals by acting on multiple elements of the RAS pathways (79). Other miRNAs targeting multiple RAS effectors include miR-143 / miR-145, previously mentioned to target all RAS genes and BRAF; miR-524-5p that can target both BRAF and ERK2. In melanoma, miR-524-5p is downregulated and affects cell migration and proliferation both *in vitro* and *in vivo* (80).

These miRNAs are potentially very important, as they can represent useful molecules to effectively restore the normal expression of multiple proteins belonging to RAS pathways.

microRNAs IMPLICATED IN RESISTANCE TO TARGET THERAPIES

Therapeutic interventions in advanced cancers include traditional chemotherapy as well as targeted/immuno-therapies. Targeted therapies make use of molecules capable of blocking aberrantly activated oncogenes that act as tumorigenic drivers. Oncogenic RAS proteins would represent outstanding targets for such therapies. But, no drug targeting RAS has been yet validated for clinical use. At present, most available targeted therapies are instead designed to block the activity of several elements of RAS-centered pathways. These include a large number of tyrosine kinase inhibitors (TKIs) or antibodies against RTKs; drugs that target BRAF V600E mutation (vemurafenib and dabrafenib), MEK (trametinib, cobimetinib and binimetinib), PI3K mutations (alpelisib), and mTOR (everolimus). The RAS pathways are therefore targeted by several drugs, with the RAS itself being a major exception.

Even more disappointing is the fact that mutant activated RAS often reduces the efficacy of targeted drugs and patients become resistant to therapies. One of the best-known mechanisms associated with the emergence of TKI resistance is indeed KRAS mutation. It is known that tumors with KRAS mutations at codons 12, 13, 61, or 146 do not respond to treatment with anti- EGFR antibodies or TKIs and therefore mutational analyses on all RAS genes are carried out on tumor biopsies before a therapeutic regimen is chosen.

Albeit not implemented for clinical use, given their important role in regulating RAS and linked pathways, it is reasonable to believe that altered miRNA expression could also affect the development of resistance to targeted therapies. To this effect, a number of experimental evidences exist (81–95).

Among miRNAs that target KRAS, the reduced expression of miR-181a was shown to be associated with gefitinib resistance in lung cancer (96, 97); in CRC patients treated with cetuximab, it was reported that low levels of miR-181a were associated with a lower overall survival, indicating a reduced efficacy of anti-EGFR therapy (98). While miR-145 was shown to synergize with cetuximab activity (99), high levels of let-7 could predict the efficacy of cetuximab therapy even in CRC patients carrying mutant KRAS (100).

Dietrich et al. (57) not only revealed an inverse correlation of KRAS and miR-622 expression but, additionally, they could

attribute KRAS-miR-622 interplay to therapy resistance since sorafenib induced further KRAS augmentation and down-regulation of miR-622. These few examples suggest that the miRNA-mediated modulation of RAS protein levels can indeed affect the response to TKIs or anti-EGFR targeted therapies.

In addition to RAS, the dysregulation of miRNAs responsible for the activation of elements of the MAPK or the PI3K pathways can also reduce the efficacy of TKIs. For example, the reduction of PTEN protein level by up-regulated miRNAs, like miR-21, miR-221, miR-23a and miR-214, can reduce efficacy of TKIs in lung cancer by activating the PI3K pathway (83, 101–106). miRNAs have also been associated with resistance to the BRAF inhibitor vemurafenib (107–110). In short, several studies have shown that the dysregulation of miRNAs has an important role in the efficacy of target therapies, thus suggesting that their levels of expression can be useful to guide the choice of therapy, alongside the more conventional mutational investigations. Furthermore, they also provide suggestions for potential therapeutic approaches useful to restore or improve sensitivity to treatments.

CONCLUSIONS

Taken together, published data provides a strong indication that altered miRNA expression represents an important mechanism for RAS activation, with various implications. First, it represents a mechanism of pathogenic relevance, responsible for the promotion of several tumor traits, irrespective of RAS oncogenic mutations. Second, considering that the activation of RAS represents a frequent mechanism of resistance for drugs directed against RTKs, it is possible that miRNA dysregulation represents a relevant aspect to consider when assessing the proper management of patients on target therapies. Third, miRNAs may represent potentially useful molecules for the control of RAS oncogenic activation, aimed at overcoming the lack of drugs targeting RAS and possibly improving the efficacy of target therapies.

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REVIEW
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Behind the Wheel of Epithelial Plasticity in KRAS-Driven Cancers

Emily N. Arner[1], *Wenting Du*[1] *and Rolf A. Brekken*[1,2]*

[1] *Cancer Biology Graduate Program, Department of Surgery and the Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, TX, United States,* [2] *Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, United States*

Cellular plasticity, a feature associated with epithelial-to-mesenchymal transition (EMT), contributes to tumor cell survival, migration, invasion, and therapy resistance. Phenotypic plasticity of the epithelium is a critical feature in multiple phases of human cancer in an oncogene- and tissue-specific context. Many factors can drive epithelial plasticity, including activating mutations in *KRAS*, which are found in an estimated 30% of all cancers. In this review, we will introduce cellular plasticity and its effect on cancer progression and therapy resistance and then summarize the drivers of EMT with an emphasis on KRAS effector signaling. Lastly, we will discuss the contribution of cellular plasticity to metastasis and its potential clinical implications. Understanding oncogenic KRAS cellular reprogramming has the potential to reveal novel strategies to control metastasis in KRAS-driven cancers.

Keywords: EMT, KRAS, metastasis, TBK1, AXL, drug resistance

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***Correspondence:**
Rolf A. Brekken
rolf.brekken@utsouthwestern.edu

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INTRODUCTION

KRAS is mutated in an estimated 30% of all cancers. In fact, the small GTPase KRAS has an activating point mutation in over 90% of pancreatic cancer patients (1), ∼35% of lung cancer patients, and ∼40% of colorectal cancer patients (2). As such, oncogenic KRAS is established as a driver of cancer initiation, progression, metastasis, therapy resistance, and immune suppression in multiple cancers (3). KRAS is an alluring therapeutic target, yet strategies targeting KRAS have been largely unsuccessful. However, understanding downstream effectors of KRAS signaling might provide alternative strategies to indirectly target KRAS and the cellular reprogramming driven by oncogenic KRAS signaling.

Recent evidence suggests that individual KRAS mutations activate distinct signaling pathways (2, 4). For example, gene expression analysis of primary human NSCLCs expressing G12C or G12V activating mutations in KRAS showed distinct gene expression profiles compared to cell lines expressing other KRAS activating point mutations (5). Similarly, Hammond et al. (6) engineered SW48 colorectal cancer cells, which are KRAS wild-type, to express KRAS point mutations: G12V, G12D, or G13D. Subsequent phosphoprotein expression analysis revealed the activation of differential signaling pathways in distinct KRAS mutational contexts. In support of these results, a large-scale screening effort using RNAi, small-molecules, and genetic analysis of cell lines and TCGA analysis revealed that KRAS binds to different effector proteins depending on the cellular context, which was determined by cell lineage, secondary mutations, and metabolic state (7). To further study context-dependent KRAS signaling in cancer, Brubaker et al. (4) developed a statistical approach to humanize multiplexed quantitative proteomic data from mouse models of colon and pancreatic cancer. Through the integration of proteomics and mutation data from human PDAC cohorts they identified synthetic lethal partners with oncogenic KRAS and

mutant KRAS tissue-specific and cross-tissue signaling. Each of these studies indicate that the signaling outcome and thus cellular phenotype driven by KRAS mutation is deeply dependent on cellular context.

Epithelial plasticity or an epithelial-to-mesenchymal transition (EMT) is a key cellular program that can be activated by KRAS. EMT contributes to tumor progression by enhancing tumor cell survival and therapy resistance and by facilitating success in the metastatic cascade. In this review, we will introduce cellular plasticity and its effect on cancer progression and therapy resistance and then summarize drivers of EMT with an emphasis on KRAS signaling. Lastly, we will discuss the contribution of cellular plasticity to metastasis and its potential clinical implications.

CELLULAR PLASTICITY AND EMT

Cellular plasticity serves as a mechanism of tissue adaptation and regeneration in normal tissues and can also predispose tissue to cancer transformation (8). In the pancreas, pancreatic epithelial and acinar cells display robust plasticity, enabling adaptation to metabolic and environmental stress. In pancreatic cancer, tumor cells alter their phenotype as a result of exposure to diverse metabolic conditions, signaling molecules, stromal elements, and therapeutic agents. This plastic state in tumor cells can facilitate tumor progression, including metastasis, chemoresistance, and immune evasion (8).

Acinar-to-ductal metaplasia (ADM) (9), describes a process where normal pancreatic acinar cells assume a duct-like state in the setting of chronic injury, such as pancreatitis. When pancreatitis resolves in normal/non-malignant pancreatic tissue, ADM lesions revert to acinar morphology. However, if KRAS-transformed acinar cells are subjected to the stress of pancreatitis, precancerous pancreatic intraepithelial neoplasia often forms (10–14). This suggests that pancreatic ductal adenocarcinomas (PDACs) may arise from acinar cells that have undergone transdifferentiation to a duct-like state. Normal pancreatic cells are sensitive to the transforming effects of mutant *KRAS* and the loss of phosphatase and tensin homolog (15), indicating that the likelihood of tumor formation and eventual histologic tumor type depends on the specific drivers that are present as well as the cellular compartments in which they are expressed (16–20).

EMT is another example of cellular plasticity program that is used by cells and tissues to adapt to cues or cellular stress. EMT classically defined is a developmental program that is instrumental in early embryo patterning during gastrulation (21, 22) and is characterized by epithelial cells losing cell-to-cell adhesion, epithelial tight junctions, and desmosomes. These changes are thought to occur through coordinated genetic reprogramming induced by EMT-transcription factors (EMT-TFs) that are activated in response to extracellular cues (21). These cues include growth factors such as transforming growth factor-β (TGFβ), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF1) (21, 23–26). This essential developmental program can be hijacked during tumorigenesis to promote increased cell migration and survival.

EMT in tumor cells can also be induced by cellular stress such as inflammation or nutrient/oxygen deprivation (27), and transforming oncogenes including oncogenic *KRAS* (28, 29). The genetic reprogramming associated with EMT in normal tissue or cancer leads to a shift from an epithelial to a mesenchymal phenotype. Epithelial cells often have polygonal shapes in monolayer culture, are polarized along their apical-basal axis and are tightly joined to one another laterally through adherens junctions. In contrast, mesenchymal cells exhibit spindle-like morphology and are loosely attached to the surrounding stroma through focal adhesions, which contributes to increased motility and invasive behavior (30) (**Figure 1**).

In epithelial tumors, the manifestation of an EMT program is associated with tumor grade. High-grade cancer is aggressive and characterized by a loss of normal tissue structure and architecture. High-grade tumors are often described as poorly differentiated and mesenchymal, displaying tumor cells that have undergone EMT. In contrast, low-grade tumors are characterized as well-differentiated cancers that retain an epithelial phenotype. Across human cancer, tumors that are high grade and poorly differentiated carry a worse prognosis with a high likelihood of metastasizing to distant organs (8).

EMT is a common feature associated with tumor progression and is thought to be critical to cancer cell dissemination in some tumors (31–33). The metastasis of epithelial tumors, such as PDAC, requires the cancer cells to escape epithelial nests, invade surrounding stroma, intravasate into blood or lymphatic vessels, survive circulation, and extravasate at the secondary site, where successful cells form micrometastases and eventually macrometastases (34). The escape of tumor cells from tumor cell nests encapsulated by a basement membrane can be facilitated by tumor cell epithelial plasticity, which results in epithelial tumor cells losing contact with the basement membrane and nearby cells while adopting mesenchymal-like features that enable cell migration and invasion. This is a common feature in mouse models of PDAC (35–37). While epithelial plasticity alters morphology and cell-cell contact it also enhances tumor cell survival under stressful environmental conditions, such as chemotherapy and radiation (32, 38–40). EMT and metastasis are generally considered to be late events in tumorigenesis; however, EMT and the metastatic cascade has been shown to occur even in "preinvasive" stages of PDAC (35). Thus, the concept that EMT is driven by the oncogenotype of a tumor is worthy of consideration.

In KRAS-driven tumors, such as PDAC, tumorigenesis and epithelial plasticity programs are often intertwined. For example, in genetically engineered mouse models (GEMMs) of PDAC harboring mutant KRAS, EMT was found to be an early event after tumor formation (35). Furthermore, co-expression of mutant KRAS and a polycomb-group repressor complex protein, Bmi1, in normal human pancreatic duct-derived cells (HPNE) induces partial EMT via upregulation of the EMT-TF Snail (28, 41–43). In addition, multiple receptor tyrosine kinases (RTKs) implicated in the induction of EMT activate RAS and the resulting signaling cascade induces the expression of EMT-TFs in a RAS-dependent manner (43–46). Other pathways have also been shown to interact with mutant KRAS to drive EMT. For example, the EMT-TF, Snail has been shown to induce TGFβ

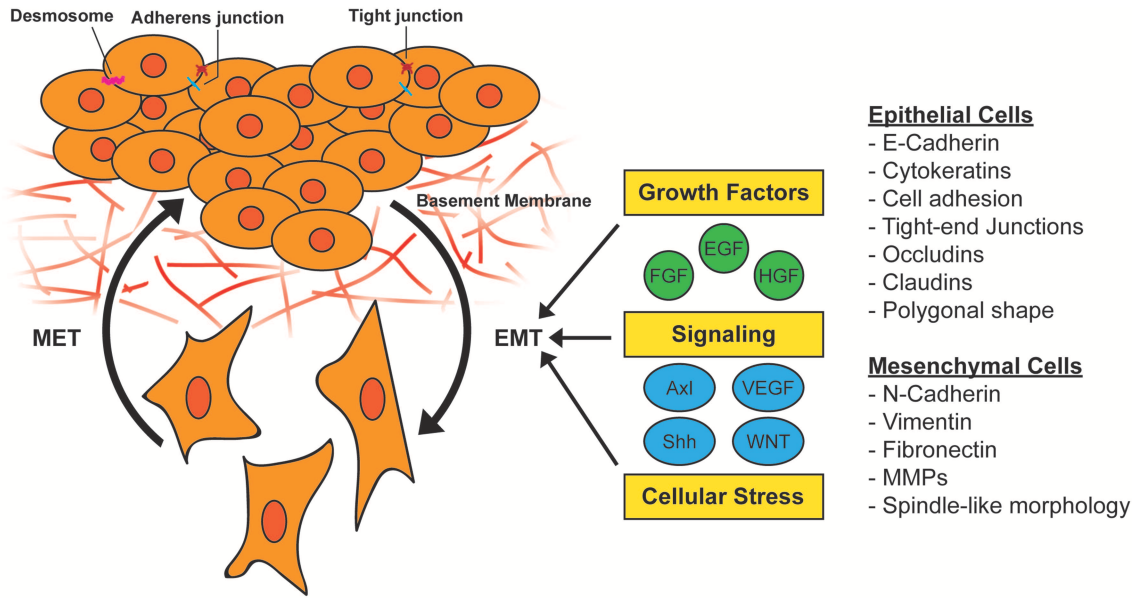

FIGURE 1 | Activation of epithelial-to-mesenchymal transition (EMT). During EMT, epithelial cells lose their cell-to-cell adhesion and adopt a more spindle-like morphology due to the expression of mesenchymal markers. This morphology change results in the ability to escape the basement membrane and invade and survive stressful situations, including therapy. EMT can be induced by a variety of growth factors, signaling pathways, and cellular stress such as hypoxia and nutrient deprivation. MET, mesenchymal-to-epithelial transition; MMP, matrix metallopeptidase.

signaling in a mutant KRAS dependent manner to drive EMT (47). Other studies revealed that signal transducer and activator of transcription 3 (STAT3) can mediate a synergistic interaction between TGFβ and RAS resulting to enhance Snail driven EMT (48). Other small GTPases, RAC, and RHO, are also activated by RAS via PI3K to drive EMT by regulating adherens junctions and focal adhesions (49). Thus, while mutant KRAS driven tumors are often dependent on RAS activity for development and maintenance (28, 41, 42) the prominent oncogenic mutation also is a critical component of epithelial plasticity.

EMT AND THERAPY RESISTANCE

Epithelial plasticity is a key chemoresistance and immune surveillance evasion strategy exploited by tumor cells (50, 51). Plastic tumor cells exhibit increased rates of resistance to therapy including radio-, chemo-, targeted, and immunotherapy (39, 40, 52–54). Stress, such as inflammation, nutrient/oxygen deprivation, and therapy can induce epithelial plasticity in cancer cells (27). A common consequence of EMT is reduced drug uptake by tumor cells. For example, the expression of equilibrative nucleoside transporter 1 (ENT1), which can transport nucleoside analog chemotherapy into cells, is often reduced in tumor cells that have undergone EMT. However, tumors engineered to lack EMT transcription factors (EMT-TFs), such as Snail and Twist, showed elevated ENT1 expression and increased sensitivity to gemcitabine, a nucleoside analog (55). Consistent with these results, Ludwig et al. (54) found that inhibition of AXL reduced epithelial plasticity in models of PDAC, increased ENT1 expression and enhanced sensitivity to gemcitabine when compared to gemcitabine alone or control treated animals. To combat chemoresistance in cancer patients, intermittent dosing or "drug holidays" have been suggested, although recent studies have revealed that resistance driven by oncogenic KRAS is not reversible (56). In human cancer cell lines, therapy resistance driven by mutant *KRAS* was found to irreversibly drive ZEB1-dependent EMT and chemoresistance through the hyperactivation of ERK1/2 (56), arguing against the use of intermittent dosing in tumors driven by oncogenic KRAS. Fischer et al. (57) showed in a spontaneous breast-to-lung metastasis model that EMT contributes to chemotherapy resistance, as mesenchymal-like tumor cells survived cyclophosphamide treatment, demonstrating reduced proliferation, apoptotic tolerance, and increased expression of chemoresistance-related genes. These observations highlight the potential increase in therapeutic efficacy that might result from combining standard therapy with strategies to combat epithelial plasticity.

The hypoxic state of pancreatic tumors increases tumor cell migration and chemoresistance (58). In fact, EMT can be driven by hypoxia often via the induction of TGFβ (59). Additionally, in human pancreatic cancer cell lines, hypoxia has been shown to drive EMT in an NFκB dependent manner through the stability of hypoxia-inducible factor 1 alpha (HIF-1α) and subsequent activation of RelA (p. 65) (60–63), a subunit of the NFκB family of transcription factors (64, 65). NFκB is considered a crucial component of drug resistance in mutant KRAS driven tumors such as pancreatic cancer and colorectal cancer, which typically expresses high levels of the protein (66). The activation of NFκB has been shown to upregulate

anti-apoptosis proteins such as Bcl-XL and Bcl-2, promoting chemoresistance (67, 68). As such, NFκB inhibition might be an approach to combat chemoresistance in tumors with KRAS-driven EMT.

Resistance to targeted therapy has also been associated with a mesenchymal state. In non-small cell lung cancer (NSCLC), the expression of an EMT gene signature, which included AXL expression, was associated with resistance to treatment with epidermal growth factor receptor (EGFR) and phosphatidylinositol 3-kinase (PI3K) inhibitors (69–73). Similarly, *in vitro* studies suggested that epithelial NSCLC cell lines are more sensitive to EGFR inhibitors than mesenchymal cell lines (74), and that when AXL is inhibited, sensitivity to EGFR inhibitors is increased (75, 76). In breast cancer patients, the EMT program also serves as a major driver of drug resistance, disease occurrence, and systemic dissemination (52, 77, 78).

In addition to targeted and chemotherapy, EMT has been associated with resistance to immunotherapy (79). In murine melanoma cells, Snail, a canonical EMT-TF, was found to be necessary and sufficient for resistance to cytotoxic T-cell–mediated killing via the induction of regulatory T cells. The effect was driven by immunosuppressive $CD11c^+$ dendritic cells, which were generated in response to Snail-expressing melanoma cells (40). Similarly, immune therapy-resistant melanomas display a mesenchymal gene signature, including the downregulation of E-cadherin and upregulation of factors involved in extracellular matrix (ECM) remodeling, angiogenesis, and wound healing (80). Additionally, the immune system is a key component of chemotherapy responses, as many chemotherapeutic agents directly affect the immune landscape of tumors (81). Therefore, identification of key signaling pathways involved in epithelial plasticity could reveal overlap with tumor immune evasion and new therapeutic targets, inhibition of which increases the efficacy of chemo- and immunotherapy.

EMT AND TUMOR METABOLISM

Metabolic alterations are associated with mutant KRAS-induced EMT. Cancer cells often increase glycolytic flux to meet the high energy demand to support rapid cell growth and division (82). In contrast to normal cells that typically generate energy via the breakdown of pyruvate, cancer cells generate energy by the non-oxidative breakdown of glucose with tumor cells displaying glycolytic rates up to 200 times higher than normal cells in the body (83). This preferential activation of glycolysis for energy supply is referred to as the "Warburg Effect" (83). In pre-clinical models as well as human patient samples, oncogenic Kras signaling can transcriptionally upregulate the glucose transporter GLUT1, as well as multiple enzymes in the glycolytic pathway [e.g., Hexokinase1 (HK1), Hexokinase2 (HK2), Phosphofructokinase1 (PFK-1), and Lactate dehydrogenase A (LDHA)] (82, 84, 85). Hypoxia, a common environmental condition in solid tumors, triggers O-linked β-N-acetylglucosamine (O-GlcNAcylation) at S529 of PFK-1, inducing glycolysis and giving a selective growth advantage to the cancer cells (86, 87). Cancer induced HIF-1α and MUC1 have also been shown to upregulate the expression of key glucose transporters and glycolytic enzymes, including GLUT1 and aldolase A, which leads to increased glucose uptake and glycolysis (82, 84, 88). In addition to glycolysis, recent evidence suggests oncogenic KRAS drives glucose into the hexosamine biosynthetic pathway (HBP), which is required for multiple glycosylation events (89, 90). Taparra et al. (91), recently showed in models of lung tumorigenesis, that KRAS and the EMT program coordinated elevated expression of key enzymes within the HBP pathway. Additionally, they showed that elevated O-GlcNAcylation of intracellular proteins such as the EMT-TF Snail results in suppressed oncogenic-induced senescence and accelerated lung tumorigenesis (91). Understanding the evident metabolic changes driven by oncogenic KRAS and reinforced by epithelial plasticity may reveal novel therapeutic targets for KRAS-driven tumorigenesis.

DRIVERS OF EMT

A variety of stimuli can induce EMT, including soluble factors, ECM components, environmental conditions, and oncogenic transcriptional programs (92). These stimuli, which include signaling factors such as TGFβ, Wnt, Notch, and Sonic hedgehog (Shh), as well as growth factors such as EGF and platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), serve as ligands for the signaling pathways they activate (**Figure 1**). EMT programs can also be activated in response to several paracrine signals in parallel (21). These networks activate signal cascades and intermediates that include mitogen-activated protein kinases (MAPKs), PI3K, AKT, Smads, RhoB, c-Fos, and RAS (93), which then regulate EMT-TFs. RTKs are common initiation sites for signaling that induces EMT-TF activity.

AXL

AXL is an archetypal RTK associated with EMT (94–96) and with worse outcomes in multiple tumor types (71, 94, 97, 98). Consistent with poor outcomes, AXL expression also is associated with metastasis and resistance to therapy (54, 96). AXL is a member of the TAM (Tyro3, AXL, MerTK) family of RTKs (99). Its ligand, growth arrest-specific gene 6 (GAS6) induces AXL signaling by stimulating the auto-phosphorylation of several tyrosine residues of AXL, which function as docking sites for multiple substrates including PI3K, phospholipase C, and c-SRC (100, 101). Additionally, AXL can be activated by forming heterodimers with non-TAM family proteins, such as EGFR, PDGFR, or another TAM family member (71). Elevated AXL expression is found in multiple cancer types, including lung, breast, ovarian, gastric, colon, pancreatic, and prostate (71–73, 94, 95, 97, 102, 103). AXL expression is induced by drivers of EMT, for example TGFβ, and is generally associated with markers of EMT including N-cadherin and vimentin (104, 105).

Our lab and others have shown that AXL expression in RAS-driven cancers, such as PDAC, maintains epithelial plasticity (96). GAS6-AXL signal transduction is required to maintain epithelial-mesenchymal plasticity traits of PDAC (96). When AXL was inhibited in GEMMs of pancreatic cancer, Ludwig et al. (54)

observed an increase of epithelial differentiated tumor cells. In addition to chemotherapy resistance, AXL has been strongly implicated in resistance to targeted therapy such as EGFR and PI3K/AKT inhibitors (72, 73).

Oncogenic KRAS

RAS genes (*HRAS*, *KRAS*, and *NRAS*) are the most frequently mutated gene family in cancer (106). Of these, *KRAS* is the most mutated (86% of all *RAS*-mutant cancers), followed by *NRAS* (12%), and *HRAS* (4%) (107). *KRAS* mutations are frequent in PDAC, lung, and colorectal cancers, and also occur in other cancers such as multiple myeloma (2, 108).

KRAS, a small GTPase, functions as a molecular switch, cycling between an active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound states (109). In non-transformed cells, RAS is typically GDP-bound and inactive, but upon activation of RTKs, there is a rapid activation of RAS-GTP, leading to the activation of intracellular signaling networks that promote growth, proliferation, and migration (110) (**Figure 2**). Because KRAS-activating mutations cluster around the nucleotide-binding pocket (2), these mutations cause RAS to be persistently GTP-bound and constitutively active, resulting in the hyperactivation of signaling networks to drive cancer growth and progression (111).

Multiple RTKs, including AXL and EGFR, can activate KRAS (112). Signaling networks downstream of RAS such as ERK/MAPK and PI3K/AKT can mediate mutant *Ras*-induced EMT, such that the inhibition of MEK1 or AKT (113) can reverse RAS-stimulated epithelial plasticity. Genovese et al. (114) completed a gene set enrichment analysis of highly metastatic and poorly metastatic clonal cells lines isolated from a GEMM of PDAC, i.e., KPfC mice ($KRAS^{LSLG12D/+}$; $Trp53^{Lox/Lox}$; $Pdx1^{Cre/+}$). Their analysis revealed that "metastasis-low" clones exhibited a downregulating of *KRAS* signature genes, whereas "metastasis-high" clones exhibited a higher expression of *KRAS* signature genes (114). After validation through *in vivo* lineage tracing, their study demonstrated that in PDAC, cells reside in a spectrum of epithelial-mesenchymal states where mesenchymal cells activate KRAS signaling at a higher level.

Other genome-sequencing studies revealed genetic heterogeneity beyond a few frequently mutated drivers in human PDAC (115–121). The heterogeneity in genomic changes makes it challenging to link definitive genomic alterations to biological, morphological, or clinical phenotypes (116, 121). Despite these challenges, Mueller et al. (37), found that the gene dosage of *KRAS G12D* in human and mouse PDAC correlated with a markedly increased metastatic potential and a mesenchymal phenotype. These results link the aggressive mesenchymal PDAC subtype with the highest dosage of mutant *KRAS* and *Ras*-related transcriptional programs. Additionally, oncogenic *Ras* is closely associated with resistance to drug therapy and pathways that drive PDAC initiation, progression, and metastasis.

TBK1

Although the majority of RAS effector-targeted therapies inhibit the RAF and PI3K signaling networks, the RALGEF pathway encompassing RALA and RALB GTPases are more consistently activated than RAF or PI3K in human PDAC (122, 123). Additionally, it has been demonstrated in human cell lines that RALGTPase activation is essential for RAS-induced transformation in a spectrum of human epithelial cells and that RALGTPase activation alone is sufficient to induce a tumorigenic phenotype in some settings (124, 125). Given that RAS signaling is a driver of epithelial plasticity and that the RALGEF pathway is a critical effector of RAS, investigating RALGEF signaling has the potential to reveal novel targets involved in epithelial plasticity, metastasis, and therapy resistance in *RAS*-mutant tumors.

The serine/threonine protein kinase TANK-binding kinase 1 (TBK1) is an atypical Ikβ kinase, that together with its homolog, IKKε, contributes to innate immunity by activating interferon regulatory factor 3/7 (IRF3/7) thereby inducing type 1 interferon gene expression in response to pathogen exposure (126, 127). Additionally, TBK1 kinase activity supports cell growth, self-renewal, pathogen clearance, and organelle function (128–131). TBK1 is a constituent of the RAL pathway and is crucial to the induction and progression of RAS-driven cancers (105, 130, 132, 133). Additionally, TBK1 has been linked to the survival of mutant KRAS-expressing cells (128) and can directly activate AKT (130). The importance of RALB and TBK1 to RAS-induced lung cancer was confirmed in a RNA inhibitor screen of synthetic lethal partners of oncogenic KRAS, where RALB and TBK1 were identified as top targets (132). Further, Cooper et al. (134) screened 100 NSCLC lines for sensitivity to TBK1 inhibitors Bx795 and compound II to tease out biological features of TBK1-dependent cell lines. Sensitivity profiles correlated strongly with profiles of multiple inhibitors of the AKT/mTOR pathway, particularly in mutant *KRAS* NSCLC lines, suggesting a mechanistic interaction between TBK1 and the mTOR pathway (134). Further analysis of TBK1 inhibitor (TBK1i)-sensitive cell lines revealed mutations in RAS family members and increased mesenchymal gene expression compared to TBK1i-resistant cell lines, which had a more differentiated gene expression profile.

In support of the contribution of TBK1 to RAS-induced EMT, we reported that TBK1 expression is associated with a poor prognosis in pancreatic cancer patients (135). Furthermore, we found that the loss of TBK1 function resulted in reduced invasion, migration, and tumor growth, and reduced metastatic events in preclinical models of mutant *KRAS* PDAC, indicating that TBK1 actively contributes to pancreatic cancer progression (105). In fact, one of the most significant and top dysregulated gene networks distinguishing *TBK1* WT and *TBK1*-mutant tumors was the cancer/cellular movement networks, including many genes involved in EMT. In comparison with *TBK1* WT tumors, tumors from *TBK1* mutant mice showed a trend toward higher expression of epithelial markers and lower expression of mesenchymal markers; this trend was confirmed at the protein level (105). Mechanistic studies established that TBK1 promotes EMT downstream of AXL in PDAC, in a RAS-RALB dependent manner (105). Although the precise mechanism of how TBK1 promotes EMT is unclear, evidence suggests that TBK1 can directly activate AKT (130), which can drive EMT via the induction of EMT-TFs (e.g., Snail and Slug) (38, 136, 137). Further studies are needed to delineate the whether the interaction between TBK1 and AKT is critical to the mesenchymal phenotype of tumor cells in PDAC. The

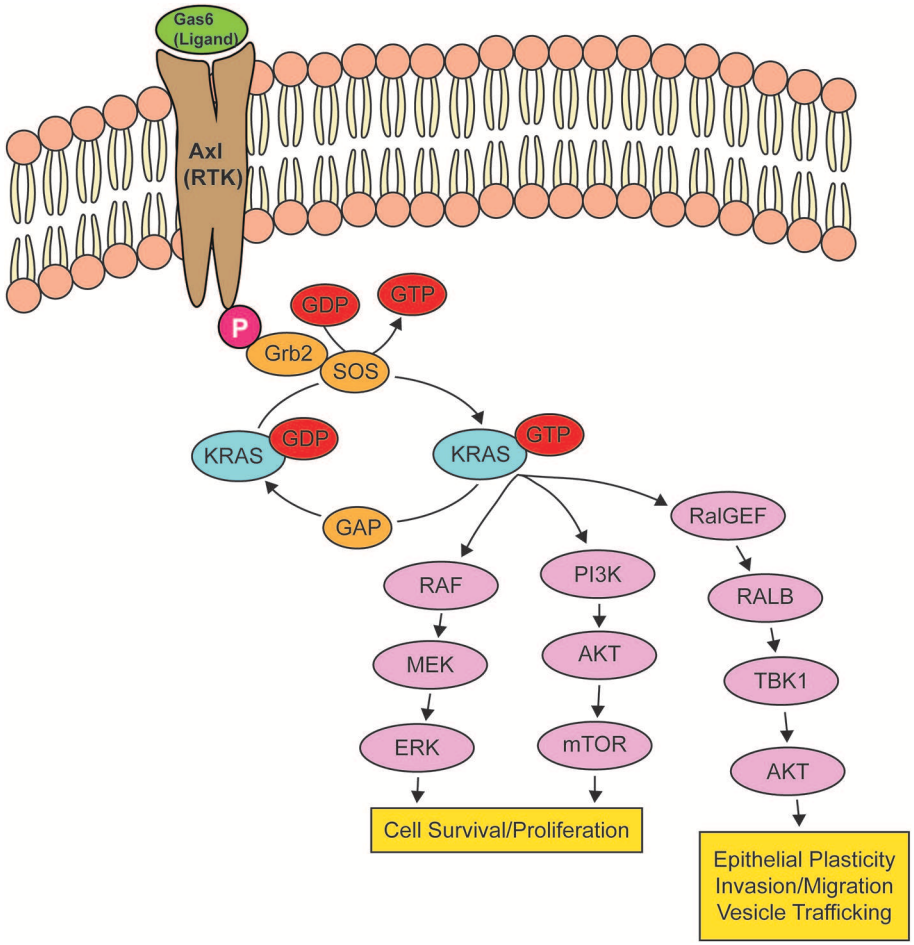

FIGURE 2 | Oncogenic KRAS effector pathways. When a receptor tyrosine kinase (RTK) is activated by its ligand, KRAS binds to GTP, rendering it active until the GTP hydrolyzes to GDP, turning KRAS off. When *KRAS* is mutated, KRAS remains bound to GTP, leading to the overstimulation of KRAS signaling pathways, resulting in cell survival and proliferation, epithelial plasticity, and migration. The activation of RTK AXL by GAS6 is shown as a potential signaling pathway that can drive an epithelial-to-mesenchymal transition via the activation of KRAS.

identification of additional TBK1 substrates that might promote EMT programs is also needed.

In contrast, knockdown of TBK1 in estrogen receptor α-positive (ERα) breast cancer cells resulted in enhanced tumorigenesis and lung metastasis in part by increasing EMT (138). Further studies are required to investigate if this pathway is dependent on oncogenic RAS. Another group observed that TBK1 is active in mutant *NRAS* melanoma and promoted migration and invasion of these cells (139), suggesting that RAS-driven epithelial plasticity may be active in the presence of other RAS isoform-driven cancers. Regardless, these studies suggest that therapies targeting TBK1 could be used to reduce EMT in *Ras*-mutant tumors.

cGAS-STING and Innate Immunity in EMT

In agreement with the concept that TBK1 loss affects antitumor immunity, studies by the Cantley (140) and Barbie (133) groups have reported that immune evasion and metastatic behavior are associated with the cGAS/STING/TBK1 innate immune pathway in cancer cells (133, 140, 141). Canadas et al. (133) revealed that mesenchymal tumor subpopulations with high AXL expression and low histone-lysine N-methyltransferase levels trigger the expression of a specific set of interferon-stimulated antisense endogenous retroviruses (ERVs). These ERVs were present in human cancer cells that produced tumors with hyperactive innate immune signaling, myeloid cell infiltration, and utilized immune checkpoint pathways. Therapeutically, this may have important implications for immune oncology drug combinations. In the second study, Bakhoum et al. (140) found that chromosomal instability (CIN) of cancer cells, promoted cellular invasion and metastasis through the presence of double-stranded DNA in the cytosol. Clustering of tumor cells via EMT genes accurately classified most cells according to their CIN status and revealed that CIN-high cells expressed mesenchymal markers. This CIN-high population also exhibited increased migratory and invasive behavior *in vitro*, underwent actin cytoskeletal reorganization, and stained positive for mesenchymal markers such as vimentin and β-catenin. Additionally, cells derived from metastases more

frequently exhibited cytoplasmic micronuclei than CIN-low or primary tumor-derived cells. These studies showed that cytosolic DNA activates the cGAS/STING pathway to mediate EMT, invasion, and metastasis (140). Under normal conditions, the cGAS-STING pathway functions as an innate cellular defense mechanism against viral infections. Once STING activates TBK1, TFs such as IRF3 and NF-κB are phosphorylated and translocate to the nucleus (142), where they mediate the transcription of inflammatory genes (143–146). In human breast and lung cancer-derived cell lines, chronic cGAS-STING activity resulting from chromosome instability has been shown to drive migration, invasion, and metastasis (140). Additionally, CIN can result in elevated mutant *KRAS* gene dosage in pancreatic cancer, which can drive higher expression of EMT genes and increase metastasis (37).

Similar to epithelial plasticity, CIN has been implicated in treatment resistance by generating heterogeneity within the tumor that enhances natural selection, thereby promoting tumor cell survival, immune evasion, drug resistance, and metastasis (37, 147–152). Given the widespread nature of CIN in human cancer, therapies targeting CIN and cGAS/STING have therapeutic potential to reduce therapy resistance and reduce metastasis.

Downstream Transcriptional Networks of Epithelial Plasticity

EMT is thought to be regulated largely through changes in the expression of genes necessary for the epithelial state, such as adherens junctions and tight junction components, which are transcriptionally repressed through the activation of EMT TFs including Snail, Twist, and Zeb (153). As previously mentioned, EMT can be induced by many signaling factors, such as TGFβ, EGF, FGF, HGF, NOTCH, and Wnt ligands. These factors initiate signaling cascades, leading to the expression of one or more EMT-TFs, which inhibit E-cadherin transcription by binding to E-boxes within the E-cadherin promoter region (154, 155).

EMT-TFs are often associated with poor patient outcomes. In resected PDAC, nearly 80% of tumors expressed moderate to strong levels of SNAI1, while only 50% showed SNAI2 expression, and very few expressed TWIST (156). Additionally, ZEB1 expression in pathologic specimens correlated with advanced tumor grade and worse outcomes (157, 158). Functions for individual EMT-TFs in different cancers have been described: for ZEB1 and ZEB2 in melanoma (159, 160), Snail and Slug in breast cancer (161), and for Sox4 (162), and Prrx (163) in PDAC. These functions can be tissue-specific, as demonstrated by the different functions of Snail in the metastasis of breast cancer (164) and PDAC (55). Such functional diversity of EMT-TFs suggests that distinct EMT programs operate in different tissues during tumor progression. With this in mind, therapeutic strategies targeting EMT-TFs should consider tissue context and target multiple factors simultaneously (112).

ZEB1 is a zinc finger/homeodomain protein that is associated with EMT and tumor progression. ZEB1 functions as a transcriptional activator by binding to CtBP co-repressors, histone acetyl-transferase TIP60, chromatin remodeling ATPase BRG1, and SIRT1, a histone deacetylase (21). Larsen et al. (165) found that ZEB1-induced EMT was crucial for the development of NSCLC but required premalignant oncogenic mutations such those for *KRAS*. Moreover, they found that ZEB1-driven EMT was a crucial early event in the progression of human bronchial epithelial cells to malignancy (165). These results supported previous *in vitro* (166) and *in vivo* (167–170) studies that established ZEB1 as a driver of EMT in lung cancer tumorigenesis. In PDAC, Krebs et al. (112) demonstrated that ZEB1 is a key driver of PDAC progression from early tumorigenesis to late-stage metastasis, highlighting the important contribution of EMT activation in these processes (112).

Beyond the levels of mRNAs, EMT-TFs can alter chromatin to achieve the stable, long-term silencing of epithelial genes required for complete EMT (171). Snail, an EMT-TF, can recruit a series of chromatin-modifying enzymes to the E-cadherin promotor to erase a mark of active transport and replace it with a trimethylated H3K9 mark that promotes the recruitment of DNA methyltransferases, causing CpG methylation of the promoter and formation of a constitutive heterochromatin resistant to transcription activation (172). Additionally, TFs of the Zeb family form a double-negative feedback loop with the miR-200 family of microRNAs (miRNA), causing this regulatory loop to operate as a switch between epithelial and mesenchymal states in a variety of tumor types (173–175). Similarly, Snail represses the expression of miR-34, a miRNA that binds to the 3′ UTR of Snail mRNA to mark it for degradation (176).

TARGETING KRAS SIGNALING AS A THERAPEUTIC APPROACH

Direct Targeting of KRAS

Targeting RAS proteins was first attempted when the proteins were shown to be modified and rendered functional by farnesylation (177–179). This initiated the launch of identifying compounds that block farnesyl transferase activity. Farnesyl transferase inhibitors were developed with impressive potency and selectivity, but they failed to show efficacy in the clinic (180). Another approach that has been considered is the development of a GTP antagonist. However, due to the picomolar affinity of GTP and RAS and the millimolar concentration of GTP in the cell, GTP antagonists had long been deemed impossible (111) until recently. In 2013, the dream of directly targeting RAS was re-imagined when Shokat and colleagues identified compounds that bind covalently and specifically to *KRAS G12C* (181). Lead compounds were further developed by Wellspring Biosciences, who showed that the compounds ARS853 and ARS1620 inhibit *KRAS G12C* effectively and specifically in cells and animals (182, 183). The first *KRAS G12C* inhibitor to enter clinical trials is Amgen 510 (**Table 1**). Multiple groups are working to create improved G12C-targeted compounds with better RAS-GTP destabilizing activity (184, 185). These studies

TABLE 1 | Clinical trials targeting KRAS, AXL, and TBK1.

Target	Drug	Disease	Trial phase	Results	Identifier
KRAS G12C	AMG 510	NSCLC	1/2	Ongoing	NCT03600883
KRAS G12C	MRTX849	Advanced solid tumors	1/2	Ongoing	NCT03785249
AXL	Bemcentinib (BGB324)	Glioblastoma	1	Ongoing	NCT03965494
AXL	Bemcentinib (BGB324)	Pancreas	1/2	Ongoing	NCT03649321
AXL	Bemcentinib (BGB324)	NSCLC	2	Ongoing	NCT03184571
AXL	Bemcentinib (BGB324)	NSCLC	1/2	Status unknown	NCT02424617
AXL	Bemcentinib (BGB324)	Malignant mesothelioma	2	Ongoing	NCT03654833
AXL	Bemcentinib (BGB324)	NSCLC	1	Ongoing	NCT02922777
AXL	Bemcentinib (BGB324)	TNBC	2	Completed	NCT03184558
AXL	Bemcentinib (BGB324)	Melanoma	1/2	Ongoing	NCT02872259
AXL	Bemcentinib (BGB324)	Acute myeloid leukemia	2	Ongoing	NCT03824080
AXL	TP-0903	NSCLC, colorectal, ovarian, melanoma	1	Ongoing	NCT02729298
AXL	TP-0903	Leukemia, lymphoma	1/2	Ongoing	NCT03572634
TBK1	Amlexanox	Type 2 diabetes	2	Finished recruitment	NCT01842282
TBK1	Amlexanox	Type 2 diabetes	2	Optimal drug dose wasn't reached.	NCT01975935

NSCLC, non-small cell lung cancer; TNBC, triple-negative breast cancer.

have reinvigorated the field and initiated research efforts, such as the NCI-supported RAS initiative.

Although this recent breakthrough suggests that targeting *KRAS G12C* may be effective, it is possible that this targetable allele may be an outlier (186). *KRAS G12C* is rarely mutated in KRAS-addicted cancers and it is likely that *KRAS G12D* and *G12V*, the most common mutant *KRAS* alleles, will be more challenging to specifically inhibit (187). As a result, the development of therapeutic strategies that either inhibit RAS effector signaling elements, such as TBK1, or inhibit elements that can activate *RAS*, such as AXL, remain an attractive therapeutic alternative.

Targeting AXL and TBK1 as a Therapeutic Strategy for KRAS-Driven Cancers

Due to its implication in metastasis, EMT, and drug therapy resistance, large efforts are focused on pharmacologically inhibiting AXL. In fact, multiple strategies are being tested clinically, including blocking GAS6 or AXL with monoclonal antibodies and small molecules (99, 188). One of the most advanced selective AXL inhibitors to date is bemcentinib (BGB324), developed by BerGenBio ASA. BGB324 has been investigated by our group in preclinical models of late-stage PDAC and shown promising therapeutic effects in enhancing gemcitabine efficacy and reducing metastasis (54). Other groups have also investigated BGB324, where it has been found to have antitumor, antimetastatic, and therapy-sensitizing effects in preclinical models of pancreatic cancer, breast cancer, glioblastoma, prostate cancer, chronic myeloid leukemia, ovarian cancer, and uterine serous cancer (189–195). Recently phase II clinical trials have begun to enroll patients using bemcentinib in multiple cancer types as a single agent or in combination with targeted or chemo- and immunotherapies (**Table 1**). Another selective AXL inhibitor is TP-0903, developed by Tolero Pharmaceuticals. In preclinical models, TP-0903 has been shown to have antitumor and therapy-sensitizing effects on multiple cancers, including neuroblastoma, leukemia, and lung cancer (196–199). TP-0903 is currently being evaluated clinically in multiple indications (**Table 1**).

For TBK1 to be a relevant target in the clinic, it will be necessary to evaluate the therapeutic efficacy of TBK1 inhibition in preclinical cancer models. Currently there are at least six distinct small molecules that inhibit TBK1, including BX795, compound II, CYT387, MRT67307, GSK2292978A, and Amlexanox, although none are highly selective. Currently, Amlexanox is the only TBK1i known to enter clinical testing, which is in a phase 2 study for the treatment of type 2 diabetes, non-alcoholic fatty liver disease, or obesity (**Table 1**). Further investigations and better inhibitors will be needed before TBK1 can be directly targeted in RAS-driven cancer in preclinical and clinical settings. Moving forward, it will be vital to understand the distinct function of TBK1 in each relevant cell type within tumors. As mesenchymal tumor cells express high levels of active TBK1 (105) and are associated with aggressive disease, metastasis, and poor patient outcomes (30), targeting TBK1 in RAS-driven cancers is a promising alternative strategy to reduce the tumor-promoting effects of KRAS-driven EMT.

CONCLUSIONS AND FUTURE PERSPECTIVES

EMT is a key cellular program that is activated by KRAS and thus contributes to tumor progression by enhancing tumor cell survival, tumor cell dissemination, and therapy resistance and has a strong association with worse clinical prognosis in many KRAS-driven cancers. Because KRAS is not currently an amenable target for many of these KRAS-driven cancers, targeting KRAS effector signaling is an attractive alternative. With this in mind, pharmacologically targeting the pathways

that contribute to KRAS-driven EMT is worth considering as a strategy to improve response to standard therapy and reduce clinical progression, therapy resistance, and metastasis.

Despite significant evidence that EMT directly contributes to tumor progression, several studies have suggested EMT is not required for the metastatic spread of PDAC and breast cancer (55, 57, 200, 201). For example, most metastatic lesions are known to exhibit epithelial features, an observation that seems to be at odds with EMT as a prerequisite for metastasis (30, 202, 203). As such, the importance of EMT in cancer biology has long been questioned (204).

Epithelial plasticity not only includes the process of EMT, but also the reverse, mesenchymal-to-epithelial transition or MET. Recent evidence suggests that MET is required for successful metastatic colonization, although it remains unknown whether the tissue-specific adaptations are acquired thorough epigenetic or genetic means. Distant metastases in carcinoma patients often present with epithelial features having a similar histology as the tissue of origin (205, 206). These observations support that epithelial plasticity lies at the heart of tumor development and progression, and that such plasticity is necessary for tumor cell survival and colonization. It has become increasingly evident that EMT encompasses a range of hybrid plastic states, a phenotype coined as "partial EMT" (36, 207, 208). Because partial EMT is not well-defined, it is unclear whether this hybrid status signifies a transitional phase during EMT or represents its own state. Similarly, using a mouse model of PDAC, the Stanger group has shown that individual tumors can activate different plasticity programs, such as "classical EMT" which involves transcriptional repression and an alternative program in which the epithelial state is lost post-transcriptionally (36). These plasticity programs were associated with either single-cell invasion or collective invasion, respectively (36). It is unclear what underlies this phenotypic heterogeneity, considering the tumors investigated in this study had the same oncogenic drivers (TP53 and KRAS). Perhaps the only difference between the states is the tumor microenvironment, as Aiello et al. found that when partial EMT cells are exposed to TGFβ, they execute a classic EMT program (36, 209). This constant plastic state may partially explain the intratumoral heterogeneity that is often seen in carcinomas such as PDAC (210–212).

The chronic activation of an EMT program within a tumor may depend on paracrine signals within the tumor microenvironment, dictating whether the tumor cells undergo EMT or MET. Because these cells exist in a plastic state, it is possible that these tumor cells readily revert their phenotype based on a microenvironment-specific context and factors (36, 205, 213, 214). One challenge impeding current *in vivo* studies is the difficulty of distinguishing carcinoma cells that have undergone EMT from fibroblasts or other mesenchymal cells that are normally found in the tumor stroma. To combat this, many labs have begun to use single-cell sequencing technology in KRAS-driven cancers such as PDAC to investigate EMT *in vivo* (215). Additionally, current *in vivo* lineage-tracing technology has not settled the debate between the importance of collective migration and/or EMT for metastatic dissemination. Additionally, the mechanisms of invasion and metastatic potential and their correlation with clinical outcome has yet to be defined. Regardless, epithelial plasticity remains as an indispensable feature in multiple phases of human cancer in an oncogene- and tissue-specific manner.

AUTHOR CONTRIBUTIONS

EA and WD wrote the manuscript. RB reviewed and revised the manuscript.

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REVIEW
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A Comparative Analysis of Individual RAS Mutations in Cancer Biology

Carmen Muñoz-Maldonado [1,2], *Yitzhak Zimmer* [1,2] *and Michaela Medová* [1,2*]

[1] *Department of Radiation Oncology, Inselspital, Bern University Hospital, Bern, Switzerland,* [2] *Radiation Oncology, Department for BioMedical Research, University of Bern, Bern, Switzerland*

In human cells, three closely related *RAS* genes, termed *HRAS*, *KRAS*, and *NRAS*, encode four highly homologous proteins. RAS proteins are small GTPases involved in a broad spectrum of key molecular and cellular activities, including proliferation and survival among others. Gain-of-function missense mutations, mostly located at codons 12, 13, and 61, constitutively activate RAS proteins and can be detected in various types of human cancers. *KRAS* is the most frequently mutated, followed by *NRAS* and *HRAS*. However, each isoform exhibits distinctive mutation frequency at each codon, supporting the hypothesis that different RAS mutants may lead to distinct biologic manifestations. This review is focused on the differences in signaling and phenotype, as well as on transcriptomics, proteomics, and metabolomics profiles related to individual RAS-mutated variants. Additionally, association of these mutants with particular targeted outcomes and rare mutations at additional RAS codons are discussed.

Keywords: RAS mutations, RAS profile, RAS-mutated cancers, treatment responses, RAS-related omics, GTP/GDP binding, RAS signaling, rare codons

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University of Kent, United Kingdom

***Correspondence:**
Michaela Medová
michaela.medova@dbmr.unibe.ch

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INTRODUCTION

RAS subfamily comprises the ubiquitously expressed human RAS proteins KRAS4A, KRAS4B (the two KRAS splice variants), HRAS, and NRAS, which are frequently mutated in cancer (1). These genes encode small GTPases that function as molecular regulators, controlling a broad spectrum of cellular activities, such as proliferation and cell survival (2).

RAS proteins are considered molecular switches because they cycle between the "on" and "off" conformations, which are given by the binding of GTP and GDP, respectively (3). The transition between both states is regulated by two different protein families. The guanine nucleotide exchange factors (GEFs) promote GDP dissociation and GTP binding while the GTPase-activating proteins (GAPs) stimulate RAS intrinsic GTPase activity to switch off this signal.

High homology is shared by the three RAS proteins, except for the C-terminus hypervariable region, which is thought to confer the specific function of each protein (2). It has been reported that up to one-third of human cancers (4) bears gain-of-function missense mutations (5) that occur in the protein region that is identical among the four RAS proteins. Forty-four different point mutations have been described and 99.2% of them are located at codons 12, 13, and 61 (2), but other non-canonical codons (such as 19, 117, or 146) are also mutated at low frequencies (6). All these canonical mutations prompt the loss of the intrinsic and/or the GAP-stimulated GTPase activity of RAS proteins, leading to a constitutively activated form of RAS. However, some non-canonical mutations, such as for example HRAS A146 mutations, do not impair RAS GTPase activity, but increase guanine nucleotide exchange.

Interestingly, the mutated isoform, as well as the codon position and the amino acid substitution varies among RAS proteins in human cancers, but the reason remains to be established (4). Regarding protein variability, *KRAS* is the most frequently mutated protein in human cancers, followed by *NRAS* and *HRAS*. Oncogenic alterations in *KRAS* are more frequent in patients with pancreatic carcinoma, colorectal tumors and lung malignancies (5). Mutations in *HRAS* can be found in dermatological malignancies and head and neck cancers, while *NRAS* mutations are common in melanomas and in some hematopoietic malignancies (**Table 1**) (5).

The mutations rates at each codon differ between the RAS proteins (2). While *KRAS* is commonly mutated at codon 12 with only few mutations occurring at codon 61, *NRAS* mutations are most frequently observed at codon 61. In addition, *HRAS* mutational rate is similar for both codons 12 and 61, displaying an intermediate mutational pattern between *KRAS* and *NRAS* (2).

Each of these codons can be substituted through a single-nucleotide change resulting in codons 12 and 13 changes from glycine to alanine, cysteine, aspartic acid, arginine, serine or valine and codon 61 from glutamine to glutamic acid, histidine, lysine, leucine, proline or arginine. In KRAS, the variations at codons 12 and 13, which are the most frequent mutations associated with this protein, result in G12D and G13D substitution, respectively. Similarly, the most common mutation in HRAS is the G12V substitution. As previously mentioned, NRAS has a mutation bias at codon 61, Q61R replacement at this position being the most frequent aberration (2).

Considering that RAS mutations are all located in the homologous amino-acid region, it could be postulated that their effect on the protein function is equivalent. Nevertheless, studies have demonstrated that different substitutions in RAS proteins distinctly modify protein GTPase activity or its affinity for downstream effectors (6–8). According to these reports, different RAS mutations may result in distinct biological manifestations. As this topic is less discussed in the literature, within this review we focus on the differences among RAS proteins mutations with respect to their preferential signaling pathways, biochemistry, specific changes in cellular phenotype, mutations-specific transcriptomics, proteomics and metabolomics characteristics, as well as their individual association with patient treatment outcome and survival.

RAS PROTEINS: FUNCTIONAL AND LOCALIZATION VARIANCES

RAS proteins were initially believed to be functionally redundant due to their high homology in structure, biophysical and biochemical properties (9). Subsequently, accumulating solid experimental evidence indicated that RAS proteins differ substantially in their function in various cell types and tissues (9). For example, while, *KRAS4A*-, *NRAS*-, or *HRAS*-deficient mice are viable, *KRAS4B* knockout mice die during embryogenesis between days 12 and term due to liver, cardiac and hematopoietic abnormalities (10–13). These findings suggest that only *KRAS4B* may be essential during development and that there might be a redundancy in signaling among the other RAS proteins in embryogenesis. Later on, Potenza et al. modified the *KRAS* gene to encode an HRAS protein, showing that HRAS can functionally replace KRAS during embryogenesis but only under the control of KRAS promoter (6). However, these adult mice displayed dilated cardiomyopathy, indicating that KRAS has a unique role in cardiovascular homeostasis (14) and that the mortality of *KRAS*-deficient mice is likely derived from the inability of other RAS proteins to be expressed in the same subcellular compartments (9).

In relation to the protein-specific role of RAS in mouse embryogenesis, some studies have pointed out also their similar specific functions in human development. It has been shown that germline mutations in RAS proteins or in RAS regulators, such as *NF1*, *PTPN11,* or *SOS1*, lead to several congenital developmental disorders, such as neurofibromatosis type 1, Noonan, or Costello syndromes, respectively (9). Therefore, these data in combination with the aforementioned animal experiments indicate that normal development is regulated by a precise pattern of RAS signaling (15).

Numerous mechanistic studies from the last two decades support the notion that each RAS protein displays specific downstream signaling (16–20). The distinct protein functionalities can be attributed to different post-translational modifications occurring at the C-termini of the RAS proteins. These modifications allow RAS proteins to anchor in different subcellular membranes from where each protein can activate different signaling pathways (21). Although plasma membrane is the major location for all the RAS proteins, they have also been found in the endoplasmic reticulum, Golgi apparatus, endosomal network, and mitochondria (21). Interestingly, the level of each protein in these subcellular compartments varies according to their total abundance and between cell types. For example, Chiu et al. reported that NRAS and HRAS maintain the highest Golgi pool, followed by KRAS4A and KRAS4B, which are mainly located in the plasma membrane (18).

Early evidences from plasma membrane perturbation studies support the idea of compartmentalized RAS protein signaling. Roy et al. reported that HRAS but not KRAS4B was able to inhibit RAF/MAPK signaling pathway (16). In addition, analysis of mutant RAS proteins revealed distinctive RAF1 (CRAF) activation, with KRAS4B and KRAS4A being more potent RAF1 activators than NRAS or HRAS (17). Moreover, the protein-specific signaling leads to different outputs depending on the RAS subcellular localization (15). For example, KRAS anchored in the plasma membrane can induce cellular transformation, while its activation when located in the mitochondria triggers apoptosis (19). In the case of HRAS, Chiu et al. demonstrated that only the endoplasmic reticulum-associated form can activate the RAF1-ERK signaling pathway, leading to fibroblast transformation (18). However, HRAS Golgi-associated form seems to be unable to induce cell transformation or proliferation (20). Taken together, these data suggest that RAS protein subcellular localization modulates signaling pathway activation and its outcome.

TABLE 1 | Most common mutations in the individual codons of RAS proteins.

RAS protein	Malignancies	Codon	Amino acid substitution
HRAS	Dermatological Head and neck cancer	Codon 12: GGC (Gly, G)	12A, 12C, 12D, 12R, 12S, 12V
		Codon 13: GGT (Gly, G)	13C, 13D, 13R, 13S, 13V
		Codon 61: CAG (Gln, Q)	61H, 61K, 61L, 61P, 61R
KRAS	Pancreatic carcinoma Colorectal cancer Lung malignancies	Codon 12: GGT (Gly, G)	12A, 12C, 12D, 12R, 12S, 12V
		Codon 13: GGC (Gly, G)	13A, 13C, 13D, 13R, 13S, 13V
		Codon 61: CAA (Gln, Q)	61E, 61H, 61K, 61L, 61P, 61R
NRAS	Melanomas Hematopoietic malignancies	Codon 12: GGT(Gly, G)	12A, 12C, 12D, 12R, 12S, 12V
		Codon 13: GGT (Gly, G)	13A, 13C, 13D, 13R, 13S, 13V
		Codon 61: CAA (Gln, Q)	61E, 61H, 61K, 61L, 61P, 61R

Amino acid substitutions identified at codon 12, 13, and 61 of each RAS protein, highlighting in red the most frequently observed. Gly and G, glycine; Gln and Q, glutamine; A, alanine; C, cysteine; D, aspartic acid; R, arginine; S, serine; V, valine; H, histidine; K, lysine; L, Leucine; P, proline; E, glutamic acid.

PHENOTYPICAL DIFFERENCES AMONG RAS PROTEINS MUTATIONS

Early studies analyzing the biochemical consequences of RAS mutations showed connections between HRAS specific mutations and cell transformation (7, 8). These reports pointed out that particular RAS mutations may modify the biochemical behavior of RAS proteins including their ability to bind GTP and GDP. Three decades later, additional differences in RAS mutations biology with respect to endpoints such as anchorage-independent growth or cell migration in many types of cancers are being continuously reported (6, 22–25), showing that RAS biological behavior is more complex than previously thought.

Transforming Potential

Seeburg et al. were in 1984 the first to assess the transforming potential of different HRAS mutations (7) by transfecting rat fibroblasts with plasmids encoding 20 different HRAS mutant variants at codon 12, which encodes for glycine. The transforming potential of these mutants was assessed by changes in colony morphology. Rat fibroblasts expressing G12V, G12L, G12I, G12R, or G12T variants showed a fully transformed colony morphology, with cells consistently round and refractile that grew to the highest saturation densities. Interestingly, the transfection with G12K- or G12Q-mutated variants displayed low transformation, with foci induction after 2 or 3 weeks and cells with almost normal morphologies. Similarly, fibroblasts transfected with G12S, G12M, G12C, G12Y, G12F, G12W, G12H, G12D, G12E, G12A, and G12N plasmids exhibited an intermediate transformation, with cells overgrowing the monolayer but less striking changes in morphology than the most potent mutations. However, similarly to glycine, no transformation was observed with the G12P variant (7). Later, HRAS mutations at codon 61 were analyzed by Der et al. (8). NIH3T3 mouse fibroblast cells were transfected with plasmids encoding 17 different amino acids at codon 61 and the transforming potential was analyzed by foci formation (8). The transfected cells displayed different transforming potential, from very strong transforming mutants (Q61V, Q61L, Q61K, Q61A, Q61C, and Q61R) to a very weak one, Q61G, which was ∼200-fold lower than Q61V. Q61H, Q61I, Q61Y, Q61M, Q61T, Q61N, Q61W, and Q61F mutants showed an intermediate spectrum between weak and strong transformation. Moreover, Q61P and HRAS WT failed to demonstrate any transformation (8). This failure is not due to the impaired expression of the mutant protein (7), but it could be explained by the fact that proline at codons 12 or 61 of HRAS displays similar biological properties as wild type (WT) HRAS (8). The overexpression of either WT HRAS or HRAS G12P or Q61P in NIH3T3 fibroblasts leads to cell transformation (8). Moreover, based on HRAS structure, proline at position 12 may cause a helix termination, resulting in a lower transforming potential (26). These early observations suggest that the transforming potential of RAS proteins also depends on the substitution that replaces the original amino acid.

Later, Smith et al. similarly compared the transforming potential of different KRAS mutations (22). NIH3T3 fibroblasts were transfected with plasmids expressing WT, G12V, G12D, G13D, and Q61H KRAS. All KRAS mutants exhibited foci formation after 21 days, however codon 12 mutations had a slightly greater transforming potential than mutations at codons 13 and 61 (G12V > G12D > G13D > Q61H) (**Table 2**) (22).

These intriguing data stimulated further studies in which the role of the same mutation in different RAS proteins properties has also been investigated. In that sense, Voice et al. compared transforming potential of the G12V mutation among HRAS, NRAS, KRAS4A, and KRAS4B proteins in different cell lines (17). The focus forming abilities of HRAS and KRAS4A in NIH3T3 and Rat-1 cells were ∼2- to 2.5-fold higher than those of KRAS4B and NRAS. Interestingly, in RIE-1 cells, HRAS and KRAS4A transforming potentials were 8.3- and 6.3-fold higher than those of KRAS4B and NRAS (17), indicating that

TABLE 2 | Phenotypical differences among RAS proteins mutations.

Characteristic/mutation	KRAS4A G12V	KRAS4B G12V	HRAS G12V	NRAS G12V	KRAS G12A	KRAS G12C	KRAS G13D	KRAS Q61L	KRAS Q61H	KRAS G12D	KRAS G13C	KRAS G12V	KRAS G12R	NRAS Q61R
Transforming potential	High (17)	Low (17)	High (17)	Low (17)			High (22)		Mid (22)	Very high (22)		Very high (22)		
GTP binding						High (6)			Very high (6)		High (6)	Yes (22) High (6)		
Instrinsic GTP hydrolysis					Very slow (25)		Slow (25)	Very slow (25)	Very slow (25)	Slow (25)		Slow (25)	Very slow (25)	Very slow (24)
GAP-mediated GTP hydrolysis				Slow (25)	Very slow (25)			Slow (25)	Slow (25)	Slow (25)			Slow (25)	Very slow (24)
Anchorage-independent growth	Yes (17)	No/Yes (17)	No/Yes (17)	Yes (17)		Yes (23) Yes (6)	Yes/No (6)		Yes (6)	No (23) Yes/No (6)	Yes/No (6)	Yes (6)		
Migration	No (17)	Yes (17) Fast (30)	Minimally (17) Slow (30)	No (17)			Yes (6)			Yes (6)		Yes (6)		

Summary of RAS mutant proteins manifestations according to different studied characteristics. GTP, guanosine-5'-triphosphate; GAP, GTPase-activating proteins.

the differences in mutant transforming potential are also cell type-dependent (**Table 2**).

In addition to *in vitro* studies that have been performed to elucidate the differences among RAS mutations functional characteristics, xenograft models and genetically-engineered mouse models have been used for that purpose as well (24, 27, 28). For example, Céspedes et al. identified the tumorigenic potential of KRAS G12V and G12D mutations *in vivo* (27). Both mutations generated tumors but cells harboring the G12V mutation grew significantly faster than cells harboring the KRAS G12D mutant variant (27). A later study by Haigis et al. analyzed the transforming potential of KRAS and NRAS G12D mutant proteins expressed in the colonic epithelium of genetically-engineered mice (28). Animals harboring KRAS G12D developed widespread hyperplasia throughout the colonic epithelium, which also happened in adult mice. However, the expression of NRAS G12D mutant variant in this tissue had no effect, suggesting that KRAS might be the only RAS protein modulating the homeostasis of the colon. Interestingly, KRAS G12D mice did not develop colon cancer, indicating that the expression of this mutant variant is not sufficient to promote neoplasia (28). In addition, using a melanoma mouse model, Burd et al. reported that homozygous NRAS G12D or NRAS Q61R $p16^{INK4a}$-deficient mice developed significantly more nevi than control mice. However, mice harboring NRAS Q61R triggered nevi formation more frequently than animals harboring NRAS G12D mutation ($p = 0.03$) (24). Moreover, the penetrance of the tumors was higher in NRAS Q61R mice than in NRAS G12D animals, results that are in accordance with the frequency of nevi formation. Nevertheless, tumor growth and histology were similar between the NRAS G12D- and the NRAS Q61R-induced tumors (24). Collectively these studies have formed a basis for the notion that the different RAS mutations display a wide variety of transforming potentials depending on various factors including the codon site, RAS protein, and cell type.

GDP and GTP Binding

As mutations at codons 12, 13, and 61 cluster around the nucleotide-binding site, amino acids exchange at these positions may alter the interactions between RAS proteins and GTP or GDP (25). In their 1986 manuscript, Der et al. also analyzed the GDP and GTP binding affinity in WT and 17 different HRAS mutants (8). Both GDP and GTP appeared to bind WT HRAS or the HRAS Q61L mutant variant with the same affinity. In addition, the kinetics of GTP hydrolysis between WT and mutant HRAS was studied. All the analyzed mutants reduced GTP hydrolysis compared to WT HRAS, which correlates with the oncogenic activation of RAS. However, Q61L, Q61W, Q61N, Q61G, Q61P, and Q61E mutants displayed indistinguishable GTP hydrolysis, with one-eighth reduction in the rate compared to WT HRAS (8). Interestingly, these HRAS mutants have different transforming potentials, suggesting that compromised GTP hydrolysis is necessary but not sufficient for a complete RAS activation.

More than 30 years later, further studies continue reporting differences in GTP binding and intrinsic or GAP-mediated GTP hydrolysis (6, 22, 25). Smith et al. detected KRAS G12V

in the GTP-bound conformation, which was consistent with its high transforming potential (22). In addition, experiments in MCF10A cells transduced with different KRAS mutations revealed that WT KRAS and KRAS G12D and G13D were able to bind GTP with a similar affinity as control cells, which only express endogenous KRAS, after EGF stimulation (6). In contrast, KRAS G12C, G12V, and G13C mutants showed an increase in GTP-binding up to 2-fold and up to 5- to 6-fold in KRAS Q61H mutant compared to control cells (**Figure 1A**, **Table 2**) (6). A similar study analysing WT KRAS and KRAS mutations G12A, G12C, G12D, G12R, G12V, G13D, Q61L, and Q61H showed that the kinetics of GDP-GTP exchange were similar between all mutant proteins and WT KRAS, with the exception of KRAS G13D (25). This mutation showed a faster GDP and GTP exchange than the WT KRAS, suggesting that KRAS G13D mutant protein might be auto-activated by nucleotide exchange easier than other mutant variants. Moreover, the fast nucleotide exchange of the KRAS G13D mutant may contribute to a more aggressive biology of tumors harboring this mutation (25).

Additionally, this study also reported that while KRAS G12A, G12R, Q61H, and Q61L decreased GTP hydrolysis speed approximately by 40- to 80-fold as compared to WT KRAS, the G12C mutation had a minimal impact in this respect. Regarding this endpoint, KRAS G12D, G12V, and G13D mutant proteins displayed an intermediate effect (25). When analyzing GAP-mediated GTP hydrolysis, all KRAS mutants showed 97–99% reduction in GAP-mediated GTP hydrolysis compared to WT KRAS. In the case of KRAS G12A and Q61L, the GAP-stimulated rate was 15- to 25-fold higher than the intrinsic GTP hydrolysis rate, which may suggest that these mutants keep part of the GAP-mediated GTP hydrolysis activity (**Figure 1A**, **Table 2**) (25).

The GAP-mediated and the intrinsic nucleotide exchange were studied in tumors derived from an *in vivo* melanoma model (24). WT NRAS and NRAS G12D and Q61R mutant proteins showed similar GDP exchange rates, but differed significantly in their GTP exchange rates, with WT NRAS showing the fastest GTP exchange and NRAS Q61R the slowest. These differences were more significant when the reaction was catalyzed by GEFs. Moreover, NRAS Q61R mutant protein also showed the slowest intrinsic GTP hydrolysis (1,150- and 2,300- times slower than NRAS G12D and WT NRAS, respectively) (**Table 2**) (24).

These data suggest that not only intrinsic GTP hydrolysis is important for mutant RAS transformation. GAP-mediated nucleotide exchange might also have an effect on RAS mutant proteins transformation, which makes it more difficult to anticipate the transforming potential of a particular RAS mutant variant.

Anchorage-Independent Growth

Anchorage-independent growth is the ability of transformed cells to grow in suspension or unattached to any matrix (6), an associated characteristic for tumor metastasis regulated by the RAS/RAF/MAPK signaling pathway (29). Seeburg et al. reported that with the exception of HRAS WT and HRAS G12P, all the HRAS codon 12 mutants were able to grow in soft agar (7), results paralleling their data on transforming potential of these mutant proteins.

Voice et al. also assessed the anchorage-independent growth of the G12V mutation of different RAS proteins in RIE-1 and Rat-1 cells (17). Unlike the KRAS4B G12V-harboring RIE-1 cells, same cells expressing the KRAS4A G12V mutants were able to grow in soft agar, correlating with the ability of these proteins to form foci. However, the HRAS G12V cells failed to grow in soft agar despite their ability to form foci in RIE-1 cells, whereas the NRAS G12V mutation enabled RIE-1 cells to grow in soft agar despite its little transforming activity. Interestingly, all these RAS G12V proteins enabled growth in soft agar when expressed in Rat-1 fibroblasts, although KRAS4B and NRAS showed reduced transforming potential in this cell line (**Table 2**) (17). This suggests that the ability to grow independently of anchorage depends on a particular cellular intrinsic milieu rather than on the RAS proteins harboring the substitution.

Later, immortalized human bronchial epithelial cells with specific shRNA knockdown of p53 mRNA expressing KRAS G12C were able to form colonies in soft agar compared to KRAS G12D- and KRAS WT-transfected cells (23), suggesting that the genetic background could also affect the phenotypical manifestation of mutant RAS variants. Moreover, Stolze et al. showed that the overexpression of KRAS G12D, G13C, and G13D in MCF10A cells yielded a very high colony number in soft agar. However, the expression of these mutants at physiological levels did not confer anchorage-independent growth (6). In the case of clones expressing KRAS Q61H, G12V, and G12C, a slight increase in colony number was observed compared to control cells expressing endogenous KRAS, which also correlated with the highest GTP-bound levels reported in the same study (**Table 2**) (6). Collectively, these results suggest that some RAS mutant proteins might have the ability to grow independent of anchorage, which may depend on cell type and genetic background.

Migration

Cell migration is controlled by several RAS downstream pathways, such as the RAS/RAF/MAPK pathway (29). As this process involves cancer cells local invasion and metastasis (6), several studies analyzed the migration abilities of distinct RAS mutant proteins (6, 17, 30). Voice et al. reported that the KRAS4B G12V variant could accelerate COS-7 cells migration while HRAS G12V had a minimal effect, compared with cells transfected with GFP alone. However, KRAS4A G12V- and NRAS G12V-expressing cells were unable to migrate, even at higher expression levels (17). A later study by Walsh et al. (30) showed that KRAS4B G12V-transfected REF-52 cells migrated at the speed of 18 μm/h, while the HRAS G12V cells at 12 μm/h (30). In addition, Stolze et al. reported that the overexpression of KRAS G12D, G12V, and G13D enabled MCF10A cells migration (6). Similarly to KRAS-overexpressing mutant proteins, control cells were able to migrate after EGF addition (**Table 2**). However, none of the studied mutations expressed at physiological levels increased migration abilities compared to WT KRAS or control cells, which expressed endogenous KRAS (6). Therefore, these results contrast with previous studies (17, 30) as only the overexpression of KRAS mutant variants leads to cell migration.

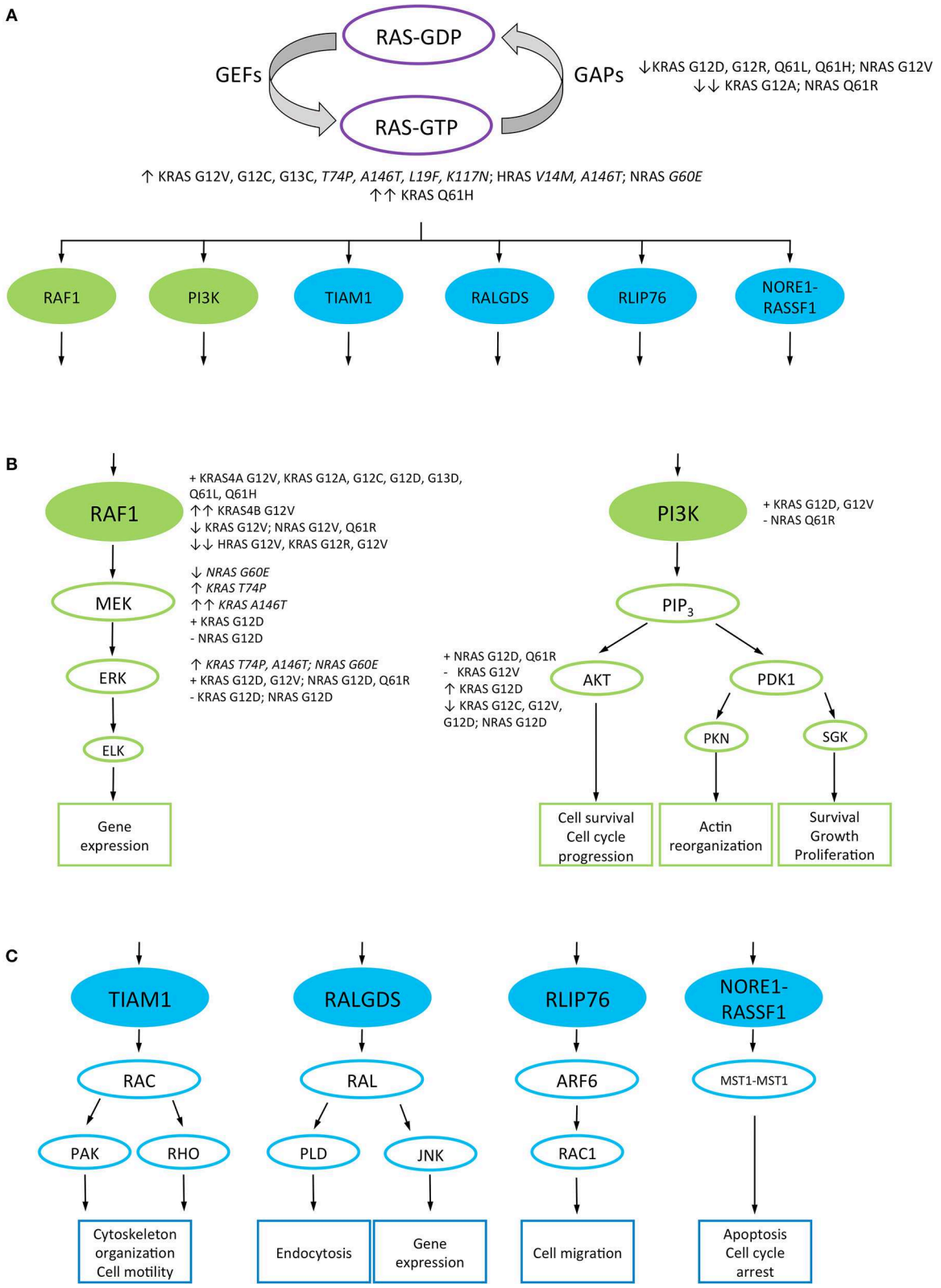

FIGURE 1 | RAS downstream signaling pathways. RAS canonical and non-canonical downstream signaling pathways are represented in green and blue, respectively. RAS proteins non-canonical mutations are highlighted in italics. **(A)** RAS proteins signal between "on" and "off" conformations, given by the binding of GTP and GDP,

(Continued)

FIGURE 1 | respectively. The transition from the inactive to the active form is catalyzed by guanine nucleotide exchange factors (GEFs), while the GTPase-activating proteins (GAPs) control the inverse reaction. RAS-GTP proteins interact with different downstream effector proteins to activate several signaling pathways. RAS mutant variants which decrease GAP-mediated GTP hydrolysis and strongly bind GTP are represented. **(B)** RAS canonical downstream pathways: RAS/RAF1/MAPK and PI3K/AKT signaling pathways, and their cellular output. The ability and relative strength of different RAS mutant proteins to interact or activate effector proteins are mentioned. **(C)** A representation of the non-canonical downstream pathways of RAS and their cellular output. GEFs, guanine nucleotide exchange factors; GAPs, GTPase-activating proteins; RAF1, rapidly accelerated fibrosarcoma 1; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; PDK, 3-phosphoinositide-dependent protein kinase; ELK, ETS Like-1 protein; PIP_3, Phosphatidylinositol (3,4,5)-triphosphate; PKN, protein kinase N1; SGK, serum and glucocorticoid-regulated kinase; RAC, Ras-related C3 botulinum toxin substrate 1; RAL, Ras-related protein Ral; TIAM1, T-lymphoma invasion and metastasis-inducing protein 1; PAK, p21-activated kinase; RHO, Ras homologous protein; RALGDS, Ral guanine nucleotide dissociation stimulator; PLD, phospholipase D; JNK, c-Jun N-terminal kinase; RLIP76, ralA-binding protein 1; ARF6, ADP-ribosylation factor 6; RASSF1, Ras associated domain-containing protein 5; MST1, serine/threonine kinase 4; ↑, high interaction or downstream proteins activation; ↑↑, very high interaction or downstream proteins activation; ↓, low interaction or downstream proteins activation; ↓↓, very low interaction or downstream proteins activation; +, interaction or activation of the downstream effector proteins; –, inability to interact or activate the downstream effector protein.

Animal model studies also evaluated metastatic capacities of tumors harboring KRAS mutations (31, 32). A recent *in vivo* study by Tang et al. analyzed tumor formation and their metastatic capacity in KRAS G12D $p53^{-/-}$ mice (31). As compared to $KRAS^{WT/WT}$ $p53^{-/-}$ and $KRAS^{WT/WT}$ $p53^{+/+}$ (wild type) mice, animals harboring both KRAS G12D and $p53^{-/-}$ alterations developed tumors with 100% penetrance and their size increased over time. Moreover, tumors from KRAS G12D $p53^{-/-}$ mice were able to metastasize to the liver, spleen and kidney whereas tumors formed in $KRAS^{WT/WT}$ $p53^{-/-}$ and WT animals were not (31). Previously, Whipple et al. studied the involvement of the heparin sulfate proteoglycan Glypican-1 (GPC1) in KRAS G12D-driven mouse model of pancreatic cancer (32). At 65 days of age, 14 of 14 animals harboring wild type GPC1 developed large pancreatic tumors that invaded the surrounding organs, whereas 16 of the 20 $GPC1^{-/-}$ mice developed smaller and non-invasive tumors (32). Moreover, four primary cancer cell lines were derived from tumors developed in $GPC1^{+/+}$ (F1015 and F1048) and $GPC1^{-/-}$ (J444 and J1032) mice. These cell lines formed tumors in $GPC1^{+/+}$ nude mice. However, *in vitro* studies revealed that J444 and J1032 cells exhibited decreased invasion capacities in response to FGF-2 compared to F1015 and F1048 primary cancer cells (32). To determine whether the loss of GPC1 was also involved in a reduction of invasion *in vivo*, tumor fragments from $GPC1^{+/+}$ and $GPC1^{-/-}$ mice were implanted into the pancreas of athymic $GPC1^{+/+}$ and $GPC1^{-/-}$ animals. Two weeks after the implantation, only 2 out of 14 $GPC1^{-/-}$ mice developed metastasis in the mesentery, while 9 out of 15 $GPC1^{+/+}$ mice developed several (over 100 per animal) mesenteric metastases and three of them also showed multiple renal metastases (32). Therefore, these two studies suggest that not only KRAS G12D-expressing tumors are able to migrate and metastasize *in vivo*, but also other RAS mutant proteins may have the capacity to invade the surrounding tissues, as reported by *in vitro* studies (6, 17, 30).

All together these findings strongly indicate that point mutations at codons 12, 13, and 61 of RAS display different phenotypical characteristics compared to WT RAS. Depending on the RAS isoform and the amino acid substitution, RAS mutant proteins differ in their transforming ability, GTP binding, anchorage-independent growth and migration capacities. But these results also suggest that RAS mutations show a different biological behavior depending on the cell type where they are expressed, adding complexity to our understanding of RAS biology.

MUTANT RAS PROTEINS DIFFER IN THEIR BIOCHEMICAL SIGNALING

Wild type RAS proteins are able to activate different signaling pathways depending on particular cell type, tissue and their subcellular localization (21). As codon 12, 13, and 61 mutations are located around the nucleotide-binding site, it has been suggested that the nucleotide exchange may alter the affinity of mutant RAS proteins for downstream effectors proteins (17, 25).

Activation of the RAF1/MAPK Pathway

The RAF1 serine/threonine kinase is one of the best characterized RAS effector proteins, located directly downstream of RAS in the MAPK pathway (25). Considering that point mutations at codons 12 and 61 of HRAS differ in their phenotypical properties as previously reported (7, 8), Voice et al. hypothesized that mutant RAS proteins might activate RAF1 differentially (17). The co-transfection of WT RAF1 and G12V HRAS, NRAS, KRAS4A, and KRAS4B in COS-1 cells confirmed that RAS proteins differ in their ability to activate RAF1. KRAS4B activated RAF1 8.4-, 4.4-, and 2.3-fold better than HRAS, NRAS, and KRAS4A, respectively, proposing the following hierarchy in RAF1 activation by these RAS proteins: KRAS4B > KRAS4A >>> NRAS > HRAS (**Figure 1B**) (17). Later, Hunter et al. analyzed the affinity of different KRAS mutants for the RAS-binding domain (RBD) of RAF1 (25). KRAS G12A, G12C, G13D, Q61L, and Q61H showed 1.2- to 2.3-fold decrease in relative affinity compared to WT KRAS and KRAS G12D, G12R and G12V displayed even more pronounced decrease in affinity for RAF1 (4.8-, 6.2-, and 7.3-fold, respectively) (**Figure 1B**, **Table 3**) (25). These results contrast with those reported by Voice et al. (17) where KRAS G12V showed a high activation of RAF1. However, these differences could be related to the method used in each study to detect RAF1 activation.

Other works analyzed the activation of the MAPK pathway by assessing ERK activation through its phosphorylation status (6, 33). For example, transduction of primary rat hepatocytes with HRAS G12V, but not with KRAS G12V, showed a strong activation of ERK2 independently of EGF stimulation

TABLE 3 | Interaction and activation of different RAS proteins downstream effectors.

Characteristic/mutation	KRAS4A G12V	KRAS4B G12V	HRAS G12V	NRAS G12V	KRAS G12A	KRAS G12C	KRAS G13D	KRAS Q61L	KRAS Q61H	KRAS G12D	KRAS G12R	KRAS G12V	NRAS G12D	NRAS Q61R
RAF1 interaction	High (17)	Very high (17)	Very low (17)	Low (17)	High (25)	High (25)	High (25)	High (25)	High (25)	Low (25) – (27)	Very low (25)	Very low (25) + (27)	High (24)	Low (24)
ERK activation			Strong (33)							–(27) + (28)		Low (33) + (27)	– (28) + (24)	+ (24)
MEK activation										+ (28)			– (28)	
PI3K interaction										+ (27)		+ (27)		Low (24)
AKT activation						Low (23)				Strong (27) Decreased (28)		Low (23) (27)	Decreased (28) + (24)	+ (24)
70S6K activation						Strong (23)				Strong (23)				
RPS6 activation							High (6)							
RAC interaction		Strong (30)	Low (30)							– (28)			– (28)	
RAL interaction						+ (23)				– (23, 28)			– (28)	

Summary of the activation of downstream signaling pathways by RAS mutant proteins. Proteins activation was assessed by phosphorylation at different residues. +, interaction or activation of the effector; –, inability to interact or activate the downstream effector protein; RAF1, rapidly accelerated fibrosarcoma 1; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; 70S6K, ribosomal protein S6 kinase 1; RPS6, ribosomal protein S6; RAC, Ras-related C3 botulinum toxin substrate 1; RAL, Ras-related protein Ral.

(33), revealing that different RAS proteins harboring the same mutation activate downstream signaling pathways differently. On the other hand, a more recent study by Stolze et al. reported no differences in ERK phosphorylation levels in MFC10A cells expressing KRAS G12D, G12V, G12C, G13D, G13C, and Q61H at low levels compared to WT KRAS or control cells, expressing endogenous KRAS (**Figure 1B**, **Table 3**) (6). These data suggest that not only the cell type but also the level of expression may influence the pattern and intensity of RAS mutations signaling pathways activation.

Mutant RAS signaling differences have also been identified in tumors derived from animals. Céspedes et al. reported that mouse tumors expressing KRAS G12V, but not G12D, were able to interact with RAF1 and showed a high phosphorylation of ERK (27). Interestingly, Haigis et al. reported different results concerning KRAS G12D (28), where KRAS G12D but not NRAS G12D could activate MEK and ERK in colonic epithelium of genetically engineered mice (**Figure 1B**). However, the activation of both KRAS G12D and NRAS G12D proteins at the same time only appeared in the differentiated cells at the top of the colonic crypt and not in the undifferentiated cells at the bottom of the crypt, suggesting that the exact activation pattern of ERK depends on the cell type (28). Recently, a study by Burd et al. revealed that NRAS Q61R bound RAF1 with lower affinity than WT NRAS or NRAS G12D in melanoma. However, both NRAS Q61R and G12D mutant proteins activated ERK at variable levels (**Figure 1B**, **Table 3**) (24), suggesting that the activation of MAPK pathway in melanoma is codon-independent.

PI3K/AKT/mTOR Pathway Activation

RAS proteins also trigger the activation of the PI3K/AKT/mTOR pathway to promote cell survival by activating survival factors and inhibiting apoptotic proteins (5). Therefore, different *in vitro* (6, 23) and *in vivo* (24, 27, 28) studies also assessed the activation of this pathway by the interaction of various RAS mutated variants with PI3K and different downstream proteins phosphorylation, such as AKT, 4EBP, or RPS6. The comparison of KRAS G12C and G12V with WT KRAS in a panel of 67 non-small cell lung cancer cell lines showed that these mutations decreased AKT activation compared to WT KRAS (**Figure 1B**) (23). Despite this low activation of AKT, cells expressing KRAS G12C or G12V showed the same phosphorylation levels of 70S6K and 4EBP proteins compared to WT KRAS in the absence of serum whereas the addition of serum to the media enabled KRAS G12C and G12V to strongly phosphorylate 70S6K compared to WT KRAS (**Figure 1B**, **Table 3**) (23). Later on, Stolze et al. reported that KRAS G12D, G12V, G12C, G13D, G13C, and Q61H expressed at low levels in MCF10A cells did not show higher phosphorylation of PDK1 and AKT compared to WT KRAS or control cells which only express endogenous KRAS (6). Nevertheless, both KRAS G13D low expression as well as overexpression were associated with a high RPS6 phosphorylation upon EGF (**Table 3**), indicating that this mutant enabled mTOR pathway activation but seemingly not through the PI3K/AKT pathway (6). Interestingly, the results reported for KRAS G12D in this study are inconsistent with those obtained by Ihle et al. (23), suggesting that the cell type and/or the genetic background may alter the activation of the downstream signaling pathways.

In mouse xenograft tumors, Céspedes et al. showed that both KRAS G12V and G12D mutants were able to interact directly with PI3K (27). However, KRAS G12V was unable to activate AKT despite its interaction with PI3K whereas KRAS G12D strongly activated AKT (27). Contrary, KRAS G12D as well as NRAS G12D expressed in the mouse colonic epithelium showed a decrease in AKT phosphorylation compared to WT animals (**Table 3**) (28), proposing once again that cell type may alter the downstream signaling pathways activation. In addition, studies employing a mouse model of melanoma revealed that NRAS Q61R binds PI3K with lower affinity than WT NRAS or NRAS G12D while NRAS G12D and Q61R activate AKT at variable levels (**Figure 1B**, **Table 3**) (24), indicating that the activation of the PI3K pathway in melanoma is codon-independent.

Other Effectors Activation

RAS proteins can also interact and activate effectors that do not belong to the MAPK and the PI3K canonical cascades. For example, RAC, a subfamily of small GTPases of the RHO family, can interact with RAS via the RacGEF called Tiam1. The RAS/RAC signaling pathway controls several cellular functions through the regulation of actin cytoskeleton, including cell morphology, locomotion, and polarity (34). Another RAS downstream effector subfamily is the RAL group of proteins, which are involved in membrane trafficking, proliferation, survival and metastasis in many types of cancer (35).

Walsh and Bar-Sagi studied the differential activation of RAC in COS-1 cells transduced with KRAS4B G12V and HRAS G12V (30). Cells expressing KRAS4B G12V activated RAC more effectively than HRAS G12V-transduced cells (30). Moreover, *in vivo* studies also analyzed the modulation of RAC (28). The expression of either KRAS or NRAS G12D in the mouse colonic epithelium did not promote RAC modulation. In addition, this study revealed that KRAS and NRAS G12D were unable to modulate RAL activation (**Table 3**), indicating the limited signal activation of these mutants *in vivo* (28). These results are consistent with a later *in vitro* study (23), in which WT KRAS and KRAS G12C, but not KRAS G12D, where able to activate RALA and RALB effector proteins (**Table 3**) (23).

Furthermore, Stolze et al. analyzed whether any of the KRAS codon 12, 13, and 61 mutations included in the study was able to increase the activation of EGFR and p53 (6). Only KRAS G13D ectopically expressed in MCF10A breast cancer cells promoted an increase in total and phosphorylated EGFR. Moreover, KRAS G13D stimulated a strong phosphorylation of p53 at serine 15, a site known to be phosphorylated by the master DNA damage response kinase ATM, which suggests that particularly the KRAS G13D mutant might induce a DNA damage response under replicative stress (6). As other KRAS mutants did not show the activation of EGFR and p53, these authors suggest that this activation could be the biological explanation of the favorable clinical outcome of colorectal cancer patients harboring KRAS G13D mutation treated with anti-EGFR therapy compared to patients with KRAS codon 12 mutations (6).

Signaling Pathway Activation and Outcome

Several studies assessing the impact of RAS mutations on cell behavior correlated the signaling pathways activated by a specific mutation with a particular outcome such as cell death or cell cycle redistribution (27, 28, 30, 33). Joneson and Bar-Sagi reported that overexpression of HRAS G12V induced apoptosis in a panel of primary and immortalized cells (36). However, the co-transfection of REF-52 fibroblasts with HRAS G12V and activated RAC blocked HRAS G12V-induced apoptosis, indicating that RAC signaling pathway is sufficient to antagonize RAS proapoptotic signals (36). As KRAS4B G12V and HRAS G12V differentially activate the RAC signaling pathway (30), Walsh and Bar-Sagi hypothesized that these mutant variants may differ in their ability to induce apoptosis (30). The overexpression of HRAS G12V in REF-52 fibroblasts induced apoptosis in 38% of the cells whereas the overexpression of KRAS4B G12V had no effect on cell viability, results that are consistent with the RAC activation levels reported in this work for each mutant (30).

Céspedes et al. described that expression of KRAS G12V in xenograft tumors enhanced Retinoblastoma (Rb) protein phosphorylation and was accompanied by an increase in cyclin B1 expression. This could be related to the high proliferation rate of these tumors and their fast G1/S and G2/M transitions (27). However, no differences in the level of procaspase 3 or 9 proteolysis were detected between KRAS G12V and G12D tumors, leading to a similar activation of apoptosis (27). In a later study, Haigis et al. exposed genetically engineered mice to 2.5% dextran sodium sulfate (DSS). Mice expressing WT KRAS or KRAS G12D in the colonic epithelium were sensitive to DSS-induced apoptosis in this tissue, whereas in mice expressing NRAS G12D little or no apoptotic effect was observed. However, NRAS G12D mice were sensitive to irradiation-induced apoptosis in the colonic epithelium, indicating that the effect of this mutation might depend on the apoptotic stimuli and the activated cell death pathway (28).

Along similar lines of investigations, Rosseland et al. studied proliferation of primary rat hepatocytes transfected with HRAS G12V or KRAS G12V (33). Compared to control cells expressing the yellow fluorescent protein, the proliferation rate of HRAS G12V, but not of KRAS G12V, was increased after EGF stimulation (33). In addition, an earlier study by Oberhammer et al. reported that TGF-βI increased the incidence of apoptosis in hepatocytes by 5-fold, suggesting that TGF-βI is involved in the initiation of apoptosis in the liver (37). Based on these results, Rosseland et al. tested whether HRAS G12V and KRAS G12V were able to induce apoptosis in rat hepatocytes after TGF-βI stimulation (33). Hepatocytes expressing HRAS G12V or KRAS G12V had reduced apoptosis compared to untransfected control cells, demonstrating that both RAS mutant proteins have a pro-survival effect (33). To further investigate the signaling pathways involved in this phenomenon, PI3K and ERK pathways were blocked with different inhibitors. In untransfected control cells, apoptosis was only slightly increased after ERK pathway inhibition while PI3K inhibition strongly increased apoptosis, indicating that both ERK and PI3K pathways are involved in survival of primary hepatocytes (33). In HRAS G12V-transfected hepatocytes, the inhibition of ERK or PI3K pathways did not reduce apoptosis after TGF-βI stimulation. However, in KRAS G12V-transfected cells, only the inhibition of PI3K pathway showed an increase in hepatocytes apoptosis (33). This suggests that apoptosis is triggered through different pathways depending on the RAS isoform and mutation.

Taken together, a single amino acid change at codons 12, 13, or 61 of RAS alters the interaction of these proteins with the downstream effectors. Depending on the RAS protein and the amino acid substitution, RAS mutants activate differently the canonical and non-canonical downstream signaling pathways *in vitro* and *in vivo*. Moreover, the amino acid substitutions have been correlated with a particular outcome, such as proliferation or cell death and hence these observations should be further exploited and considered for the choice of treatment of patients.

RAS MUTATED VARIANTS DIFFER IN THEIR TRANSCRIPTOMIC, PROTEOMIC AND METABOLOMIC PROFILES

Protein and metabolic stress are two recognized hallmarks of cancer in which different cellular signaling pathways are altered to confer an advantage to cancer cells and sustain their growth and proliferation (38). To get insights into global cellular networks that underlie various RAS mutated variants, various works have been assessing the transcriptomic, proteomic/phosphoproteomic and metabolic profile of RAS mutant variants to possibly associate and understand the basis of their phenotypic disparities (6, 39–41).

Transcriptomics

Roberts et al. analyzed whether the expression pattern of 2,100 genes involved in cancer progression differ between KRAS G12V- and HRAS G12V-expressing Caco-2 colorectal adenocarcinoma cells and found 71 differentially regulated genes (42). KRAS G12V significantly up-regulated the expression of genes in the cytokine/chemokine family, for example *CD40L*, *CD27L*, *CD30L,* and *TRAF-5* and regulated processes related to immune response, development, nucleotide excision repair, cell proliferation, transcription and cytokine signaling (42). HRAS G12V-expressing cells up-regulated vimentin and down-regulated villin and fibronectin, correlating with the main biological processes controlled by HRAS G12V such as cell-matrix and cell-cell adhesion, protein biosynthesis, integrin-mediated signaling, cell motility and cell cycle checkpoint control, most of them involved in the epithelial-mesenchymal transition (42). In addition, this work assessed changes in the transcriptome profile *in vivo*, revealing 26 genes differentially expressed between KRAS G12V and HRAS G12V tumors. Up-regulation of Notch signaling, cell motility or microtubule cytoskeleton were detected in KRAS G12V whereas genes involved in cell adhesion and motility were deregulated and those involved in organogenesis/angiogenesis and cytokinesis processes down-regulated in HRAS G12V tumors (42). Later, to provide insights into the differential response of KRAS G12D and KRAS G13D mutant variants to anti-EGFR therapy, Stolze et al. compared the gene expression of these mutants and

WT KRAS (6). The analysis of 2,487 genes demonstrated that WT KRAS and control MCF10A cells, expressing endogenous KRAS only, had a similar expression profile, while KRAS G12D- and G13D-expressing cells showed a different one, clustering them separately from each other and from the WT KRAS and control cells (6). Moreover, this work identified, 11,207 and 1,011 genes significantly up- and down-regulated, respectively, in KRAS G13D compared to KRAS G12D-expressing cells (6). The analysis of the top 300 up- and down-regulated genes in both mutants and their comparison to luminal and basal/mesenchymal breast cancer gene expression profiles reported previously (43, 44), associated KRAS G13D with the basal/mesenchymal and KRAS G12D with the luminal breast cancer subtype. Thus, KRAS G13D mutant variant highly expressed genes such as those encoding for integrins, collagens, and proteases, compared to KRAS G12D (6). Furthermore, Stolze et al. were able to identify mutation-specific signaling networks: 87 out of 300 top up-regulated genes were included in a cluster associated with cytokine-induced cell migration. In this cluster, the top up-regulated cytokines were *CXL1*, *IL1B,* and *IL8*, which showed >10-fold increase in their transcription in KRAS G13D-expressing MCF10A cells compared to KRAS G12D-expressing cells (6).

Recently, KRAS G13D transcriptomic profile has been reported also by Charitou et al. for the isogenic HKe3 colorectal cancer cell line expressing WT KRAS or KRAS G13D (40). More than 6,000 genes were identified to be differentially expressed between WT KRAS- and KRAS G13D-expressing cells. Pathway analysis of up-regulated genes revealed that ribosome biogenesis, mRNA translation, regulation of gene expression and metabolism were among the most significantly enriched processes in cells expressing KRAS G13D (40). Metabolic stress is a recognized hallmark of cancer. To respond to the high energetic demand, cancer cells increase ribosome biogenesis to translate mRNAs into proteins in response to their high metabolic rate. In this respect, some metabolic pathways were also up-regulated in KRAS G13D-expressing cells compared to WT KRAS-expressing cells. These pathways include glycolysis/gluconeogenesis, steroid biosynthesis and glycine, serine and threonine metabolism (40). The steroid biosynthesis pathway has cholesterol as its final product. It has been reported that oncogene-transformed cells require high levels of cholesterol to support their rapid growth (45, 46). These results suggest that KRAS G13D-expressing cells might have a higher metabolic rate compared to cells expressing other KRAS mutant variants. Moreover, among the down-regulated genes in the KRAS G13D-expressing HKe3 cells, the most enriched pathways were the type I interferon signaling pathway and the antigen processing and presentation pathway, which may help cancer cells to evade the host immune response (40).

Jiang et al. analyzed the differences in both protein and microRNA (miRNA) gene expression of NRAS Q61K-, Q61L-, and Q61R-driven melanomas compared to those expressing WT NRAS (47). One thousand one hundred fifty protein-coding genes were significantly differentially expressed, with 469 and 681 up- and down-regulated, respectively, in NRAS Q61K, Q61L, and Q61R samples compared to the WT NRAS samples. In the case of miRNAs, the expression of 49 miRNAs was altered, with 26 and 23 up- and down-regulated, respectively (47). Moreover, this work identified pathways associated with these deregulated genes and miRNAs; the most significant ones in both deregulated genes and miRNAs were the MAPK signaling pathway, followed by the PI3K/AKT and the CDK/4/6/Rb pathways (47). The MAPK pathway is altered in most melanomas, while PI3K/AKT pathway is involved in melanoma initiation and its therapeutic resistance (48). In addition, CDK4 is a regulator of the G1/S cell cycle checkpoint, and its targeting using Palbociclib has demonstrated antitumor activity in melanoma (47). Other signaling pathways were also enriched in NRAS-mutated melanoma, including pathways involved in calcium, TGF-β, and WNT signaling, actin cytoskeleton, focal adhesion and axon guidance, suggesting them as novel candidate pathways for melanoma treatment (47).

Proteomics and Phosphoproteomics

Hammond et al. investigated proteomics and phosphoproteomics signatures of isogenic SW48 colorectal cancer cell lines expressing either WT KRAS or KRAS G12D, G12V, or G13D variants (49). Hierarchical clustering of proteomic and phosphoproteomic data revealed that KRAS G12D- and G12V-expressing cells had similar signatures, but these were different from KRAS G13D-expressing cells. KRAS G13D showed more proteins and phosphopeptides up-regulated (around 50% compared to WT KRAS) than KRAS G12D-expressing cells (<10% compared to WT KRAS) (49). These findings suggest that specific mutated codons define different proteomic and phosphoproteomic signatures. In addition, same authors assessed in this work proteins and phosphoproteins differentially expressed in KRAS G12D and G13D to determine whether a codon-specific signature could be found (49). The analysis of the proteomes revealed that the expression of mitochondrial proteins involved in oxidative phosphorylation was decreased in KRAS G13D-expressing SW48 cells compared to KRAS G12D-expressing cells. Moreover, KRAS G13D showed a decrease in 5 members of the cytochrome bc1 complex (complex III) and succinate dehydrogenase of complex II of the mitochondrial respiratory chain. In contrast, the expression of aldehyde dehydrogenase (ALDH3A1) was increased in KRAS G13D-expressing cells and decreased in KRAS G12D-expressing SW48 cells (49). Regarding the phosphoproteomic data, MET Thr995 and Caveolin-1 Ser37 sites exhibited >10-fold increased abundance in KRAS G12D as compared to KRAS G13D, explained by an increase in protein expression, while BRAF Ser729 phosphorylation was decreased in KRAS G12D vs, G13D-expressing cells. These results were further confirmed in a panel of 275 lung, pancreas and colon cancer cell lines harboring KRAS codon 12 and 13 mutations or WT KRAS (49). In addition, this work identified the doublecortin-like kinase 1 (DCLK1) protein levels to be at least 8-fold up-regulated in KRAS G12D-expressing SW48 cells compared to WT KRAS-expressing cells. However, qPCR analysis revealed that the increased levels of DCLK1 are due to transcriptional up-regulation, and this increase in the mRNA level is reversed upon KRAS knockdown, indicating that KRAS directly regulates DCLK1 expression (49). DCLK1 is frequently

overexpressed in colorectal cancer (50) and has been identified as a colorectal cancer stem cell specific marker, whose depletion promotes polyps regression (51). Moreover, a KRAS synthetic lethal screening previously identified the related kinase DCLK2 as a hit in the colorectal DLD-1 cell line (52), suggesting DCLK1 as a potential target for combination therapy in the context of KRAS-mutated colorectal cancer.

Concerning HRAS mutant variants, Doll et al. profiled the proteomic and phosphoproteomic changes in HRAS G12V-transformed normal human astrocytes (53). Two hundred and seventy-eight phosphosites in 154 proteins and 245 phosphorylation sites in 160 proteins were up- and down-regulated, respectively, in WT HRAS- vs. HRAS G12V-expressing cells. The analysis of these up-regulated phosphosites revealed that the MAPK, PI3K/AKT and mTOR pathways were significantly up-regulated in HRAS G12V-expressing astrocytes as compared to WT HRAS cells (53). In the MAPK pathway, Sprouty 4, whose expression is induced by this pathway, showed 10-fold upregulation at protein level. Regarding PI3K/AKT, the Niban protein (FAM129A), which regulates the phosphorylation of the transcription factor EIF2A, showed 2-fold upregulation in HRAS G12V-expressing cells. Moreover, the phosphorylation of RPTOR on Ser863 showed a 2.6-fold upregulation (53). This phosphosite is involved in mTORC1 activation, whose signaling activates different transcription factors involved in transcription of cell proliferation and survival proteins (54). This work also identified other deregulated proteins downstream of HRAS. For example, six of the 13 RAL direct downstream effectors of RAS involved in endocytosis and gene expression (**Figure 1**), including RALA and RALB, showed 2-fold or higher upregulation at protein level (53). Collectively, these results indicate that HRAS G12V mainly activates the canonical downstream pathways of RAS, triggering changes in gene expression that facilitate cancer cells proliferation and survival.

Interestingly, Santra et al. recently reported differences in HRAS G12V signaling according to its subcellular localization in HeLa cells (41). Three hundred and ninety-seven proteins that interact with HRAS G12V were identified across plasma membrane (PM), lipid rafts (LR), endoplasmic reticulum (ER) and Golgi apparatus (GA), out of which 341 were new interactors. Only 5% of the interactors were identified in all subcellular localizations, whereas ∼53% were specific for one of the localizations (41). The pathway enrichment analysis revealed that HRAS G12V not only regulates receptor tyrosine kinase (RTK) signaling, but also biosynthesis and metabolic pathways mainly from the ER, while immune signaling is triggered from the GA. Additionally, lipid biosynthesis pathways were also enriched (41), a finding which might be related to changes in cellular metabolism. This work also assessed changes in the phosphoproteome of HRAS G12V-expressing cells according to its subcellular localization (41). One thousand four hundred sixty-one phosphosites in 1,078 proteins were differentially phosphorylated, with 74% of the phosphosites activated at LR and PM (41). The analysis of the enriched pathways showed that HRAS G12V-expressing cells regulate RTK signaling and other signaling pathways, such as WNT, MAPK, or insulin signaling pathways (41). The results of this work confirm previously described findings that apart of subcellular localization-specific differences in RAS WT proteins signaling (20, 21), also RAS mutant variants may signal differently depending on the particular cellular membrane where they are anchored, thus increasing the complexity of RAS signaling.

With respect to NRAS mutant variants, Posch et al. analyzed the differences in the phosphoproteomic profile of primary human melanocytes (PHMs) transfected with WT NRAS and NRAS G12V or Q61L (55). One hundred and sixty-three phosphosites in 132 proteins were differentially phosphorylated between NRAS G12V and WT NRAS, with 83 and 80 phosphosites up- and down-regulated, respectively. PHMs expressing NRAS Q61L showed 202 phosphosites in 150 proteins differentially regulated compared to PHMs expressing WT NRAS, with 73 and 129 phosphosites up- and down-regulated, respectively. Posch et al. also identified 126 proteins and 163 phosphosites 2-fold differentially regulated between NRAS G12V- and NRAS Q61L-expressing cells (55), indicating that both NRAS G12V and Q61L have different phosphoproteomic profiles. Moreover, this work assessed the enriched canonical pathways regulated by each NRAS mutant. Whereas, NRAS Q61L-expressing cells showed an overrepresentation of phosphopeptides related to the MAPK signaling pathway, NRAS G12V had an enrichment of the "14-3-3-mediated"- pathway, which is related to the PI3K/AKT signaling pathway due to the modulation of PI3K signaling by 14-3-3 protein (55). To confirm these results, changes in the phosphorylation level of AKT, RPS6, MEK and ERK were determined. While NRAS G12V-expressing cells showed an increase in AKT and RPS6 phosphorylation levels, NRAS Q61L-expressing cells showed an increase in MEK and ERK phosphorylation levels (55). These data suggest that NRAS G12V preferentially signals through the PI3K/AKT pathway while NRAS Q61L activates the MAPK pathway. In addition, Posch et al. determined kinases differentially expressed between NRAS G12V and Q61L cells. PHMs expressing NRAS G12V showed an overrepresentation of phosphosites associated with the PIM2 kinase and other kinases related to the PI3K/AKT signaling pathway, which correlates with the pathway enrichment reported in this work, while NRAS Q61L-expressing cells showed enriched CK2α kinase-related sites (55). This *in silico* prediction was later confirmed by analyzing clinical samples of NRAS mutant melanoma. Sixteen out of 18 NRAS Q61 mutated melanomas and one out of 2 NRAS G12 mutant melanomas showed a positive expression for CK2α, with higher expression levels in the NRAS Q61 mutant samples (55). Moreover, the TCGA data set for skin cutaneous melanoma was analyzed to determine whether CK2α was differentially expressed between NRAS Q61 and NRAS G12 mutant melanomas. The comparison of CK2α mRNA levels between both NRAS Q61 and G12 mutant melanomas showed a higher expression of CK2α in NRAS Q61 mutant samples (55), confirming thus the *in silico* prediction. CK2α is a constitutively active serine/threonine protein kinase involved in many cellular processes, such as cell growth, proliferation, and survival (56). Recently, its role in antitumor drug resistance has been reviewed, pointing to the modulation of PI3K/AKT, β-catenin and other signaling pathways directly involved in drug

resistance by CK2α. Moreover, the available CK2α inhibitors (56) are under evaluation to determine whether this kinase is a potential target in cancer treatment.

Metabolomics

Brunelli et al. characterized the metabolic profile of the isogenic NCI-H1299 NSCLC cell line overexpressing WT KRAS or KRAS G12C, G12D, or G12V (38). The majority of metabolites identified were common to all three KRAS-mutated lines (G12C, G12D, and G12V), although these mutants harbored 74, 58, and 48 unique metabolites, respectively, compared to WT (38). Moreover, the deregulated metabolites between WT and mutant KRAS variants were classified into biochemical groups. The two most abundant classes for KRAS G12C, G12D, and G12V were glycerophospholipids and amino acids. KRAS G12C and G12D mainly affected phosphatidylcholines (PC) and phosphatidylinositols (PI), whereas KRAS G12V influenced PI and phosphatidylserine (38). In addition, the report by Brunelli et al. provided further insights over the biology of the deregulated metabolites. KRAS G12C, G12D, and G12V variants showed an increase of metabolites related to protein biosynthesis, glutathione, glutamate metabolism and ammonia recycling (38). Regarding the protein synthesis pathway, all these mutants displayed greater levels of tryptophan and lower levels of the rest of the amino acids compared to WT KRAS, with the exception of the high amount of phenylalanine found in KRAS G12D-expressing cells (38). Moreover, KRAS G12C, G12D, and G12V had lower levels of glutamate, glutamine, asparagine and proline, amino acids interconnected in the glutamate synthase cycle, and lower levels of NAD^+, an essential coenzyme involved in many cellular metabolic pathways (38). Glutamate and glutamine are two amino acids involved in glutaminolysis, one of the central cellular pathways that fuel cancer cells growth and proliferation, which also support the production of antioxidant molecules such as glutathione. Considering the low levels of glutamine reported in this work (38), Brunelli et al. studied glutathione cellular levels. All analyzed KRAS mutant variants showed low levels of reduced glutathione (GSH) and pyroglutamic acid, both involved in glutathione metabolism. However, the GSH level was slightly higher in KRAS G12C than in KRAS G12D and G12V, but not different from WT KRAS (38).

Following on these results (38), the group of Roberta Pastorelli continued studying the metabolic profile of KRAS G12C, as it is the most representative KRAS mutation in NSCLC patients. In this work, the NCI-H1299 NSCLC cell line expressing WT or KRAS G12C and xenograft tumors generated from this cell line were analyzed (39). Brunelli et al. identified 26 and 23 deregulated metabolites *in vitro* and *in vivo*, respectively, between WT KRAS and KRAS G12C. The enriched pathway analysis of these deregulated metabolites showed that KRAS G12C alters the same metabolic pathways *in vitro* and *in vivo*, including pathways involved in protein biosynthesis, ammonia recycling, and urea cycle (39). Focusing on the deregulated metabolites whose abundance changed significantly *in vitro* and *in vivo* between WT KRAS and KRAS G12C, 11 and 16 metabolites were significantly altered, respectively. Moreover, in both *in vitro* and *in vivo* models, KRAS G12C decreased the levels of glutamine and glutamate, two amino acids involved in nitrogen balance maintenance, supporting the central role of glutaminolysis and nitrogen anabolism to provide energy for cancer cell growth and proliferation. This indicates that cells expressing the KRAS G12C variant use glutaminolysis as a source of energy (39). In addition, KRAS G12C mutation induced a significant increase in the levels of carnitine, acetyl-carnitine and butyryl-carnitine, which are involved in the oxidation of fatty acids (39). This increase could be associated with the mitochondrial fatty acid beta oxidation to respond to the increasing energy demand triggered by KRAS G12C to fuel cell or tumor growth and proliferation (39). Moreover, the same group previously reported that KRAS G12C-expressing cells mainly affected PC and PI (38), showing later a down-regulation of some PC species *in vitro* but not *in vivo* compared to WT KRAS. These changes have been reported to be an important source of second messengers that could play a role in the MAPK and PI3K/AKT signaling pathways that are commonly altered in cancer (57).

In addition to the transcriptomic profile, Charitou et al. also assessed the metabolic differences between WT KRAS- and KRAS G13D-expressing HKe3 colorectal cancer cells to confirm the results predicted in their RNAseq analysis (40). The analysis of 188 endogenous metabolites revealed that 97 of them were significantly changed between WT KRAS- or KRAS G13D-expressing cells, showing different metabolic profiles (40). The metabolic data revealed that KRAS G13D-expressing cells have an increased abundance of almost all amino acids, results that are consistent with the pathway analysis of up-regulated genes (40). In addition, this work showed a decrease in PC levels and an increase in carnitine and its esters in KRAS G13D-expressing cells (40). These findings are consistent with those previously published by Brunelli et al. concerning KRAS G12C (39), suggesting that these changes are not a codon-specific signature.

The results provided by omics profiling studies indicate that the differences in biological properties or downstream signaling pathways activation of distinct RAS proteins mutations are presumably consequences of their very specific transcriptomic, proteomic/phosphoproteomic and metabolomic profiles. The large amount of data provided by such profiles allows the comparison of different RAS mutant variants to determine their differences in a particular cancer or to provide important insights in the response to a specific treatment. Moreover, these studies identify hits that might be potential targets in therapy, as they are involved in numerous pathways previously described to be altered in cancer.

RAS MUTATED VARIANTS AT NON-CANONICAL CODONS

The most studied mutations in RAS genes are located at the canonical codons 12, 13, and 61. However, other mutations at non-canonical codons of RAS, such as 19, 22, 59, 117, or 146, have been described (6, 58–61). Both somatic as well as germline mutations at these codons have been reported. For example, NRAS A146T can be found in the leukemic cell lines NALM6 and ML-216, while HRAS K117N and A146T germline

mutations have been identified in a small number of patients with Costello syndrome (62) and KRAS V14I in patients with Noonan syndrome (60). In addition, point mutations at codon 59 are commonly identified in the viral forms of HRAS and KRAS (58).

As non-canonical mutations have also been identified in patients' samples (59, 60, 62) and thus may be relevant for oncogenesis, functional and biochemical evaluation of these mutant protein have been performed in comparison with wild type RAS or other canonical RAS mutations (6, 59, 60).

Transforming Potential

Feig and Cooper described two different HRAS non-canonical mutations, V14M and A146V, and assessed their transforming potential by their ability to form foci (58). Whereas, NIH3T3 fibroblasts expressing HRAS V14M had an indistinguishable foci formation ability compared to WT HRAS, HRAS A146V showed an increase in foci formation (58). This work also compared the transforming potential of WT HRAS and HRAS A59T and A59I, both of them identified as viral HRAS mutants defective in their autophoshporylation. HRAS A59T and A59I showed higher and lower transforming potential, respectively, compared to WT HRAS (58). The results concerning HRAS A59T are consistent with those previously published by Fasano et al. (63) and Lacal et al. (64), where HRAS A59T mutant protein was able to fully transform NIH3T3 mouse fibroblasts (64) and form foci compared to WT HRAS (**Table 4**) (63).

Later, the sequencing of different types of cancers revealed new mutations at the non-canonical codons 22, 60, 74, and 146 (60, 65). Tsukuda et al. analyzed the transforming potential and the proliferation rate of KRAS Q22K *in vitro* and *in vivo* (65). NIH3T3 mouse fibroblasts transfected with WT KRAS or KRAS Q22K were able to form few foci compared to the well-characterized activating mutation KRAS G12V. However, KRAS Q22K-expressing fibroblasts showed typical transformed cell morphology: small, spindle-shaped cells with no tight adherence (65). Moreover, cells expressing KRAS Q22K were able to grow under starving, while WT KRAS cells ceased to grow within 10 days under the same experimental conditions. However, neither WT or mutant KRAS showed tumor formation *in vivo* in 15 days (**Table 4**), whereas fibroblasts expressing KRAS G12V formed progressive tumors (65). These results indicate that KRAS Q22K is able to change mouse fibroblasts morphology but its transforming potential is not sufficient to develop tumors *in vivo*. In addition, Tyner et al. transfected A31 fibroblasts and murine bone marrow cells with WT or different KRAS and NRAS mutants (60). Whereas, WT KRAS- or NRAS-expressing cells exhibited few foci, indicating contact inhibited growth, NRAS G60E and KRAS T74P and A146T were able to form numerous foci (**Table 4**) (60).

Furthermore, Akagi et al. reported another non-canonical mutation at codon 19 of KRAS (59). To assess its transforming potential, three different characteristics were measured: cell morphology, proliferation and saturation density (59). The transfection of NIH3T3 fibroblasts with plasmids encoding WT KRAS or KRAS L19F showed that clones expressing this mutant protein were smaller and more rounded than those expressing WT KRAS. Moreover, whereas WT KRAS expressing cells ceased

TABLE 4 | Phenotypical and signaling differences among RAS proteins non-canonical mutations.

Characteristic/mutation	HRAS V14M	HRAS A146T	HRAS A59T	HRAS A59I	KRAS Q22K	NRAS G60E	KRAS T74P	KRAS A146T	KRAS L19F	KRAS K117N	KRAS R164Q	KRAS A18D
Transforming potential	No (58)	Yes (58)	High (58,63,64)	Low (58)	Low (65)	Yes (60)	Yes (60)	Yes (22,60)	High (59) Low (22)	Yes (22)	No (22)	
GTP binding	High (58)	High (58)	As WT (58)	As WT (58)	Yes (62)	High (60)	High (60)	High (60)	High (59) Yes (22)	Yes (22,62) High (6)	No (22)	As WT (6)
Instrinsic GTP hydrolysis		As WT (58)	Low (58)	Low (58)				Low (61)				
Nucleotide exchange rate		Fast (58)	Very fast (58,66)	As WT (58)				Very fast (61)				
Anchorage-independent growth					No (65)				Yes (59)	Yes (6)		No (6)
Migration										No (6)		No (6)
MEK activation						Low (60)	High (60)	Very high (60)				
ERK activation						High (60)	High (60)	High (60,61)		As WT (6)		As WT (6)
AKT activation										As WT (6)		As WT (6)
PDK activation										As WT (6)		As WT (6)

Summary of RAS mutant proteins manifestations according to different studied characteristics and their ability to activate downstream pathways. Proteins activation was assessed by phosphorylation at different residues. WT, wild type; GTP, guanosine-5'-triphosphate; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; AKT, protein kinase B; PDK, 3-phosphoinositide-dependent protein kinase.

to grow under starved conditions, KRAS L19F clones were able to grow and had greater density that could be due to their small cell size and loss of contact inhibition (59). This work also studied the ability of the mutant KRAS L19F to form tumors *in vivo*, reporting that 75% of the KRAS L19F injected clones developed tumors in contrast to 13% of WT KRAS clones (**Table 4**) (59). Therefore, these results indicate that KRAS L19F has a higher proliferation capacity *in vitro* and *in vivo* compared to WT KRAS.

Smith et al. also assessed the transforming potential of the previously studied non-canonical mutations KRAS L19F and A146T and two new KRAS mutations, K117N and R164Q (22). The transduction of NIH3T3 fibroblasts with plasmids expressing these KRAS mutations and WT KRAS revealed that KRAS K117N and A146T enabled foci formation, whereas KRAS L19F only formed isolated foci (**Table 4**) (22). These findings contrast with those previously described by Akagi et al. (59) for codon 19, but in agreement with those published by Tyner et al. (60) for KRAS A146T.

These observations indicate that, similarly to mutations at codons 12, 13 and 61, mutations at non-canonical codons of the different RAS proteins display a diverse phenotype regarding their transforming potential.

GTP Binding

Feig and Cooper determined the nucleotide binding affinities of WT HRAS and HRAS V14M and A146V (58). Whereas, WT HRAS showed affinity of for both GTP and GDP, the affinity of HRAS V14M and A146V for GTP and GDP were higher compared to WT HRAS (**Figure 1A**) (58). Moreover, this work also assessed the GDP-GTP exchange and the GTPase activity of HRAS A146V. This mutation showed a fast nucleotide exchange compared to WT HRAS but the same GTPase activity (**Table 4**), indicating that the transforming potential of HRAS A146V reported in this work was due to an increase in the speed of nucleotide exchange rather than any alteration in its GTPase activity (58). Moreover, the nucleotide binding affinity, nucleotide exchange rate and GTPase activity were also studied for HRAS A59T and A59I mutant proteins. Both HRAS A59T and A59I mutations and WT KRAS bound GTP and GDP. Regarding the nucleotide exchange, whereas HRAS A59I exhibited nearly the same exchange rate as WT HRAS, HRAS A59T mutation showed a rate 10-fold greater than WT HRAS. However, both HRAS A59T and A59I mutant proteins showed a reduction in their intrinsic GTPase activity (**Table 4**) (58). The results concerning HRAS A59T are consistent with the ones previously published by Lacal and Aaronson (66), who determined that HRAS A59T showed 3- to 9-fold greater nucleotide exchange than WT HRAS (66). All together, these results indicate that the transforming potential of HRAS A59T is due to a reduction in GTPase activity and an increase in nucleotide exchange. However, the inability of HRAS A59I to form foci reported in this work indicates that a reduction of the GTPase activity is not sufficient to confer transforming capacity (58), suggesting that changes in the nucleotide exchange rate are also important at this codon to acquire transforming capacities.

Akagi et al. studied the ability of RAS non-canonical mutations to bind GTP (59). KRAS L19F showed elevated RAS-GTP levels compared to WT KRAS, which was consistent with the *in vitro* and *in vivo* transforming potential of this KRAS mutant (59). Later on, experiments in HEK 293T/7 cells transfected with WT or mutants KRAS and NRAS revealed that NRAS G60E, KRAS T74P, and A146T had increased RAS-GTP levels compared to WT NRAS and KRAS (**Figure 1A**, **Table 4**) (3). The increase in KRAS T74P-GTP levels could be explained as the substitution of proline may disrupt the protein conformation involved in GTP hydrolysis, thus impairing GTP-GDP exchange (60). In addition, Janakiraman et al. showed that HEK 293FT cells expressing KRAS Q22K, E31K, K117N, and A146T were able to bind GTP (**Table 4**), with KRAS Q22K mutant variant showing the highest levels and KRAS E31K levels similar to WT KRAS, establishing the following hierarchy Q22K >> K117N ≈ A146T >> E31K (62). Later on, Smith et al. showed that KRAS L19F, K117N, and A146T were able to bind GTP, but WT KRAS and KRAS R164Q were not (**Figure 1A**, **Table 4**). These results are consistent with the transforming potential of these mutants reported in this work (22) and with the previously observed ability of KRAS L19F and A146T to bind GTP (59, 60, 62). In addition, Stolze et al. reported that KRAS A18D has a similar GTP-binding to WT KRAS and control cells, which only express endogenous KRAS, following EGF stimulation (6). In contrast, KRAS K117N mutant protein showed an increase in GTP-biding up to 5 to 6-fold compared to control cells (**Figure 1A**, **Table 4**) (6), which is consistent with the data reported by Janakiraman et al. (62).

Recently, in a 2019 study, Poulin et al. compared the nucleotide exchange and GTP hydrolysis between WT KRAS and KRAS A146T (61). The authors reported in this work that KRAS A146T had ∼12-fold higher GDP dissociation rate than WT KRAS, a difference that was further increased by the addition of the GEF protein SOS1. The intrinsic GTP hydrolysis of KRAS A146T was reduced compared to WT KRAS (**Table 4**), while GAP-mediated GTP hydrolysis was only mildly impaired (61). These results are consistent with those published previously (22, 60, 62). Therefore, the ability of KRAS A146T to form foci reported by Tyner et al. and Smith et al. (22, 60) might be due to an increase in the intrinsic and GEF-mediated nucleotide exchange rather than a loss of GAP-mediated exchange (61).

Anchorage-Independent Growth and Migration

Using colony formation as a measurement of anchorage-independent growth, Tsukuda et al. have shown that KRAS Q22K formed only few colonies in soft agar, similar to WT KRAS (**Table 4**) (65), indicating that this mutation cannot grow independent of anchorage, results that are in agreement with its inability to form tumors *in vivo*. Later, Akagi et al. reported that 9.2% of NHI3T3 cells expressing KRAS L19F were able to form colonies, while fibroblasts expressing WT KRAS failed to do so (**Table 4**) (59), consistently with the transforming potential

assessed in this study. In addition, Stolze et al. reported that MCF10A breast cancer cells ectopically expressing KRAS A18D at physiological levels were unable to form colonies in soft agar (6). However, KRAS K117N expressing cells displayed a slight increase in colony formation compared to control cells expressing endogenous KRAS (6). In addition, KRAS A18D- and K117N-expressing cells showed no increase in their migration abilities compared to WT KRAS or control cells when they are expressed at physiological levels (**Table 4**) (6).

Downstream Pathways Activation and Outcome

Several studies assessed the activation of RAS downstream pathways by non-canonical mutations (6, 60, 61). For example, Tyner et al. studied the activation of the MAPK pathway by MEK and ERK phosphorylation status (60). Compared to WT NRAS, HEK 293T/17 cells expressing NRAS G60E showed an increase in ERK but not in MEK phosphorylation. In the case of KRAS T74P and A146T, both mutant proteins increased ERK phosphorylation levels in comparison to WT KRAS, but KRAS A146T showed higher MEK activation than KRAS T74P and WT KRAS (**Figure 1B**, **Table 4**) (60). In addition, Stolze et al. reported that MCF10A cells expressing KRAS A18D or K117N at physiological levels did not show higher phosphorylation levels of ERK, PDK, and AKT compared to WT KRAS or control cells expressing endogenous KRAS (**Figure 1B**, **Table 4**) (6). However, either the physiological expression or overexpression of KRAS K117N increased the activation of RPS6 compared to WT KRAS and control cells after the addition of EGF (**Table 4**) and thus, this mutant enabled the activation of the mTOR pathway (6). In addition, Poulin et al. analyzed the activation of the RAS downstream pathways assessing the phosphoproteome of WT KRAS and KRAS A146T (61). KRAS A146T mutant protein expressed in the colon increased the phosphorylation level of ERK1/2 compared to WT KRAS, but less than KRAS G12D (**Table 4**) (61). Therefore, KRAS A146T seems to activate the MAPK pathway less strongly compared to KRAS G12D. However, the inhibition of this signaling pathway reduced the proliferation in the colonic epithelium, indicating that the activation of the MAPK pathway at low levels is sufficient to increase the proliferation rate in this tissue (61).

In addition to *in vitro* studies which analyzed RAS mutations at non-canonical codons, *in vivo* xenograft models have also been employed to study the activation of downstream signaling pathways and related outcomes (61, 62). For example, Janakiraman et al. showed >95% decrease in ERK phosphorylation 6h after the inhibition of the MAPK pathway. Moreover, this inhibition was also associated with a downregulation of cyclin D1, an increase in p27 expression and hypophosphorylation of Rb (62).

Poulin et al. studied the phenotype that ensues from the expression of KRAS A146T in the colonic epithelium, hematopoietic stem cells and pancreas of genetically engineered mice (61). In the colonic epithelium, KRAS A146T caused a moderate hyperplasia and an intermediate proliferation between KRAS G12D and WT. The expression of KRAS A146T in hematopoietic stem cells led to a myelodysplastic syndrome/myeloproliferative neoplasm with a delayed onset compared to mice expressing KRAS G12D in the same cells, and these animals died with severe anemia and splenomegaly at an older age than KRAS G12D-expressing mice (61). However, when KRAS A146T was expressed in the pancreas, mice showed no evidence of pancreatic intraepithelial neoplasia at 2 months of age. Even the induction of acute pancreatitis was not sufficient to induce pancreatic neoplasia, suggesting that this mutation does not alter pancreatic homeostasis (61).

Transcriptomics and Proteomics

Differences in overall mRNA and protein expression among RAS non-canonical codons have been described (22, 61). Smith et al. performed a hierarchical clustering of transcriptomic data of WT KRAS, KRAS canonical mutations G12V, G12C, G12D, and G13D and KRAS non-canonical mutations L19F, K117N, A146T, and R164Q (22). The analysis revealed two different clusters: WT KRAS and the codon 12 mutations clustered in one group ("cluster one") while the codon 13 and non-canonical mutations clustered in a second group ("cluster two"), indicating that non-canonical mutations displayed similar gene expression profile to KRAS G13D. Despite previous results of this study showing that KRAS R164Q had a similar transforming potential to WT KRAS (22), this mutation was grouped in the "cluster two," suggesting an attenuated transforming potential. In addition, KRAS L19F and R164Q formed a transcriptomic subcluster within the "cluster two," suggesting that these two mutations are different from KRAS G13D, K117N, and A146T (22). Furthermore, Smith et al. analyzed the expression of genes involved in signal transduction, cytoskeleton remodeling and cell adhesion (22). Despite the few changes in gene expression induced by KRAS R164Q, there were examples of genes whose expression was induced by all mutants, such as the protein tyrosine phosphatase *PTPRE* and the RHO GTPase-activating protein *ARHGAG6* (22). Moreover, genes induced or repressed by all of the mutants except KRAS R164Q were identified, including the MAPK phosphatases *DUSP4* and *DUSP6*, the RHO guanine-exchange factor *NGEF*, the cell adhesion molecule *CEACAM1* and the plasminogen activator inhibitor *SERPINB2*. Interestingly, "cluster one" but not "cluster two" KRAS mutants differentially expressed some genes, for example *VEGFA*, *PAK3,* or *PIM1*; and "cluster two" but not "cluster one" mutants showed a different expression of *IGF1R* and *CREB1* among other genes. Additional genes, such as *E2F2*, *SLC2A1,* or *JUN*, were differentially regulated by all the analyzed mutants (22).

Poulin et al. studied the proteome and phosphoproteome of colon, pancreas and spleen from mice expressing WT KRAS or the mutant proteins KRAS G12D and A146T (61). The data derived from each tissue revealed that the two mutant variants and WT KRAS clustered separately. The collective analysis of all data showed that samples from the same tissue cluster together regardless of the KRAS mutation and samples expressing KRAS A146T tended to cluster closer to the ones expressing WT KRAS (61), suggesting that WT KRAS and KRAS A146T display similar proteomic and phosphoproteomic profiles in these tissues. Moreover, the same authors uncovered the

enriched biological pathways in KRAS G12D or A146T using the dataset of each tissue analyzed (61). In the colon dataset, KRAS G12D and A146T differentially regulated the majority of the enriched pathways, such as the calcium signaling pathway. Similar to the colon-associated data, the majority of pathways enriched in the pancreas dataset were discordantly regulated by both mutations. Interestingly, whereas the nitrogen metabolism pathway was up-regulated by KRAS G12D and A146T in colon, the same pathway was down-regulated in the pancreas. In the spleen dataset, KRAS G12D and A146T showed no pathways differentially regulated by the two mutants compared to WT KRAS (61). Conclusively, KRAS G12D and A146T differentially regulate downstream signaling pathways, depending also on the tissue where these mutant proteins are expressed.

All together, these results suggest that, similarly to mutations at codons 12, 13, and 61, mutations at non-canonical codons of RAS proteins display different biological manifestations that relate to their transforming potential and GTP binding. Moreover, these mutations activate differently RAS downstream signaling pathways and alter genes and proteins expression compared to the WT protein. However, non-canonical mutations are less studied compared to mutations at codons 12, 13, and 61, despite the fact that they have been described in patients' samples. Therefore, it is of an immense interest to continue studying their biological characteristics *in vitro* and *in vivo* to uncover more over the properties of those uncommon variants and their relevance to RAS-related oncogenesis.

RAS PROTEINS MUTATIONS AFFECT TREATMENT RESPONSES

KRAS is the most frequently mutated RAS protein in cancer (5) and therefore the most studied in clinical trials for different therapy regimen [1]. The association between treatment responses and survival in patients carrying KRAS mutant variants has been studied since the late 1990s (67). For example, Keohavong et al. reported that lung cancer patients carrying the KRAS G12V or G12R mutations had a shorter overall survival (OS) compared to those with WT KRAS tumors, while KRAS G12D-carrying patients showed longer survival (67). Regarding treatment response, Petrelli et al. reported in a meta-analysis of 12 colorectal cancer clinical trials that patients carrying WT KRAS had a better response rate (RR) to chemotherapy plus bevacizumab than those harboring KRAS-mutated tumors (68). Of particular importance, Allegra et al. reported in their retrospective study that colorectal cancer (CRC) patients carrying KRAS mutant variants do not benefit from the anti-EGFR antibodies cetuximab and panitumumab (69). However, it has also been demonstrated that about 10% of the patients with KRAS-mutated tumors can respond to anti-EGFR therapy and about 15% have long-term disease stabilization (70). This subchapter discusses in detail the relation between KRAS mutant variants and survival and treatment response of patients with various cancer types that are particularly prone to carry KRAS mutations.

[1]https://clinicaltrials.gov

Colorectal Cancer

Approximately 40% of CRC cases harbor KRAS mutations at codons 12, 13, and 61, resulting mainly in the KRAS G12D, G12V, and G13D variants (2, 5). Almost already 10 years ago, De Rock et al. analyzed whether the presence of the KRAS G13D mutant variant is associated with treatment response or survival of CRC patients (70). As KRAS G13D has been reported to exhibit weaker transforming potential than KRAS codon 12 mutant variants (6, 22, 25), De Rock et al. hypothesized that patients harboring KRAS G13D mutation might have a better outcome after cetuximab treatment compared to patients carrying other KRAS mutant variants. To confirm this hypothesis, 579 patients with varying KRAS status who had chemotherapy-refractory metastatic colorectal cancer (mCRC) were divided into two different treatment lines: cetuximab only and cetuximab plus chemotherapy (70). Compared to other KRAS mutant variants, patients carrying KRAS G13D mutant variant who received cetuximab treatment, either alone or in combination with chemotherapy, had longer OS (median 7.6 months vs. median, 5.7 months, HR, 0.50) and progression-free survival (PFS) (median 4.0 months vs. median, 1.9 months, HR, 0.51). However, no significant differences in OS or PFS were identified in KRAS G13D patients compared to those carrying the WT KRAS (70). Similarly, patients with KRAS G13D-expressing tumors receiving the combination treatment of cetuximab plus chemotherapy showed longer OS and PFS than patients carrying other KRAS mutant variants (OS: median, 10.6 months vs. 7.4 months, HR, 0.46; PFS: median, 4.1 months vs. 2.8 months, HR, 0.49). No differences between KRAS G13D and WT KRAS regarding OS and PFS were identified, results also reported in the "cetuximab only" group (70). These results confirmed that patients carrying KRAS G13D benefited from cetuximab treatment compared to those carrying other KRAS mutant variants, which may be explained by the weak transforming potential showed *in vitro* (70). This work also compared the RRs of KRAS G13D patients in the different treatment groups (70). Patients carrying KRAS G13D mutant variant receiving the combination of cetuximab plus chemotherapy showed higher but not statistically significant RR compared to patients with other KRAS mutations. However, patients carrying WT KRAS in the cetuximab plus chemotherapy treatment arm showed higher RR than those with tumors expressing KRAS G13D, but this difference is not statistically significant when WT KRAS patients are compared to KRAS G13D patients receiving cetuximab only (70). In addition, De Rock et al. studied the *in vivo* response to cetuximab (70). Cetuximab inhibited the growth of tumors harboring WT KRAS or KRAS G13D, showing a similar response to the treatment; however, the treatment did not affect the growth of KRAS G12V-expressing tumors (70), suggesting that KRAS codon 12 mutant variants are resistant to cetuximab.

Later, Tepjar et al. combined the data of 1,378 mCRC patients included in the previous clinical trials (71). In this work, patients carrying the KRAS G13D mutant variant had additional benefit from chemotherapy plus cetuximab than from chemotherapy alone. These patients showed higher PFS (median 7.4 vs. 6.0 months; HR, 0.47) and tumor response (median 40.5 vs. 22.0% months; OR, 3.38), but not OS (median 15.4 vs. 14.7 months; HR,

0.89), when cetuximab was added to the chemotherapy regimen. These results could partially be explained by the worse prognosis of KRAS G13D patients in the control arm (71). Opposite to KRAS G13D, patients carrying the KRAS G12V mutant variant receiving chemotherapy plus cetuximab showed worse PFS than those receiving chemotherapy only (71). When only the chemotherapy plus cetuximab treatment arm was considered, patients carrying G13D or G12V mutant variants showed a similar OS, which was markedly lower than those patients with WT KRAS tumors (71). Within the chemotherapy only arm, patients with KRAS G13D mutant tumors tended to have worse, but not statistically significant, PFS and OS compared to those harboring tumors with other KRAS mutant variants (PFS: HR, 1.49; OS: HR, 1.25). However, patients with KRAS G12V tumors did not showed worse PFS or OS compared to other KRAS mutant tumors (PFS: HR, 0.77; OS: HR, 1) (71).

Similarly, Fiala et al. studied KRAS mutations at codons 12 and 13 of mCRC patients treated with the anti-angiogenic antibody bevacizumab who previously have received different chemotherapeutic regimen (72). Patients carrying mutant variants at codons 12 or 13 had shorter OS and PFS than those with WT KRAS (72). When each KRAS mutant variant was analyzed independently, patients carrying KRAS G12V or G12A had shorter PFS and OS compared to WT KRAS patients (PFS: HR = 2.18; OS: HR = 2.58) (72).

The impact of KRAS mutant variants have been analyzed not only in relation to chemotherapy, but also regarding surgery as a treatment strategy (73, 74). Mangonis et al. studied the outcome of CRC patients carrying KRAS mutations after curative intent liver resection due to liver metastasis (73). Patients carrying KRAS mutant variants at codon 12 (G12V, G12D, G12C, G12S, and G12A) or KRAS G13D had no significant differences in 5-year recurrence-free survival (RFS) compared to WT KRAS patients ($p = 0.57$). Moreover, none of the aforementioned mutant variants were associated with worse RFS than WT KRAS (73). In addition, patients carrying any KRAS codon 12 mutant variant had worse 5-year OS compared to WT KRAS patients (HR, 1.7), while KRAS G13D patients had no differences in OS compared to those carrying WT KRAS (HR, 1.47) (73). When each KRAS mutant variant was analyzed independently, KRAS G12V and G12S were associated with 2- to 3-fold increase risk for long-term death compared to WT KRAS patients. In addition, patients carrying KRAS G12V, G12S, and G12C had a higher risk of death after recurrence compared to those harboring WT KRAS who recurred (73). Recently, Hayama et al. analyzed 200 CRC patients who underwent curative resection (74). Analysis of relapse-free survival revealed that a small proportion of patients carrying KRAS mutant variants G12D, G12V, G12C, G12A, G12S, or G13D reached the 3-year relapse-free survival endpoint compared to those carrying WT KRAS (69.7 vs. 82.1%, respectively; $p = 0.01$). Moreover, patients carrying KRAS G12V or G12C had a higher risk of long-term recurrence than those with WT KRAS tumors or KRAS G12A, G12D, or G12S-mutated CRC (74).

Independently of the type of treatment (chemotherapy or surgery), CRC patients carrying KRAS G13D mutant variant seem to show no significant differences in PFS, OS or RFS as compared to WT KRAS across these various studies. However, patients harboring KRAS codon 12 mutant tumors generally had worse PFS, OS, RFS and RR compared to patients with WT KRAS tumors. This could be potentially explained by the fact that KRAS codon 12 mutant variants, especially the G12V and G12D mutations, have been reported to have a very high transforming potential and a low GTP intrinsic and GAP-mediated GTP hydrolysis (6, 22, 25). Importantly, this also correlates with the *in vivo* animal models findings reported by De Rock et al. regarding KRAS G12V and its resistance to cetuximab (70) and with preclinical results by Leiser et al. on KRAS G12V, G12D, and G13D mutant variants conferring resistance to two different MET inhibitors (75).

Non-small Cell Lung Cancer (NSCLC)

KRAS mutations, mainly KRAS G12C, G12D, and G12V, are observed in 20–30% of NSCLC patients, predominantly in patients with adenocarcinomas (2, 76). Subsequently, the association between KRAS mutant variants and treatment survival in NSCLC has also been extensively studied (23, 76, 77). For example, Ihle et al. reported that patients with refractory NSCLC carrying KRAS G12C or G12V mutant variants showed a statistically significant decrease in PFS (median survival = 1.84 months; $p = 0.046$) compared to other KRAS mutant variants (G12A and G12D) (median survival = 3.35 months) or WT KRAS (median survival = 1.95 months) (23). This association was more pronounced in patients receiving sorafenib whereas no statistically significant association was identified between patients harboring KRAS G12C or G12V mutations and PFS in either erlotinib or bexarotene plus erlotinib treatment groups (23). Later on, Mellema et al. analyzed whether there was an association between KRAS codon 12 mutant variants and OS, PFS and RR in 464 advanced NSCLC patients who received platinum-based chemotherapy as a first-line treatment (76) Patients in this study were treated with different agents (pemetrexed, gemcitabine, taxane or bevacizumab) in addition to the previously administrated platinum treatment (76). Interestingly, patients carrying KRAS G12V mutant variant showed a higher RR when treated with taxanes than those harboring the same mutant variant but treated with pemetrexed or gemcitabine. However, KRAS G12V patients in the taxanes group had longer, but not statistically significant, PFS and OS compared to patients carrying the same mutant variant but treated with pemetrexed or gemcitabine. Moreover, patients carrying KRAS G12C or G12D mutant variants had similar RR, PFS and OS within all treatment groups (76). In addition, this work assessed the PFS and OS among KRAS G12C, G12V, and G12D mutant variants independently of the received treatment, showing no differences (PFS: median 4.9, 4.8, and 4.3 months for G12C, G12V, and G12D, respectively ($p = 0.45$); OS: median 10.4, 8.0, and 8.3 months for G12C, G12V, and G12D, respectively ($p = 0.46$) (76).

Interestingly, Renaud et al. also analyzed KRAS-mutated patients with NSCLC who received platinum-based chemotherapy as first-line treatment, with similar treatment arms: pemetrexed, vinorelbine, gemcitabine, taxane, or bevacizumab (77). Amino acid substitutions in KRAS did

not affect patients' OS even when differences in treatment were considered (77). When assessing the time to progression (TTP), treatments with vinorelbine and taxane were associated with a better TTP (HR, 0.76 and 0.32, respectively) compared to gemcitabine and pemetrexed treatment arms. However, KRAS mutational status was not a significant predictor of TTP, despite the fact that patients carrying KRAS G12D or G12V mutant variants tended to have a better, but not statistically significant, TTP compared to those carrying KRAS G12C. This tendency was also observed in patients carrying KRAS G13D mutant variant when treated with taxane. Interestingly, all KRAS mutant variants identified in this study were associated with worse TTP when treated with bevacizumab compared to other treatment regimen (77).

To summarize, in the case of NSCLC, contradictory results addressing the impact of KRAS different mutations in clinical settings are being reported. While Ihle et al. and Mellema et al. reported that patients carrying KRAS G12V mutant variant showed longer OS and PFS when treated with taxanes compared to other treatment regimen (23, 76), Renaud et al. reported no differences among the codon 12 amino acid substitution considering the various treatment arms, including taxanes (77). Moreover, this work showed that patients with KRAS G12D or G12V-expressing tumors tended to have better TTP than those with KRAS G12C tumors (77), which contrasts with the higher PFS and OS reported by Mellema et al. for KRAS G12C patients (76). Additional studies would thus be needed to allow for consistent conclusions and potential clinical implementation of such findings.

Pancreatic Ductal Adenocarcinoma

Pancreatic adenocarcinoma is considered one of the most aggressive forms of cancer. KRAS mutations are carried approximately by 90% of the patients and can be detected at both early and chronic stage of the disease (5). Among all the possible KRAS mutant variants, the most frequently observed in the pancreatic ductal adenocarcinoma (PDAC) are KRAS G12D and G12V (2).

As previous studies in colorectal and lung cancer have demonstrated that KRAS status influences patients prognosis (23, 71), Bournet et al. studied whether KRAS codon mutant variants were associated with the OS in 219 patients with metastatic or locally advanced PDAC (78). This work reported no differences in OS between patients carrying WT KRAS or codon 12 mutations (KRAS G12D, G12V, and G12R). However, KRAS G12D patients showed a decreased OS compared to KRAS G12V or G12R patients (78). These results are in agreement with the previously published data by Boeck et al. who reported median OS of 5.3 months for patients carrying KRAS G12D, 6.6 months for those with the KRAS G12V mutation and 7.7 months for patients with tumors harboring the G12R KRAS mutation (79). Moreover, Bournet et al. showed that among all the 162 patients who received chemotherapy, those carrying the KRAS G12D mutant variant had a worse prognosis compared to those with KRAS G12V or G12R. However, patients carrying KRAS codon 12 mutant variant in the chemotherapy treatment subgroup showed no difference in OS compared to WT KRAS patients. Similar results were also reported in a subgroup of 119 patients who received gemcitabine as first-line treatment (78).

Collectively, data summarized in this chapter suggest that CRC patients carrying the KRAS G13D mutant variant show a similar treatment response as those carrying WT KRAS and, thus, will benefit from anti-EGFR therapy. Interestingly, KRAS codon 12 mutants showed different outcome results depending on the cancer type and treatment employed. These results are reflected in KRAS G12V patients, who showed worse PFS when treated with cetuximab but an increase in this endpoint with taxanes treatment. However, of course, such generalization of the results should be taken into consideration only very carefully due to the possible differences in basic patients' characteristics, the sample size and the treatment regimen in each study.

FINAL REMARKS

In these emerging times of personalized medicine, it is highly anticipated that detailed knowledge of cancer genomic landscapes may improve treatments, resulting ultimately in a significant increase of patients' survival. The members of the RAS subfamily of GTPases, which includes KRAS, HRAS, and NRAS, are frequently mutated in cancer. KRAS is often altered in pancreatic carcinoma, colorectal tumors and lung malignancies and *HRAS* mutations are common in dermatological malignancies and head and neck cancers whereas *NRAS* alterations in melanomas and in hematopoietic malignancies. Despite the differences in mutations rates at each codon, the three RAS proteins are usually mutated at the canonical codons 12, 13, and 61. However, other mutant variants have been described at non-canonical codons such as 19, 59, 117, and 146, illustrating the complexity that is affiliated with thes oncogenes. As canonical codons are located in the homologous amino-acid region, shared by all RAS proteins, it could be postulated that their effect on the protein function is equivalent. However, various experimental lines of evidence summarized in this review have demonstrated that not all RAS mutant variants display the same biological and biochemical properties, suggesting that tumors harboring different RAS mutations may behave differently according to the expressed RAS mutant variant. Therefore, detailed knowledge about the biological and biochemical properties of each RAS mutant variant *in vitro* and *in vivo* seems to be essential to help understand the biology of the particular treatment and possibly predict patients' treatment response and survival.

At the same time, the vast majority of preclinical as well as clinical studies are mostly focused on RAS canonical mutations. However, rare RAS mutant variants seem to display similar differences in their biological properties and downstream signaling activation and, thus, their more extensive studying could help to better understand the behavior of RAS-expressing

tumors. Screening and additional biologic characterization of these non-canonical RAS mutations should also be considered in clinical practice as mutational analyses of codons 12, 13, and 61 only may misclassify patients that could benefit from particular anti-cancer therapies.

AUTHOR CONTRIBUTIONS

CM-M, YZ, and MM designed the study, edited, and approved the final version. CM-M searched the literature and drafted the manuscript. YZ and MM supervised the study.

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RAS as Supporting Actor in Breast Cancer

*Mirco Galiè**

Department of Neuroscience, Biomedicine and Movement, University of Verona, Verona, Italy

Oncogenic activation of RAS isoforms leads tumor initiation and progression in many types of cancers and is gaining increasing interest as target for novel therapeutic strategies. In sharp contrast with other types of cancer, the importance of RAS in breast tumorigenesis has long been undermined by the low frequency of its oncogenic mutation in human breast lesions. Nevertheless, a wealth of studies over the last years have revealed how the engagement of RAS function might be mandatory downstream varied oncogenic alterations for the progression, metastatic dissemination, and therapy resistance in breast cancers. We review herein the major studies over the last three decades which have explored the controversial role of RAS proteins and their mutation status in breast tumorigenesis and have contributed to reveal their role as supporting actors, instead of as primary cause, in breast cancer.

Keywords: RAS, breast cancer, oncogene, mutations, signal transduction

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Rosalind Franklin University of Medicine and Science, United States
Miriam Martini,
University of Turin, Italy

***Correspondence:**
Mirco Galiè
mirco.galie@univr.it

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INTRODUCTION

RAS G-proteins mediate the signal transduction through the transmembrane receptors. In humans, there are four highly homologous ≈21 KDa RAS isoforms: HRAS, encoded by the *Harvey rat sarcoma viral oncogene homolog (HRAS)*, NRAS, encoded by *neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS)*, and KRAS4A and KRAS4B, alternative splice variants of the *Kristen rat sarcoma viral oncogene homolog (KRAS)*. RAS proteins function as binary switches that cycle between an active ("on") GDP-bound to an inactive ("off") GTP-bound state depending on the activation status of the upstream receptors. The switch between "on" and "off" states is modulated by the complementary action of enzymes that promote either the GDP to GTP exchange (guanine exchange factors, GEFs) or the conversion back to GDP-bound form (GTPase-activating proteins, GAPs). The multiplicity of GTPases and GAPs allows the function of RAS to be finely regulated depending on the variety of extracellular and intracellular signal inputs. RAS proteins activate a hierarchical cascade of intersecting pathways which modulate biological functions such as cell proliferation, apoptosis, motility, metabolism, immune evasion. Dysregulation of RAS function is largely associated with tumorigenesis. This may rely either on genomic mutations which alter the RAS-intrinsic structure or on alteration of RAS regulating factors, which enhances RAS expression and activity.

This review is an effort to recapitulate more than 30 years of studies on RAS oncogenes and breast cancer, with the aim to reconcile two apparently conflicting evidences arisen by these studies: (1) experimental studies on cancer cells and murine models have demonstrated that RAS oncogenes and their mutations have a strong potential in breast cancer initiation and progression as it does in other type of cancers; (2) clinical studies have demonstrated that, actually, the incidence of tumorigenic RAS mutations in human breast cancers is marginal, in sharp contrast with other types of cancer.

ONCOGENIC MUTATIONS OF RAS IN HUMAN CANCERS

RAS genes were the first mutated genes identified in human cancer (1–3). The discovery in the late 1970s that their gain-of-function mutations were able to trigger tumorigenesis inaugurated the modern molecular oncology and posed the basis of the molecularly targeted anticancer drug discovery (4). To date, hundreds of genes have been identified which harbor oncogenic mutations, but the RAS genes still remain amongst the most frequently mutated oncogene families in cancer. The oncogenic activation of RAS genes is usually caused by a single point mutation (5–7) which impair the RAS responsiveness to the GAP-mediated modulation and locks RAS and their downstream pathways in a persistently active state. Traditional studies (first carried out for the HRAS oncogenic allele) have shown that a single oncogenic RAS gene could transform cells *in vitro* and could provide them with the ability to induce tumors in mice (3). Twenty five percent of human cancers display missense gain-of-function mutations in at least one of the RAS genes and in 98% of the cases mutations are found at one of the mutational hotspots G12, G13, and Q61 (COSMIC v75). Not all RAS isoforms are mutated equally, with KRAS displaying the highest frequency. Also, mutations of specific RAS isoforms exhibit marked preferences for different tumor types and different impact on clinical outcome (**Figure 1**).

ONCOGENIC ACTIVATION OF RAS IN BREAST CANCER

Mammary cell lines have served as tumor models for many seminal studies which demonstrated the tumorigenic potential of RAS oncogenes. These studies have shown that oncogenic RAS mutations constitutively enhance mammary cell interaction with basement membrane, alter the tridimensional growth in collagen gel, induce anchorage-independent phenotype, invasiveness, tumorigenic potential, secretion of TGF-β and IGF-1, activation of EGFR, mitogen-activated protein kinase (MAPK), and estrogen-insensitivity (9–18). Single copies of mutant KRAS cooperate with mutant PIK3CA to induce tumor transformation in immortalized human epithelial cells (19). Conditional expression of Ki-RasG12V in the mammary cells induces estrogen receptor alpha (ERα)-positive adenocarcinoma in mice (20), while HRAS Q61 drives breast adenomyoepitheliomas (21).

Several pathways and downstream effectors have been identified which mediate the tumorigenic phenotype induced by oncogenic RAS mutations in mammary cells. Activated NRAS oncogene and its homolog NRAS proto-oncogene act through the same pathway for *in vivo* tumorigenesis (22). Oncogenic RAS mutations support cancer progression and metastatic dissemination through the modulation of the ΔNp63, a amino-terminal truncated isoform of p63, a member of the p53 family of transcription factors (23, 24). Oncogenic RAS mutations promotes TFG-β-induced epithelial-mesenchymal transition through the activation of leukotriene B4 receptor-2-linked cascade (25). Mutated RAS associates with the induction of cyclooxygenase-2 (COX-2) expression in human breast cancer cell lines (26). Activated HRAS induces the invasive phenotype in breast epithelial cell lines through the recruitment of p38 (27, 28). Invasion of breast carcinoma cells also relies on activated Ras-mediated stimulation of E2F and a consequent E2F-mediated modulation of integrin α6β4 (29). Oncogenic RAS mutation regulates the activity of CXCL10 and its receptor splice variant CXCR-B (30). Id1 and activated RAS cooperate to subvert the cellular senescence response and to induce metastatic dissemination in mammary carcinoma (31). Focal adhesion kinase signaling is required for activated RAS and PI3K-dependent breast tumorigenesis in mice and humans (32). Dominant negative Ras activates the Raf-Mek-Erk signal transduction pathway and induces lactogenic hormone-induced differentiation (33). Activated RAS signals centrosome amplification through cyclin D1/Cdk4 and Nek2 (34). Autophagy is critically implicated in malignant transformation by oncogenic KRAS mutations and is promoted by the reactive-oxygen species-mediated JNK activation through up-regulation of ATG5 and ATG7 (35). RAS oncogenesis is accelerated by p21WAF1/CIP1 depletion in mammary cancer (36), while p21CIP attenuates RAS- and c-MYC-dependent epithelial-to-mesenchymal transition and cancer stem cell-like transcriptional profile *in vivo* (37). Gadd45a induces apoptosis and senescence in Ras-driven mammary cancers through activation of c-jun NH2-terminal kinase and p38 stress signaling (38). HMGA1a regulates genes involved in the RAS/ERK mitogenic signaling pathway, including KIT ligand and caveolin 1 and 2 (39). Oncogenic RAS mutations induce metabolic rearrangement in breast cancer as part of their tumorigenic program. Activated c-ha-Ras induces loss of fatty-acid delta desaturating ability in human mammary epithelial cells (40). Moderate restriction of energy intake hampers v-Ha-ras-induced mammary tumorigenesis (41). PI3K and KRAS cooperate to stimulate *de novo* lipid synthesis through mTORC1 and SREBP (42).

RAS HYPERFUNCTION IN BREAST CANCER

After the first identification of the tumorigenic potential of oncogenic RAS mutations *in vitro*, a great effort has been made in search for RAS mutations in human cancers, and their role in driving tumorigenesis (43). The most remarkable finding was the discovery of the stricking incidence of oncogenic KRAS mutations in colon (44, 45), lung (46), and pancreatic carcinomas (47) (**Figure 1**). According to what found in other tumor types, KRAS confirms to be the most frequently mutated RAS isoform in breast cancer (**Figure 2A**) and its mutation, unlike mutations of HRAS and NRAS, is strongly associated with the poor clinical outcome (**Figure 2B**). Nevertheless, the frequency of RAS mutations in human breast cancer proven to be much lower than expected (49) (**Figures 1**, **2**). This stands against a critical role of RAS oncogenic activation as the primary driver of the breast cancer initiation and progression in humans and has discouraged for many years the effort to investigate RAS proteins as potential targets for breast cancer therapies (**Figure 2A**). Also, human RAS

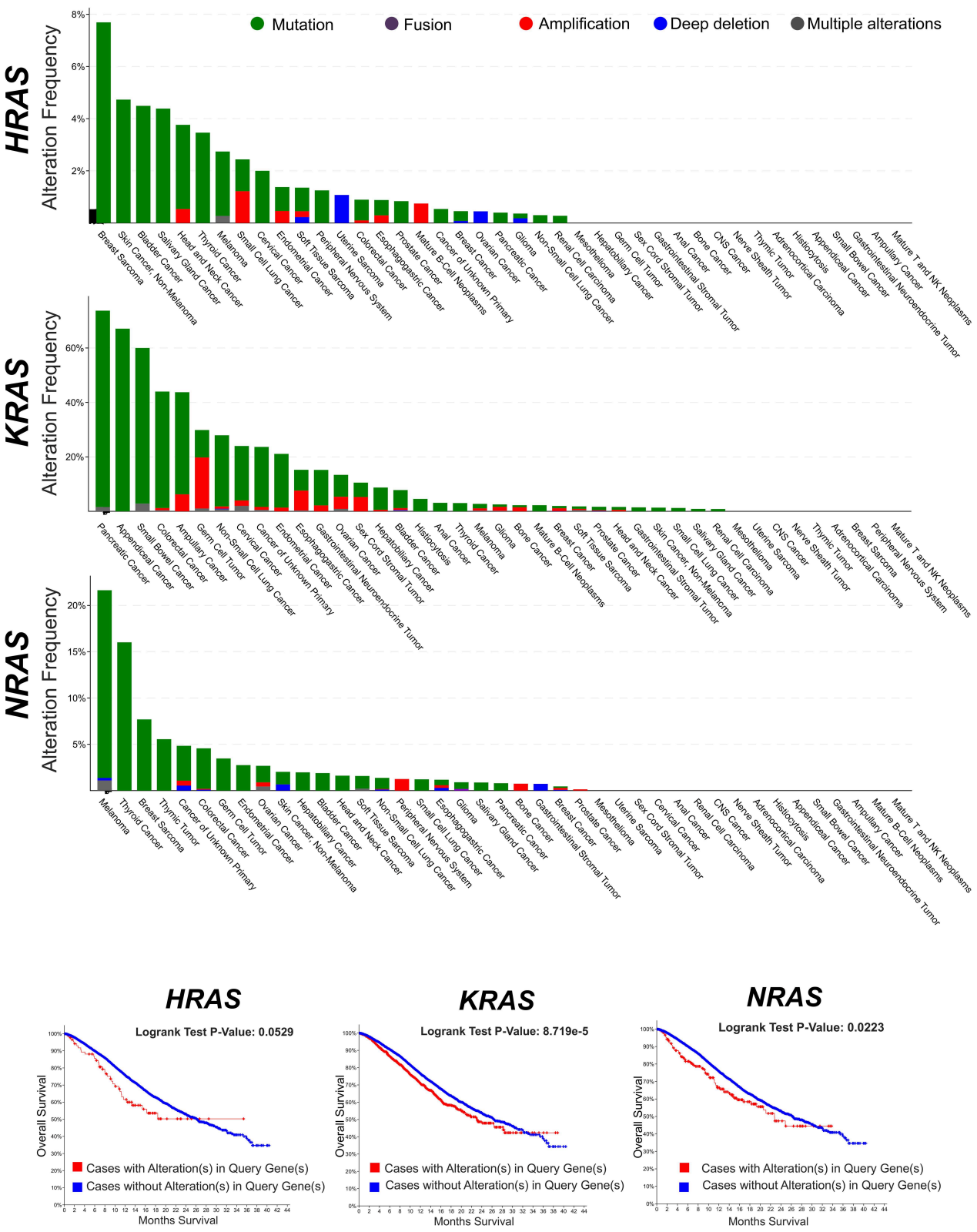

FIGURE 1 | Frequency of genomic alterations (mutation, fusion, amplification, deep deletion, multiple alterations) of the RAS genes (*HRAS, KRAS, NRAS*) across different tumor types of the MSK-IMPACT Clinical Sequencing Cohort (8). Data have been accessed through cBioportal for Cancer Genomics website (https://www.cbioportal.org).

oncogene, unlike their retroviral counterpart, cannot transform primary cells without the cooperation by a second oncogene such as MYC or adenovirus E1A.

However, the oncogenic function of RAS proteins does not rely completely on gene mutations. RAS protein overexpression, hyperactivation of upstream RAS activators, such as receptor tyrosine kinases, perturbation in the activity of RAS regulators, such as GEFs and GAPS, all may contribute to promote and sustain tumorigenicity (50, 51).

RAS Hyperfunction Induced by Upstream Tumorigenic Effectors

There are a wealth of evidence that stratified over the last 3 decades which have established a role of RAS as supporting actor in breast cancer downstream the dysregulated action of oncogenic pathways and effectors. RAS proteins serve as hub of the major intracellular signaling pathways which govern cell growth, motility, angiogenesis, immune escape. Hence, it is quite clear that the engagement of RAS function is mandatory for many oncogenic factors to be able to propagate their signals and execute their aberrant programs, while its inhibition may dampen upstream tumorigenic signals. Studies in the early 1990s reported that in 71% of human breast cancers the expression of RAS proteins was higher than in normal breast tissues and correlated with that of p185/HER-2. Interestingly, NRAS and HRAS result to be overexpressed in basal-like and HER2 tumors, the most aggressive subtypes of breast cancer (52, 53) (**Figure 2A**). HER2, as well as its cognate epidermal growth factor receptor (EGFR), is coupled to the Ras signaling by interaction with the adaptor protein Grb2, and Sos, a Ras GDP-GTP exchange factor. The overexpression of these receptors in breast cancer cells amplifies the RAS signaling pathway (54). Consistently, the tyrosine kinase inhibitors have been shown to hamper breast cancer cell proliferation at least in part by the inhibition of signal transduction processes potentially mediated through RAS (55). RAS overexpression associates with p53 loss, HER2 amplification/overexpression and aneuploidy in infiltrating ductal carcinomas (56). RAS is required also for the mammary tumorigenesis induced by the oncogene MYC, although in an inducible mouse model of c-MYC-driven mammary tumorigenesis the spontaneous occurrence of secondary RAS mutations was necessary to prevent the full regression of tumors upon c-MYC deinduction (57). Pin1, a prolyl isomerase which regulates the conformation of a subset of phosphorylated Ser/Thr-Pro motifs, is overexpressed in human tumors and interacts with Ras signaling in increasing c-Jun transcriptional activity toward cyclin D1 (58). Breast cancer displays the downregulation of the RAS/MAPK inhibitor proteins sprouty 1 and 2 (59). RAS functions downstream Rab-coupling protein RCP (also known as RAB11FIP1), a breast cancer-related oncogene (60). Bone Morphogenetic Protein 1 (BMP1) cooperates with HRAS to induce metastatic breast cancer (61). RAS signaling amplification has been reported to play a crucial role in metastatic progression and poor clinical outcome of luminal breast cancer patients (62). MicroRNA-382-5p accelerates breast cancer progression by modulating the RERG/RAS/ERK signaling axis (63). Pharmacological inhibition of SHP2 phosphatase has been recently shown to reduce the proliferation rate of receptor-tyrosine-kinase-driven human cancer cells *in vitro* and *in vivo* through the inhibition of the RAS-MAPK signaling (64). BCL-XL directly modulates RAS signaling to favor cancer cell stem-like phenotype (65).

RAS Hyperfunction Induced by Altered Activity of RAS Regulators

RAS hyperfunction with tumorigenic effects can be induced by the altered activity of RAS-specific regulators. R-RasGTPase activating protein mediates the interaction between estrogen and insulin signaling pathways in breast cancer cells (66) and affects the motile phenotype of breast epithelial cells through the modulation of Rho/Rho-kinase (67). On the other hand, RAS-GTPase inhibition promotes apoptosis in tumor cells (68). The RasGAP gene, RASAL2, functions as a tumor and metastasis suppressor in human luminal breast cancer (69) but promote triple-negative breast cancer progression through RAC1 activation (70). The Rho GTPase Rnd1 dampens mammary tumor progression and EMT by restraining RAS-MAPK signaling (71, 72). R-Ras2, a transforming GTPase that shares downstream effector with Ras proteins, promotes tumor progression in a PI3K-dependent and signaling autonomous manner although its prometastatic role requires other priming oncogenic signals and downstream effectors (73). Transposon insertion in one of two RASGAP genes, neurofibromin1 (Nf1) and RAS p21 protein activator (Rasa1), might function as the causal role of the mammary tumor development in a tumor mouse model generated by the activation of a mutagenic T2Onc2 transposon via expression of a transposase driven by the keratin K5 promoter in a $p53^{+/-}$ background (74).

RAS IN TRIPLE-NEGATIVE/BASAL-LIKE BREAST CANCER

Triple negative breast cancer (TNBC) is a heterogeneous group of tumors defined on the basis of their negativity for Estrogen receptor, Progesterone Receptor and HER2. They account for ≈15% of breast tumors and are statistically associated to poor prognosis. TNBC phenotype and clinical outcome partially overlap those of the basal-like breast cancer subtype previously identified on the basis of the gene expression profiling (52, 53), although the identification between these two categories of breast tumors is controversial (75). RAS activity and its regulators have been reported to play a role in the progression of TNBC/basal-like tumors. A 3′-untranslated region of KRAS variant has been identified which regulates the development of TNBC (76). KRAS(G12D) provides human mammary basal cells and luminal progenitors with the ability to produce serially transplantable, polyclonal, invasive ductal carcinomas into immunodeficient mice, which display a dramatic clonal diversification (77). miR-143/145 loss-of-function amplifies the tumorigenic potential of PTEN-deficient basal-like breast tumor cells at least partially

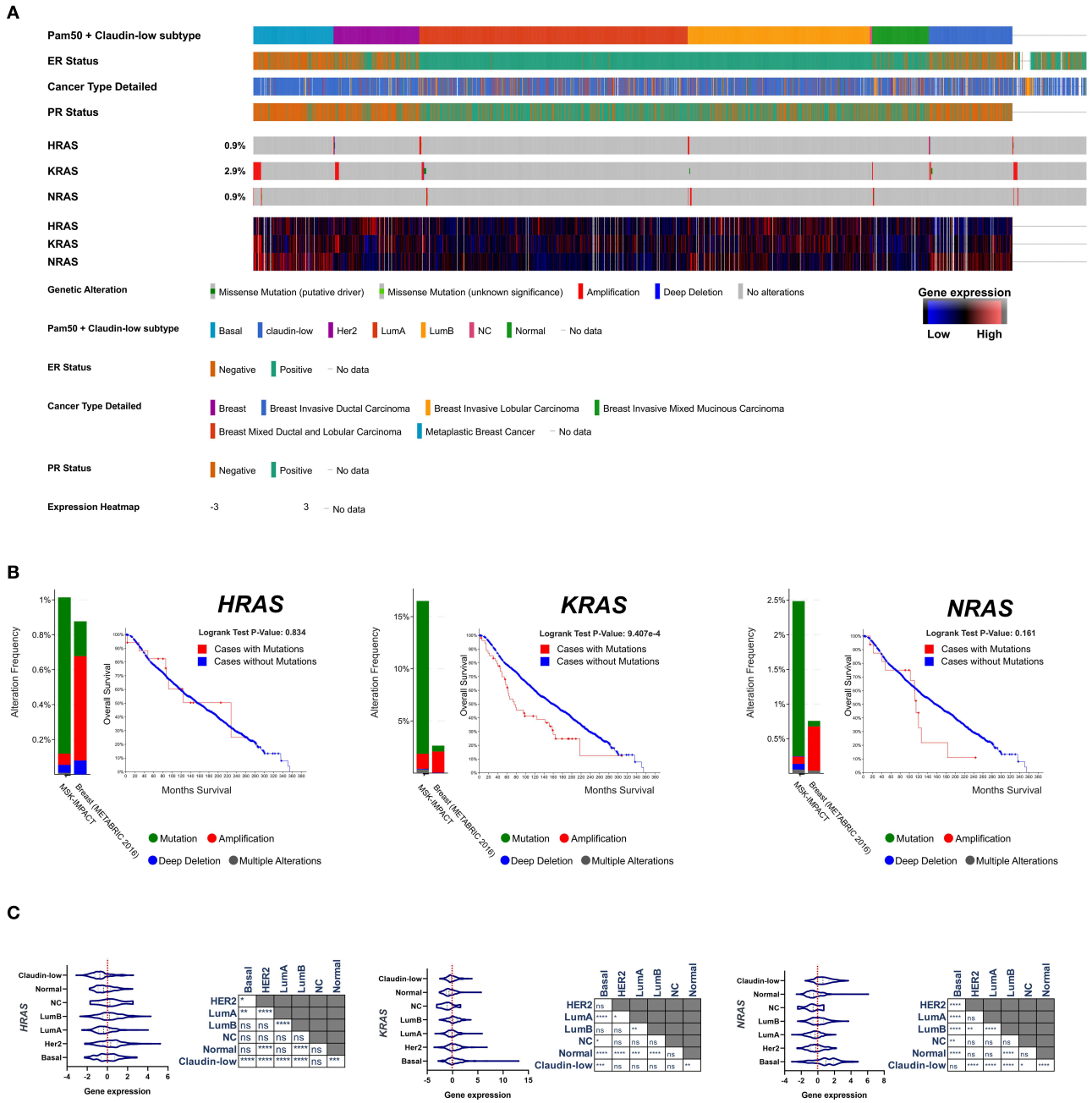

FIGURE 2 | (A) Frequency of genomic alterations and heatmap of gene expression of the RAS genes (*HRAS, KRAS, NRAS*) across 2,509 breast tumor samples of the METABRIC cohort (48), assigned to the major intrinsic subtypes (on the basis of the PAM50 profile). **(B)** The fraction of breast tumors (cases) with genomic alterations (METABRIC cohort, 2,509 breast tumors) of the RAS genes are reported in comparison with the fraction of tumor with genomic alteration in a pan-tumor cohort (MSK-IMPACT, 10945). Kaplan-Mayer curve are reported for each RAS isoform comparing the overall survival of the breast tumors with or without genomic alterations (METABRIC cohort, 2,509 breast tumors). **(C)** Gene expression level of RAS isoforms across the different subtypes of breast cancer are compared and statistically evaluated by ANOVA *t*-test (Turkey *post-hoc*) $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. Data have been accessed through cBioportal for Cancer Genomics website (https://www.cbioportal.org).

through the induction of RAS signaling. In humans, miR-145 deficiency correlates with enhanced RAS-pathway activity in basal-like breast cancer, and patient with combined PTEN/miR-145 loss or PTEN-loss/high RAS-pathway activity exhibit poor clinical outcome (78). Also, wild-type NRAS, upregulated in basal-like breast cancer (**Figure 2A**), promotes tumorigenesis through IL-8 secretion via JAK2 activation (79). RAS-MAPK pathway activation promotes immune-evasion in triple negative breast cancer (80). High level of ERK1/2 phosphorylation, a readout of Ras signaling activation, has been found in

metastatic sites relative to primary breast tumors and is more common in TNBC/basal-like cancers (81). Transcriptional signature of RAS/MAPK pathway activation is highly prevalent in TNBC/basal-like cancers compared to other subtypes of breast cancer (82, 83).

RAS IN BREAST CANCER THERAPY

Other than being a potent mediator of tumor transformation and progression, RAS might also confer resistance to therapies in breast cancer (84). RAS induces resistance to Cis-platinum by increasing GST-pi expression (85) and ERCC1 (86). Oncogenic RAS mutations cause resistance to the growth inhibitor insulin-like growth factor binding protein-3 (IGFBP-3) (87). Also, RAS induces resistance to lapatinib which might be overcome by MEK inhibition (88). RAS/Raf-1/MAPK pathway affects response to tamoxifen but not chemotherapy in breast cancer patients (89). Raf-1 functions as an effector of RAS in the radiation-response (90).

The role of RAS in breast tumorigenesis and resistance to therapies provides the rationale to assess RAS as target in breast cancer treatment. Three decades of studies contributed to rise the notion that RAS oncogenes are "undruggable," due to its conformational architecture, which lacks of pockets to facilitate the binding of small inhibitors, and its picomolar affinity for the nucleotide substrate. However, recent technologies and approaches have renewed the challenge to thwart cancer by targeting RAS directly or through its downstream signaling pathways. Direct approaches currently under investigation are addressed to enhance GTP hydrolytic activity of RAS, to inhibit its nucleotide exchange function or to prevent its interaction with downstream effectors (91, 92). These approaches are providing encouraging results at preclinical stages, but none of them have entered clinical practice thus far.

A reliable alternative approach consists in blocking the RAS downstream pathways (93). As for breast cancer, it holds great promise the therapeutic use of inhibitors of the Ras/MAPK pathway. FDA-approved Inhibitors of MEK, a central node in the Ras/MAPK pathway, specifically inhibit proliferation of TNBC/Basal-like cancer cell lines (83) and may complement chemotherapeutic treatments in xenograft models (82). MEK inhibition has been shown to prevent epithelial-mesenchymal transition and metastatic potential of tumor cells by targeting cancer stem cell compartment (94). Although the phase I studies have shown a scarce efficacy of MEK in humans, the combination with neoadjuvant or post-operative treatments might represents a promising alternative (95, 96).

CONCLUDING REMARKS

Decades of studies have contributed to unveil the primary role of RAS oncogenes in leading tumor initiation in many types of cancers. For reasons that are still unknown, breast cancer is not amongst them. Although oncogenic RAS is able to transform mammary cancer cell lines *in vitro*, the marginal incidence of RAS mutations in clinics does not support a primary role of RAS proteins in breast tumor etiology. Nevertheless, a wealth of studies over many years have demonstrated the importance of RAS function in the progression, metastatic dissemination and therapy resistance in breast cancers, regardless the molecular trigger they are initiated by, thus contributing to draw for RAS proteins a crucial role as supporting actors in breast tumorigenesis.

AUTHOR CONTRIBUTIONS

MG: conceptualization, writing, and financial support.

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Does Ras Activate Raf and PI3K Allosterically?

Ruth Nussinov[1,2]*, *Chung-Jung Tsai*[1] *and Hyunbum Jang*[1]

[1] *Cancer and Inflammation Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, National Cancer Institute at Frederick, Frederick, MD, United States,* [2] *Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel*

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

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

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***Correspondence:**
Ruth Nussinov
NussinoR@mail.nih.gov

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INTRODUCTION

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

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

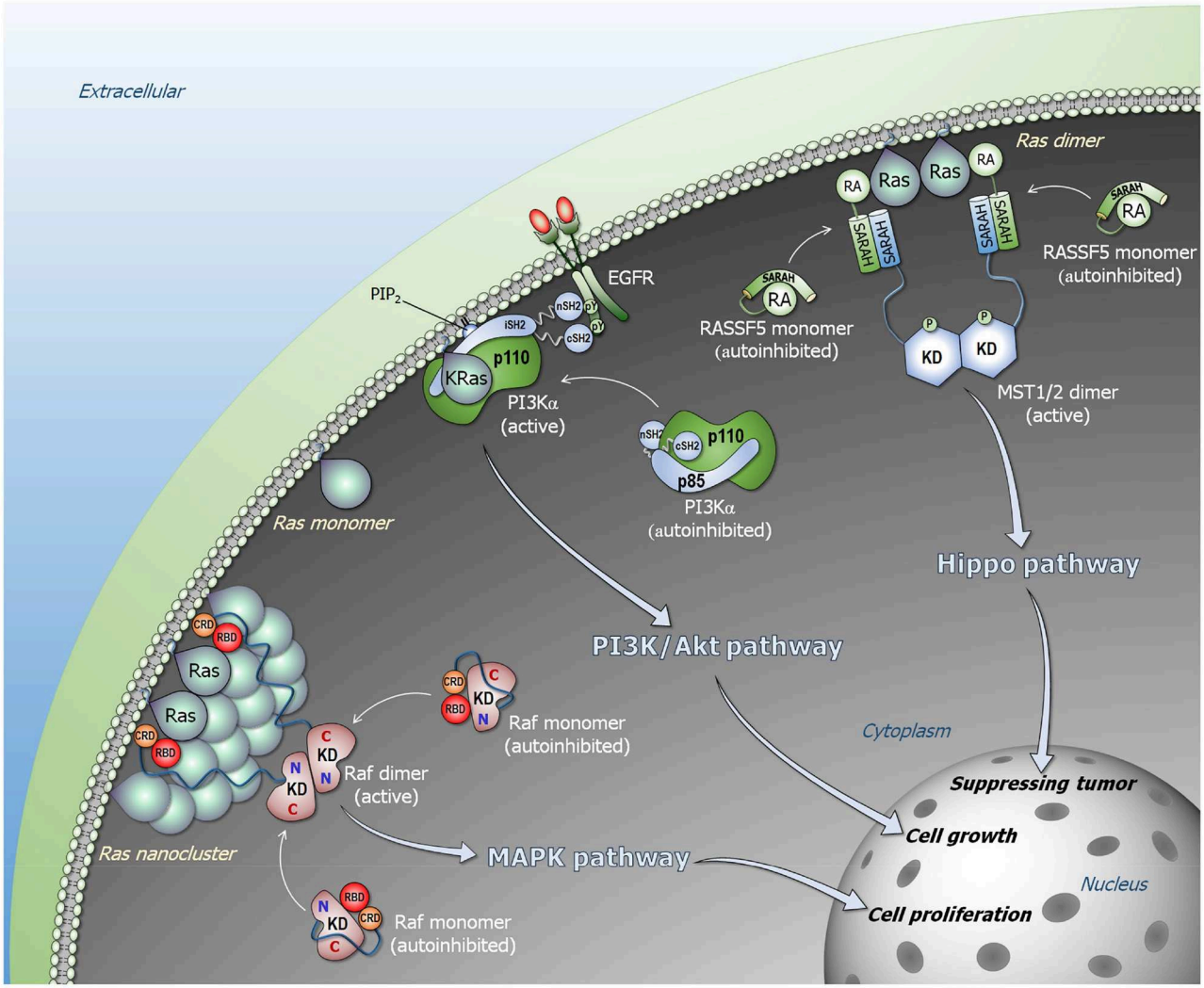

FIGURE 1 | Ras signaling pathways. Ras forms nanoclusters and promotes Raf dimerization in the Raf/MEK/ERK (MAPK) pathway (lower left). Monomeric Raf is autoinhibited in cytosol, and the high-affinity Ras–RBD interaction releases the autoinhibition, activating Raf through side-by-side dimerization. PI3Kα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

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

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

ALLOSTERIC ACTIVATION: DEFINITION AND BACKGROUND

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

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

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

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

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

ACTIVATION OF RAS EFFECTORS RAF, PI3K AND NORE1A

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

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

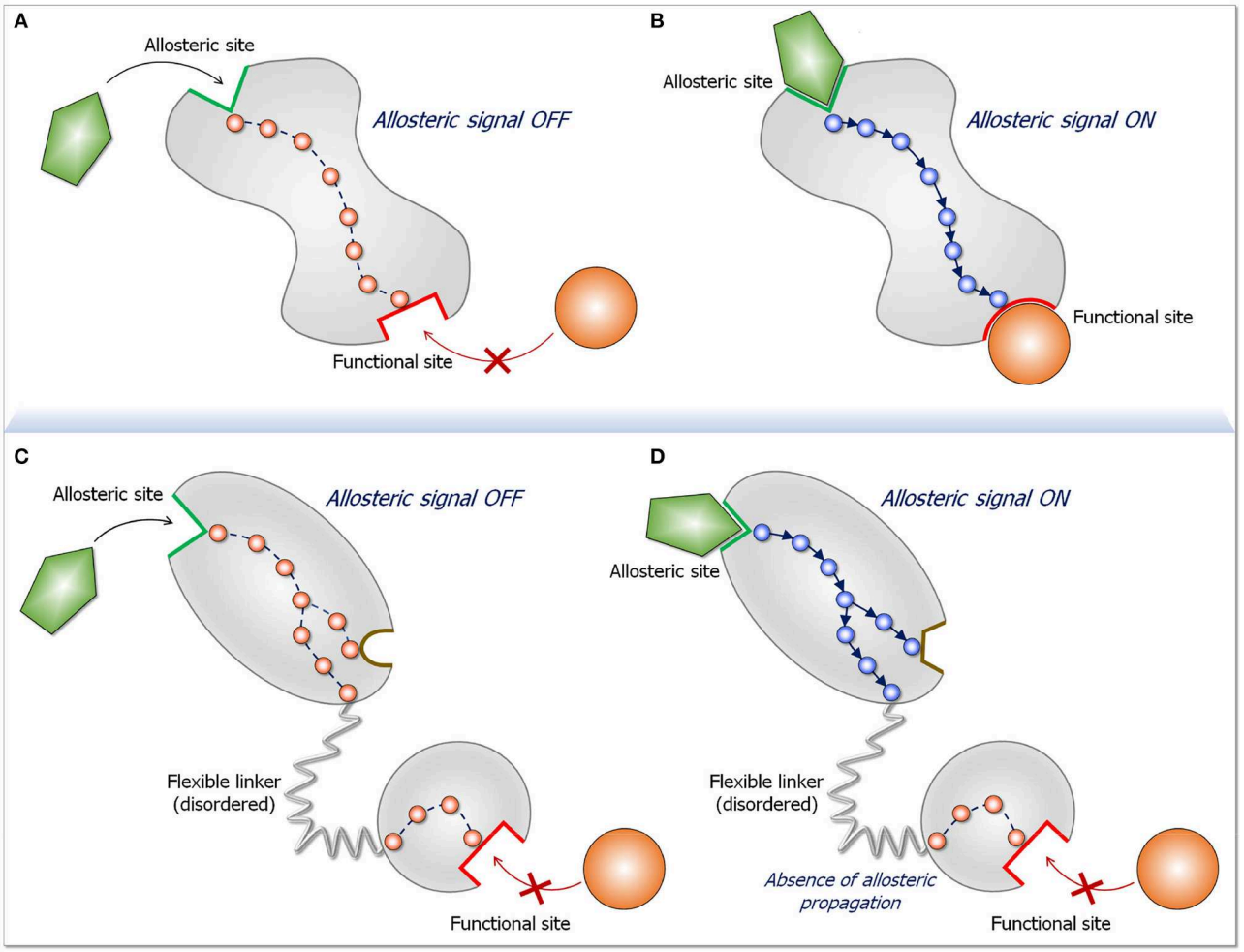

FIGURE 2 | Schematic diagram for an allosteric propagation pathway and its absence in long disordered linkers. The top two panels display a two-state dynamic allosteric switch. Both states pre-exist in the population. In the absence of the ligand **(A)** the protein populates a conformation in the ligand-free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

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

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

If Not Allostery, How Does Ras Activate Raf?

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

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

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

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

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

If Not Allostery, How Does Ras Activate NORE1A?

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

CONCLUDING REMARKS

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

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

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

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REVIEW
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RAS, Cellular Plasticity, and Tumor Budding in Colorectal Cancer

Valeria Maffeis[1], *Lorenzo Nicolè*[1] *and Rocco Cappellesso*[2]*

[1] Department of Medicine, Surgical Pathology and Cytopathology Unit, University of Padova, Padova, Italy, [2] Pathological Anatomy Unit, Padova University Hospital, Padova, Italy

The high morbidity and mortality of colorectal cancer (CRC) remain a worldwide challenge, despite the advances in prevention, diagnosis, and treatment. RAS alterations have a central role in the pathogenesis of CRC universally recognized both in the canonical mutation-based classification and in the recent transcriptome-based classification. About 40% of CRCs are *KRAS* mutated, 5% *NRAS* mutated, and only rare cases are *HRAS* mutated. Morphological and molecular correlations demonstrated the involvement of RAS in cellular plasticity, which is related to invasive and migration properties of neoplastic cells. RAS signaling has been involved in the initiation of epithelial to mesenchymal transition (EMT) in CRC leading to tumor spreading. Tumor budding is the morphological surrogate of EMT and features cellular plasticity. Tumor budding is clinically relevant for CRC patients in three different contexts: (i) in pT1 CRC the presence of tumor buds is associated with nodal metastasis, (ii) in stage II CRC identifies the cases with a prognosis similar to metastatic disease, and (iii) intratumoral budding could be useful in patient selection for neoadjuvant therapy. This review is focused on the current knowledge on RAS in CRC and its link with cellular plasticity and related clinicopathological features.

Keywords: RAS, colorectal cancer, plasticity, epithelial to mesenchymal transition, tumor budding

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***Correspondence:**
Rocco Cappellesso
rocco.cappellesso@gmail.com

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INTRODUCTION

Colorectal cancer (CRC) is a malignant epithelial tumor originating in the large bowel and in almost all cases it features as an adenocarcinoma, a neoplasia with glandular characteristics (1). Despite the big efforts of the last decades resulting in the widespread implementation of screening programs, that have proved effective in reducing the burden of the disease in the population, and in the advances of the surgical and systemic treatments, that have improved the outcome of the patients, CRC is still the third cancer for incidence and the second for mortality in both sexes worldwide (2–4). This highlights the urgent need to identify novel diagnostic, prognostic, and predictive markers and to develop new strategies for CRC prevention, early detection, and therapy to drastically reduce CRC morbidity and mortality. Indeed, the identification of circulating markers would allow to anticipate the identification of CRC in the population, to early detect interval cancers, and to better select patients really needing colonoscopy. The current categorization based on tumor histology, grade, and stage provides limited understanding of CRC biology and often fails to recognize the true high-risk population after surgery. Consistent prognostic markers would allow to tailor the treatment according to the aggressiveness of the tumor. The development of reliable sentinel lymph node methods would modify the surgical management of the disease. The discovery of mechanisms impairing the response to current drugs and of novel targetable molecular alterations would allow a more appropriate therapy in specific subgroups of patients.

In the past, pioneering morphological, and molecular studies allowed to disclose the chain of events underlying the "adenoma to carcinoma cascade" theorized by Fearon and Vogelstein characterized by chromosomal instability (CIN) and sequential mutations of Adenomatous Polyposis Coli (*APC*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and tumor protein p53 (*TP53*) genes (5). The importance of this model is such that it is the foundations on which CRC secondary prevention is based. However, it was soon clear that this model of carcinogenetic progression was not applicable to all cases of CRC since it is a heterogeneous disorder with a great variability in response to the therapies and presumed to arise from distinct precursor lesions (6). Subsequent molecular studies led to the identification of various subtypes of CRC, then grouped into a mutation-centered classification (6). However, even this approach partially failed to grasp the biological behavior of CRC and was inadequate in explaining the diversity in patient outcomes (7). More recently, research focused on gene expression profiling and characterization of tumor microenvironment pressures and stimuli to try to fill the gap in the understanding of the disease. Such strategies deepened the knowledge about cellular mechanisms of tumor progression, allowed to discover novel morphological clues of cancer aggressiveness, and provided a huge amount of data finally condensed in a new molecular classification (8).

In this article, we summarize the most meaningful molecular classifications of CRC highlighting the role of RAS in this tumor and its link with cellular plasticity, invasion, and migration at both molecular and morphological levels.

MOLECULAR CLASSIFICATIONS OF COLORECTAL CANCER

In the "adenoma to carcinoma" model, CRC carcinogenesis is presented as a stepwise process based on the accumulation of molecular alteration contributing to the malignant transformation of the mucosa. In this cascade, *APC* inactivation initiates the evolution of the mucosa into the adenoma and subsequent *KRAS* and *TP53* mutations drive the emergence of increasingly aggressive subclones (5). However, the evidence that a consistent number of CRCs lacks *APC* and *KRAS* mutations has slowly eroded the foundations of this linear theory. Thus, a different categorization was needed because tumor classification is not just to give a name to the entities, but to differentiate them according to the clarification of the clinicopathological correlations, the determination of the etiologies, and the understanding of the evolution of the disease to achieve the best response to treatment.

The first attempt to organize CRC subgroups based on correlation of clinical, morphological, and molecular features used two main molecular alterations: genetic instability and DNA methylation (6, 9–11). Genetic instability can occur in two mutually exclusive forms, one affecting whole chromosomes or portions of chromosomes (namely CIN), the other affecting small repetitive sequences of DNA [namely DNA microsatellite instability (MSI)] (12). Thus, a CRC with CIN is DNA microsatellite stable (MSS). MSI was further stratified in MSI-high (MSI-H), and MSI-low (MSI-L) depending on the frequency of the mutations in the repetitive DNA sequences throughout the genome (13). These two conditions are also linked to different onset mechanisms. While MSI-H is related to the loss of expression of one or more members of the DNA mismatch repair machinery (namely MLH1, MSH2, MSH6, and PMS2), MSI-L is connected to extensive DNA methylation of the genome due to partial methylation and loss of expression of MLH1 or loss of expression of 0-6-Methylguanine DNA Methyltransferase (MGMT) (14–16). Epigenetic instability due to aberrant promoter CpG island hypermethylation is the second cornerstone on which CRC classification is based. According to the frequency of methylation of CpG loci, CRCs are separated into negative, low, and high CpG island methylator phenotype (CIMP) groups (17–20). The combination of these features results in a classification outlining five molecular subgroups of CRC whose alterations can be found also in definite precancerous lesions (**Figure 1**). The first subtype is the conventional CRC originating from adenoma. The tumor may be sporadic or associated with inherited conditions such as familial adenomatous polyposis (FAP) and *mutY DNA glycosylase* (*MUTYH*)-associated polyposis (MAP) (21). It is the most common type of CRC accounting for ∼57% of cases and is molecularly characterized by CIN, CIMP negativity, and MSS. *APC*, *KRAS*, and *TP53* genes are usually mutated, accordingly to the "adenoma to carcinoma" sequence (6). Another CRC subtype following this mutational cascade is represented by tumors developing from adenomas in the context of Lynch syndrome (accounting for about 3% of CRCs). Indeed, these tumors are chromosomal stable and CIMP-negative, but have a hypermutator phenotype due to MSI-H caused by the inherited mutation affecting one or more components of the DNA mismatch repair system (22). *BRAF* gene is typically wild type, as opposed to the so-called sporadic MSI-H CRC that is characterized by chromosomal stability, CIMP-H, *MLH1* methylation, MSI-H, and *BRAF* mutation (23). This sort of CRC accounts for about 12% of cases and is thought to derive from sessile serrated adenoma (6, 23, 24). Another subgroup of CRC (about 8% of cases) originating from sessile serrated adenoma has chromosomal stability, CIMP-H, only partial methylation of *MLH1*, MSS or MSI-L, and harbors more commonly mutation of *BRAF* than of *KRAS* (6). The last subtype of CRC may develop from both conventional adenoma and

Abbreviations: CIN, chromosomal instability; CSS, chromosomal stability; CIMP-N/L/H, CpG island methylator phenotype-negative/low/high; MSS, microsatellite stability; MSI-L/H, microsatellite instability-low/high; EMT, epithelial to mesenchymal transition; ECM, extra-cellular matrix; TB, tumor budding; APC, adenomatous polyposis coli; KRAS, Kirsten rat sarcoma viral oncogene homolog; NRAS, neuroblastoma RAS viral oncogene homolog; HRAS, Harvey rat sarcoma viral oncogene homolog; TP53, tumor protein p53; TGF-β, transforming growth factor β; FAP, familial adenomatous polyposis; MUTYH, mutY DNA glycosylase; MAP, MUTYH-associated polyposis; CRCSC, CRC Subtyping Consortium; CMS, consensus molecular subtypes; GDP, guanosine diphosphate; GTP, guanosine-5′-triphosphate; RASGEF, recruit guanine nucleotide exchange factor; PI3K, phosphoinositide 3-kinase; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; DCLK1, doublecortin like kinase 1; ZEB, zinc finger E-box binding homeobox; TrkB, tyrosine receptor kinase B; EGFR, epidermal growth factor receptor.

sessile serrated adenoma, includes about 20% of tumors, and is characterized by CIN, CIMP-L, MSS, or MSI-L due to MGMT methylation, and always *KRAS* mutations (6). In general, CRCs with CIN are relatively more aggressive than those with MSI (25–27) and CIMP-H tumors has a less favorable prognosis than CIMP-L ones, but if CIMP-H is associated with MSI-H the outcome is slightly better (28, 29). Moreover, MSI CRCs are known to be not responsive to adjuvant fluorouracil-based therapy but may benefit of immune checkpoint blockade with anti-PD1 immunotherapy (30, 31). The major limit of this categorization is that tumors in each subgroup are considered to be a homogeneous entity from a therapeutic point of view, however they show profound differences in drug response and prognosis.

For this reason, more recent approaches shifted from the mutation-based toward the transcriptome-based classification thinking that it can better describe the behavior of the tumors. Indeed, several of such categorizations found CRC gene expression profiles more adherent to the outcome of the patients than the previous system (7, 32–37). These patient stratifications could be useful for the therapeutic decision-making process and are attractive for a rapid translation into the clinic, thus there are many expectations in this regard (7). However, several inconsistencies have emerged by the comparison of the results of these new classification systems. Indeed, each study has attained its own taxonomy including a different number of CRC subtypes. These substantial discrepancies were mostly due to the different CRC populations investigated, the various analysis platforms used, the distinct methods of bioinformatic analysis applied, and the interpretation of data performed (7, 32–37). To clear these hurdles, the CRC Subtyping Consortium (CRCSC) was formed with the purpose of evaluating potential overlaps among the different transcriptome-based CRC classifications to identify core subtype patterns (**Figure 1**) (8). Four consensus molecular subtypes (CMSs) were delineated using a network-based meta-analysis method of six different taxonomies followed by comprehensive multi-omic and clinical characterization (8). The CMS1 sort of CRCs accounts for about 14% of cases and corresponds to the "MSI immune subtype" characterized by MSI, CIMP-H, *BRAF* mutations, and intense and widespread immune infiltrate (8). CSM2, the so-called "canonical subtype," is the most common subtype of CRC accounting for ∼37% of tumors. Epithelial characteristics, CIN, activation of WNT and MYC signaling pathways, and upregulation of the miR-17-92 cluster feature this CRC (8). About 13% of CRCs are included in the "metabolic subtype" or CMS3 group, characterized by loss of regulation of metabolic pathways, CIN, CIMP-L, heterogeneous MSI-status, *KRAS* mutations, and let-7 miR family downregulation (8). Overexpression of epithelial to mesenchymal transition (EMT) markers, miR-200 family downregulation, activation of TGF-β pathway, neoangiogenesis, and stromal infiltration feature the CRC subgroup related to the worst prognosis: the "mesenchymal subtype," namely CMS4 (8). This subtype accounts for about 23% of CRC cases. Of note, ∼13% of CRCs are not classifiable in any of these categories because of intratumoral heterogeneity or a phenotype mixing molecular features of several CMS subtypes (8). The frequency of *KRAS* mutation varies among the CRC subtypes (23% in CMS1, 38% in CMS2, 28% in CMS3, and 68% in CMS4) and this could explain the different behavior of mutated tumors (7).

RAS IN COLORECTAL CANCER

The human *RAS* gene family includes three members, namely *KRAS*, neuroblastoma RAS viral oncogene homolog (*NRAS*), and Harvey rat sarcoma viral oncogene homolog (*HRAS*), encoding four proteins: KRAS4A and KRAS4B (secondary and prevalent isoforms, respectively, deriving from alternative splicing of the RNA), NRAS, and HRAS (38). By means of their GTPase enzymatic site, these small proteins play as molecular switches transducing extracellular signals, such as growth factors, differentiation factors, and mitogens, to transcription factors and cell cycle proteins in the nucleus thus triggering cell growth, differentiation, proliferation, and survival. This site cycles between the guanosine diphosphate (GDP)-bound inactive and the guanosine-5′-triphosphate (GTP)-bound active forms. In normal conditions, extracellular cues stimulate transmembrane tyrosine kinase receptors which recruit guanine nucleotide exchange factors (RASGEFs) promoting activation of the RAS GTPase through the hydrolysis of GDP to GTP (39). In turn, RAS recruits and activates several downstream effectors in different pathways, mainly the phosphoinositide 3-kinase (PI3K)-AKT pathway and the cascade comprising RAF kinase, which activate mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2), and subsequent activation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), thus promoting cell survival, proliferation, invasion, and migration (39, 40). Missense gain-of-function mutations in members of the *RAS* family have been found in about 25% of all human cancers. Usually, these are single nucleotide point mutations involving few hotspot regions: the codons 12 and 13 in exon 2, the codons 59–61 in exon 3, and the codons 117 and 146 in exon 4. Such mutations result in a conformation of the RAS active site having intrinsic hydrolytic capability (39). Thus, in mutated cells occurs an accumulation of constitutively GTB-bound active RAS proteins able to trigger downstream signaling even in the absence of extracellular stimuli.

KRAS is the most frequently mutated isoform accounting for about 20% of all human cancers. NRAS and HRAS mutations, instead, are found in about 8 and 3% of cancers, respectively (39). Interestingly, different cancer types are related to mutation of a precise RAS isoform, suggesting that the carcinogenetic role of RAS is tissue-specific (39). Indeed, *KRAS* mutations are usually detected in colorectal, pancreatic, biliary tract, and lung carcinomas, *NRAS* mutation in malignant melanomas, and *HRAS* mutation in head and neck carcinomas (41, 42). This feature has been investigated in an *adenomatous polyposis coli (APC)*-deficient mouse model where mutations of *KRAS* were able to promote the development of colorectal cancers, while *NRAS* mutations were ineffective (43).

About 40% of colorectal cancers are *KRAS* mutated, 5% NRAS mutated, and rarely *HRAS* mutated. Of note, mutations in different RAS isoforms seems to be mutually exclusive. For this reason, from now on we focus mostly on *KRAS*. *KRAS* mutations are considered to play a pivotal role both in the early

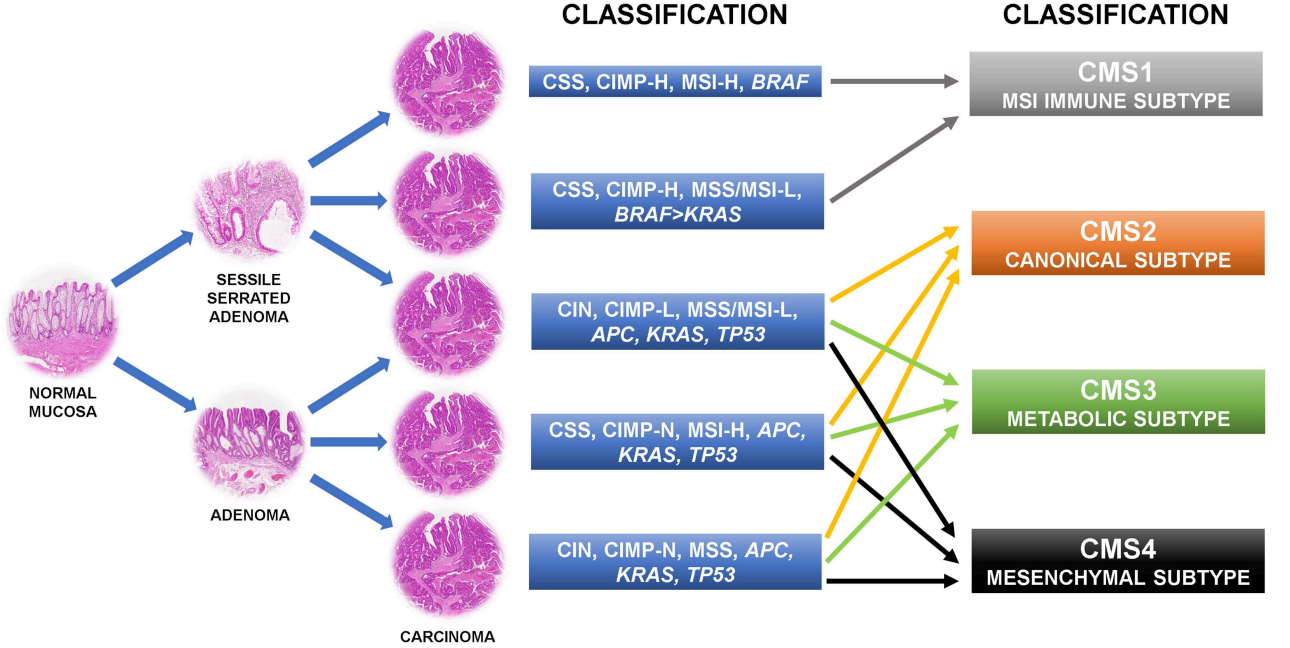


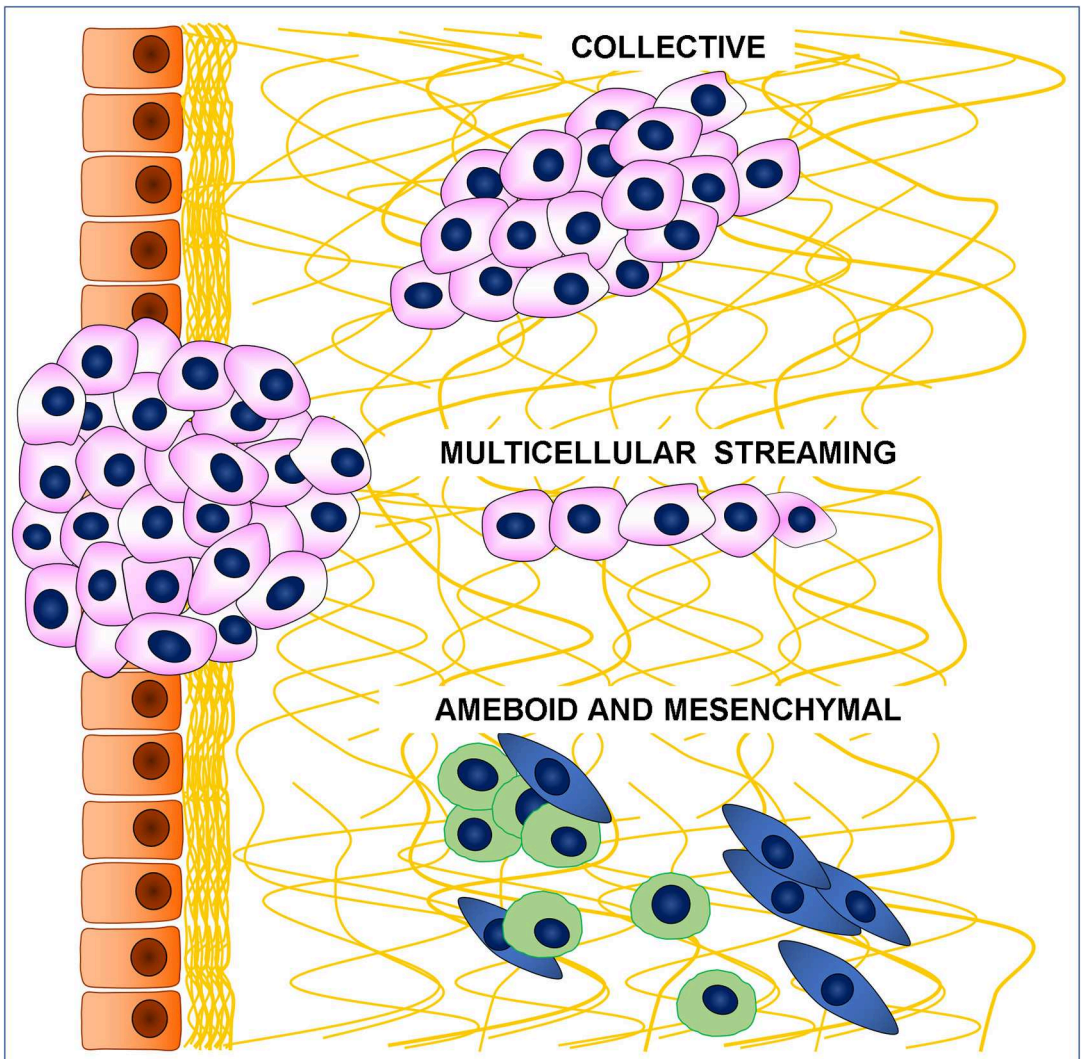
FIGURE 1 | Colorectal cancer molecular classifications recently shifted from the mutation-based toward the transcriptome-based approach because this can better describe the behavior of the tumors. CIN, chromosomal instability; CSS, chromosomal stability; CIMP-N/L/H, CpG island methylator phenotype-negative/low/high; MSS, microsatellite stability; MSI-L/H, microsatellite instability-low/high.

phases of malignant transformation of colorectal cells and in the advanced metastatic disease (44). In colorectal cancer, most KRAS mutations are in the codons 12 (about 80%) and 13 (about 15%) of exon 2 and in the codon 146 of exon 4 (about 4%); the remaining are in the codons 59-61 of exon 3 and in the codon 117 of exon 4 (45). Mutation frequency in each hotspot varies significantly among the diverse cancer types, exactly as it happens for the *RAS*-mutated isoforms (38). This could underlie that also the functional consequences of *RAS* mutation could be divergent in different cancer settings, up to assume paradoxical effects as the induction of cellular senescence as reported by Serrano et al. (46). Moreover, in the same cancer type the effects of a *RAS* mutation could vary depending on the codon involved. Indeed, a proteomic study found that in colorectal cancer cells a *KRAS* mutation in codon 12 leads to the overexpression of doublecortin like kinase 1 (DCLK1) and tyrosine-protein kinase MET, while in codon 13 brings to the overexpression of tight junction protein ZO-2 (47).

FORMS OF CELL MIGRATION AND INVASION

Metastatic dissemination results from tumor cell invasion and migration through the tissues and represents a major challenge in cancer management (48). The cornerstones of these cancer cell characteristics are deregulation of cell-cell adhesion, acquisition of cytoskeletal deformability, gaining of cellular motility, turnover of cell-matrix interactions, and extracellular matrix (ECM) breakdown (49). Cancer invasion and migration are heterogeneous and adaptive processes based on changes in the usual morphology of the cells, generation of new cell polarization, and cell body displacement that finally leads to the translocation of the entire cells. This may happen in different ways (48, 50). Indeed, tumor cell migration may be either individual, with loss of cell-cell junctions, or collective, with retention of intercellular bonds (**Figure 2**) (49). Two main types of individual cell motility have been recognized: elongated-mesenchymal and rounded-amoeboid modes. As for collective cell migration, it can happen as multicellular streaming or collective invasion. All these patterns of migration are closely linked to the ECM features, resulting from the coordinated actions of actin cytoskeleton, actomyosin contraction, cell polarity, and cell surface receptors interacting with the surrounding cells and ECM structures. Collective and individual invasion may also coexist, enhancing the efficiency of the metastatic process (51).

Individual migration patterns are featured by the absence of tumor cell-cell interactions and are strongly linked to the ECM structure. In the elongated-mesenchymal mode, the high ESM stiffness stimulates the cell to produce actin-rich protrusion, thus the cell assumes a spindle morphology with strong focal adhesion, matrix proteolysis, and actomyosin contractility localized at the rear (52). If the ECM surrounding the tumor is loose, the preferential individual invasion mode is the rounded-amoeboid pattern. The cell in this case forms small, unstable cellular protrusions (blebs or spikes) throughout its surface (53). These result from increased intracellular pressure, low degree of integrin-mediated adhesion, and reduced cell-cell interactions



	COLLECTIVE	MULTICELLULAR STREAMING	AMEBOID AND MESENCHYMAL
E-cadherin	↑↑↑	↑	↓
Proteinase	↑↑↑	↑↑↑	↑
ZEB1/2	↑	↑	↑↑↑
TWIST1/2	↑	↑	↑↑↑
SNAIL/SLUG	↑	↑	↑↑↑
miR-200 family	↓	↓	↓↓↓
miR-34	↓	↓	↓↓↓

FIGURE 2 | Tumor cell migration mode and main associated markers.

(54). Cyclic expansion and retraction of the cellular protrusion at the leading front of the cell are responsible for the cell progression (55).

In multicellular streaming migration mode, the cells move forming single cell files following the same path and are attracted by chemokines gradients or constrained by the ECM structure (56). Streaming cells can display rounded-amoeboid or spindle-mesenchymal phenotypes and advance by generating traction force on the surrounding ECM with weak and short-lived cell-cell interactions (57).

In collective migration pattern, the tumor advances through the neighboring tissues in compact clusters, strands, or cords of connected cells (49). These patterns are determined by a combination of parameters, such as cellular morphology, cell-cell adhesion, and ECM features. Unlike multicellular streaming migration, the collective migration mode is featured by cohesive cells forming solid strands or cords lined up for two or more cells, even to create broad clusters (58). This pattern is supported by long-lived cell-cell interactions, while the morphology varies according to cell nature, ECM features, and host tissue types (56). Main feature of an invasive multicellular mass is the specialization of the leading edge cells that express a mesenchymal phenotype, generate an integrin-mediated forward traction and ECM rearrangement by enzyme-mediated proteolysis of the surrounding structures (59). Interestingly, this invasion pattern has been described as the slowest migration mode (60), conferring some advantages to the tumor, such as secretion of higher amount of pro-invasive factors and immune escape (61).

MOLECULAR REGULATION OF CELLULAR PLASTICITY IN COLORECTAL CANCER

The cellular plasticity needed to allow migration of cancer cells is achieved through complex mechanisms finely governed by several genes, most of them encoding for transcription factors. In CRC, the best delineated of these molecular programs driving cellular migration is EMT, that is characterized by the acquisition of a mesenchymal phenotype through tight junction dissolution, disruption of apical-basal polarity, and reorganization of the cytoskeletal architecture (62). A huge amount of studies has shown that EMT plays a pivotal role in cancer progression and metastasis in several tumor types, including CRC (63). EMT requires a precisely regulated cooperation of a complex molecular network, which comprises factors categorized into three groups: the extracellular cues activating EMT (EMT inducers), the transcription factors orchestrating the EMT program (EMT core regulators), and the effector molecules executing the EMT-related cellular transformation (EMT effectors) (64). The best characterized external inducers are the transforming growth factor- β (TGF-β) signaling and the WNT/β-catenin pathway. Both these pathways may induce the expression of the three main family of EMT regulators: (i) the SNAIL family of zinc-finger transcription factors comprising *SNAIL* and *SLUG*; (ii) the zinc finger E-box binding homeobox (ZEB) family of transcription factors including *ZEB1* and *ZEB2*; (iii) the TWIST family of basic helix-loop-helix (bHLH) transcription factors encompassing *TWIST1* and *TWIST2*. The roles of these transcription factors in EMT have been well-established in a variety of cancers including CRC, and most of them showed correlation with the prognosis (65, 66). Final effects of EMT regulators are the overexpression of genes encoding for proteins linked to mesenchymal phenotype, such as vimentin, fibronectin, α-smooth muscle actin, and N-cadherin, and the down-regulation of epithelial markers, such as E-cadherin, claudins, and occludins (64). Post-transcriptional regulation of gene expression by EMT-related miRNAs showed a great impact in promoting epithelial or mesenchymal phenotype targeting specific mRNA (67). Members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) promote epithelial phenotype preventing the translation of *ZEB1* and *ZEB2* mRNA (68–70) that, in turn, act in a negative feedback loop down-regulating the miR-200 family expression (71). Moreover, *ZEB2* is also identified as a direct target of miR-132, miR-192, and miR-335. Downregulation of these miRNAs is usually associated with the acquisition of an aggressive mesenchymal phenotype leading to distant metastasis and dismal prognosis (72, 73). MiR-34a/b/c is another caretaker of the epithelial phenotype through the down-regulation of *SNAIL, SLUG,* and *ZEB1* (74). Suppression of miR-34a/b/c causes up-regulation of SNAIL resulting in the enhanced expression of EMT markers, mesenchymal features, and improved cell invasion and motility.

As above mentioned, *KRAS* mutation is common in CRC and activates several effector pathways involved in cell proliferation, invasion, and migration. In particular, RAS signaling has been reported to play a crucial role in EMT initiation (75, 76). It has been shown that in CRC cell lines mutated KRAS can activate downstream effectors of the PI3K pathway, such as Ras homolog gene family member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division cycle 42 (Cdc42), and in synergy with TGF-β signaling can promote EMT inducing a decrease of E-cadherin expression and an increase of vimentin expression (**Figure 3**) (40, 77, 78). Thus, it seems that *KRAS* mutation alone is not able to modify the epithelial morphology of CRC cells but requires the cooperation of growth factor cues to accomplish the cell transformation.

RAS activation is a crucial connector between receptor and cytoskeleton during chemotaxis in normal conditions (79). Indeed, PI3K-triggered RAS acts on F-actin forming a coupled excitable system that leads to short-lived RAS-F-actin patches that anticipates the extension of cellular protrusions (80).

Moreover, the activation of MEK1 in the RAS-RAF-MEK cascade allows the enrollment of the downstream effectors Egr-1 and Fra-1 that can promote the expression of SNAIL and SLUG, which in turn downregulate E-cadherin expression (81). In EMT, the pathways that regulate actomyosin and cytoskeleton dynamics drive plasticity and *KRAS* mutation can determine the mode and effectiveness of migration by means RhoA and Rac1 signaling (82, 83).

Several miRNAs were linked to K-RAS-driven tumorigenesis. In experimental models down-regulation of miR-1, Let-7a, miR-16, miR-18a, miR-30a, miR-217, miR-622 results in increased

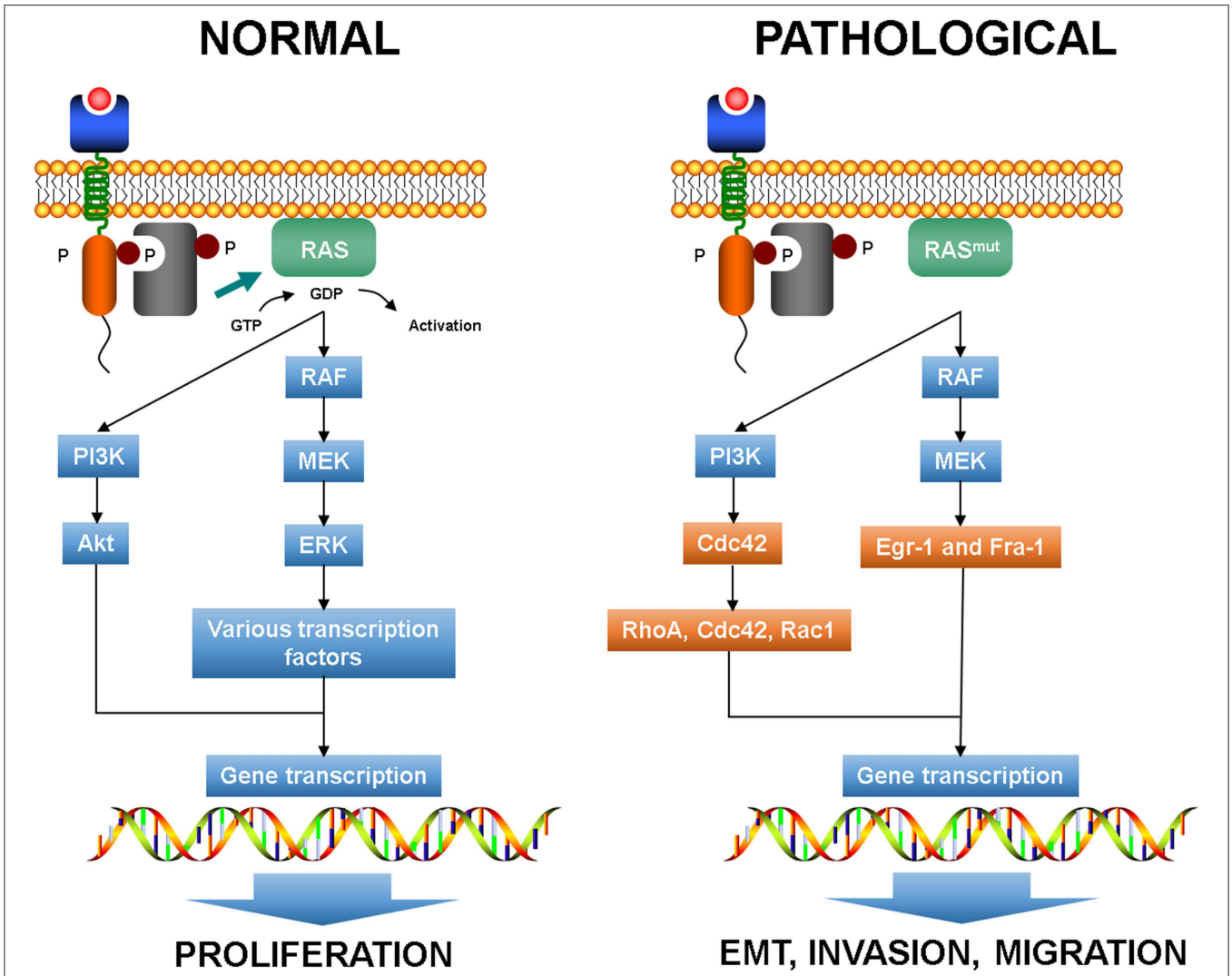


FIGURE 3 | Normal RAS pathways and plasticity-related aberrant pathways. EMT, epithelial to mesenchymal transition.

K-RAS expression (84). In particular, miR-30a directly targets KRAS and PI3K inhibiting anchorage-independent growth, cell migration and invasion, and *in vivo* tumorigenesis by KRAS-mutant CRC cells (85, 86). Moreover, low expression of miR-30a has been found in highly metastatic CRC cell lines and liver metastases (86). Clinically, down-regulation of Let-7a was correlated with increased risk of nodal metastasis and with shortened overall and disease-free survival (87).

TUMOR BUDDING AND MECHANISMS OF CELLULAR PLASTICITY IN COLORECTAL CANCER

According to the definition of the International Tumor Budding Consensus Conference (ITBCC) proposed in 2016 (88) and then validated in 2018 (89, 90), CRC tumor budding (TB) consists of single neoplastic cells or cell clusters of up to four neoplastic cells at the invasive front of the tumor (peritumoral TB) (**Figure 4**) or within the tumor mass (intratumoral TB) (88). In Western countries, these recommendations were incorporated into the College of American Pathologists (CAP) cancer protocol for patients with primary CRC (91), in the 8th edition of the American Joint Committee on Cancer (AJCC) staging manual (92) and in the European Society for Medical Oncology consensus guidelines (93). This acknowledgment derives from the increasing and established evidences of TB as reliable and independent prognostic factor in CRC, regardless of the scoring method applied for the evaluation (3, 90, 94–96). However, the inclusion of TB in the pathologist report is not yet mandatory, but merely recommended. This is due to its apparent poor reproducibility along with the lack of a standardized scoring system before the ITBCC (97–99). Indeed, TB definition and evaluation method have been controversial throughout its development and different diagnostic criteria are present in the literature (3, 100). The recent agreement reached upon the

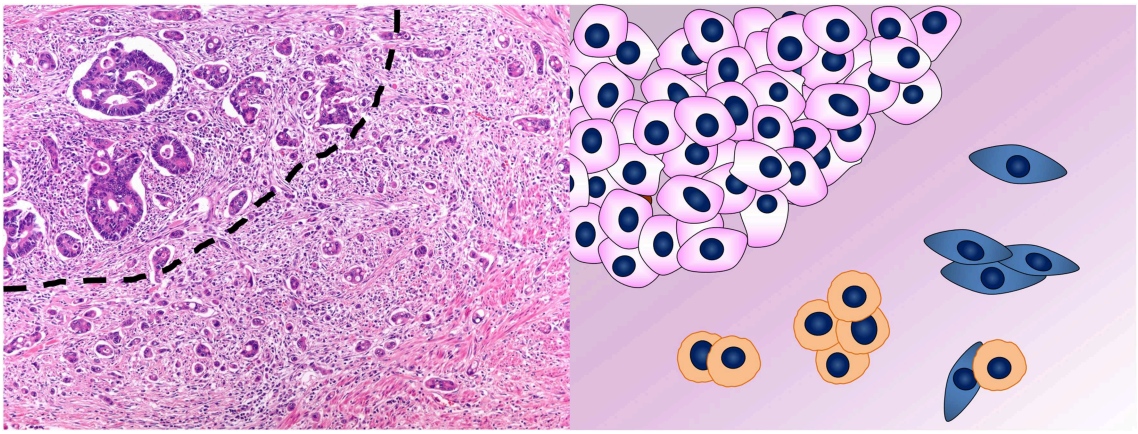


FIGURE 4 | Tumor budding in colorectal cancer. In the photomicrograph, the *dashed line* separates the tumor mass on the top left from the tumor buds on the bottom right. This phenomenon is depicted in the cartoon where single cells or aggregates of up to four cells detach from the mass of neoplastic cells in the top left and infiltrate. Hematoxylin & eosin stain. Original magnification 200x.

definition and scoring system method (89) is an essential step to implement TB in the routine CRC assessment.

The morphological feature now called TB was originally described in Japan by Imai in 1949 (101) and firstly reported in the English language literature by Gabbert in 1985 (102). Histologically, TB cells show a more marked atypia than their counterparts in the tumor bulk, thus TB was initially termed "tumor dedifferentiation" (102). Imai, instead, proposed the term "sprouting" to describe the tumor cells detaching from the tumor mass along its invasive edge. Moreover, he suggested to use this feature, peritumoral stromal reaction, and lymphovascular invasion in a prognostic system for gastric cancer (101). Some Japanese researches observed the same phenomenon in CRC (103–105) and it was called TB by Morodomi in 1989 (106). In the last decades, a growing number of data reinforced the value of TB as CRC prognostic marker (107–114). Besides CRC, TB has been found in a variety of other solid tumors, such as oral squamous cell carcinoma (115, 116), invasive ductal breast cancer (117), pancreatic (118), and esophageal cancer (119).

Invasion and metastasis are some of the hallmarks of cancer (120), which requires the ability of tumor cells to detach from the primary tumor, move through the ECM, invade lymphovascular vessels, and finally reach and colonize lymph nodes and distant organs (121, 122). TB is the histological demonstration of this ability, which is intrinsically dynamic. Thus, it is conceivable that tumor buds possess cellular plasticity properties, such as cytoskeletal deformability, motility, and full or partial EMT characteristics (122).

Tumor buds often show typical features of EMT (**Table 1**): loss of E-cadherin expression, β-catenin translocation in the nucleus (sign of WNT pathway activation), and acquisition of vimentin expression (122). The motile and invasive phenotype of TB cells is depicted by the loss of cell adhesion molecules (such as E-cadherin), overexpression of proteins involved in ECM degradation and cell invasion (such as MMP2, MMP9, and cathepsin B), and cell migration (such as laminin, fascin, and α-smooth muscle actin) (121, 138, 142, 143). However, some studies failed in confirming the expression of the classic EMT-related transcription factors ZEB1, TWIST, SNAIL, and SLUG in tumor buds (131).

Tumor buds and their corresponding tumor bulk share the same driver mutations (125). De Smedt et al. found 296 differentially expressed genes by the comparison of neoplastic cells in the tumor mass and those microdissected from the tumor buds (126). TB cells undergo phenotype switching while detaching from the main tumor, with upregulation of genes related to cellular motility and downregulation of genes involved in cell growth and proliferation (126). This is consistent with the hypothesis that migration and proliferation are spatially and temporally exclusive (122). Regarding the CRCSC categories, TB cells showed a gene expression profile consistent with the "mesenchymal phenotype" (CMS4), while the cells in the main tumor had a molecular signature similar to the "canonical subtype" (CMS2) (126). This finding is supported by the results of another study in a large series of CRCs highlighting the association of TB with CMS4 phenotype—a greater number of tumor buds was found in CMS4 than in CMS2 and CMS3 tumors—and *KRAS* mutations (90). A significant association between *KRAS* mutations and the presence of high-grade TB has been reported in CRC (**Table 1**) (122, 131, 138, 142, 143). *In vitro*, *KRAS* mutations can induce expression of ZEB1, which promotes EMT, invasion, and metastasis (71, 132). Moreover, TB cells in CRC patients show increased expression of ZEB1 and a concomitant reduction of miR-200b and miR-200c, supporting the association between miR-200 family members and EMT (133). Resistance to anoikis, the cell death mechanism that occurs to non-neoplastic cells when detach from ECM, is a prerequisite for TB cells to survive during invasion. Neurothropic tyrosine receptor kinase B (TrkB) is a potent anoikis suppressor, which is overexpressed in tumor buds and in

TABLE 1 | Studies which investigated the *KRAS* status and/or TB in relation to cell morphology and/or cellular plasticity, also considered as EMT, or partial-EMT phenotype.

References	Markers	Materials	Methods	Results
Alamo et al. (123)	TB[a], CXCR4, 5β-integrin, VEGFA, Serpine-1, and Akt	FFPE from primary CRC and metastasis induced in mice	H&E/IHC/ ELISA	Higher LN metastasis and TB, CXCR4, 5β-integrin, VEGFA, and Serpine-1 overexpression in *KRAS* G12V than KRAS G13D CRC, supporting the higher aggressiveness of CRC harboring this specific mutation
Hammond et al. (47)	DCLK1, proteome, and phosphoproteome	Colon cancer cell lines	–	DCLK1 is amplified and highly overexpressed (mRNA) in *KRAS* G12D cells (transcriptional up-regulation); its amplification is reversed upon suppression of KRAS expression: KRAS has a direct role in regulating DCLK1 expression
Cho et al. (124)	E-cadherin, VIM, RAS, β-catenin	KRAS mutated CRC cell lines/mice	IHC (TMA)/immunoblotting/real time imaging/flow cytometry	KY1022[§] prevent spindle cell morphology, E-cadherin loss, and VIM over-expression, inhibiting development of metastatic CRC
Centeno et al. (125)	Pan-CK, TB, 50 oncogene, and tumor suppressor genes	FFPE CRC	IHC/NGS	No difference in driver mutations between TB and main tumor (isolated by laser capture microdissection); *KRAS* mutation is not acquired in TBs
De Smedt et al. (126)	Pan-CK, TB, gene expression profile (mRNA), CSM	FFPE CCR	IHC/RNA seq/pathway analysis/clustering	EMT signature (CMS4, mesenchymal phenotype), upregulation of CSC related genes and cellular movement/survival genes, and downregulation of cell growth/proliferation genes in laser microdissected TB compared to tumor bulk, in relation to the CMS taxonomy of CRC
Trinh et al. (90)	TB, CSM	Patient cohorts (AMC-AJCCII-90, LUMC, CAIRO, and CAIRO2) FFPE	H&E/IHC (TMA)	TB is related to CMS4 phenotype (vs. CMS3/2) and with *KRAS* and *BRAF* mutations
Prall et al. (127)	CK18 positive TB, β-catenin, SMAD4, pSTAT3, pERK1/2, KRAS, BRAF [molecular analysis (128)]	FFPE CRC/fresh human CRC tissue for subcutaneous xenografting in T- and B- deficient mice	IHC/ morphometric studies (image J)	In the xenografts TB is reduced, tumor cells are pSTAT3 negative (indicating absence of cytokine/chemokine signaling), some are partially positive for pERK1/2, with maintenance of nuclear β-catenin and SMAD4 immunostainings, and WNT and BMP pathway activation. *KRAS/BRAF* mutational status did not correlate with TB or podia formation in the xenografts
Smit et al. (129)	TrkB, E-cadherin, TWIST, SNAIL, MAPK pathway	Cell culture	Immunoblotting/IF/qRT-PCR/…	TrkB induces an EMT- like transformation in epithelial cells through a Twist-Snail signaling axis, which is dependent on the MAPK pathway. Furthermore, Snail plays a critical and specific role in TrkB-mediated metastasis
Dawson et al. (130)	TrkB, Ki-67, caspase-3, TB	FFPE CRC	IHC (TMA)	Overexpression of TrkB in TB in comparison to main tumor, and association with *KRAS* mutation. High expression of membranous TrkB is an independent adverse prognostic factor. Inverse correlations between Trkb expression and Ki-67 as well as Caspase-3
Yamada et al. (131)	E-cadherin, ZEB1, TWIST, SNAIL, SLUG, TB	FFPE CRC	IHC (TMA)	Absent expression of these EMT markers in TB, but great expression in stromal cells surrounding high grade-TB than in low grade-TB areas

(Continued)

TABLE 1 | Continued

References	Markers	Materials	Methods	Results
Gibbons et al. (132)	Mir-200 family, ZEB1, ZEB2, CDH1, CDH2, and VIM, (…)	Lung cancer cell lines (3D- culture) derived from mice (*KRAS* and *p53* mutant)	mRNA and miRNA expression profile/qRT-PCR/IF/migration and cytogenetic assay	These tumor cells have a marked plasticity [transit reversibly between epithelial and mesenchymal states, forming highly polarized epithelial spheres in 3D culture that underwent EMT, which is dependent on miR-200 family (decrease during EMT)]. Forced expression of miR-200 abrogated the capacity of these tumor cells to undergo EMT, invade, and metastasize, and conferred transcriptional features of metastasis-incompetent tumor cells. Tumor cell metastasis is regulated by miR-200 expression, which changes in response to contextual extracellular cues
Liu et al. (71)	RAS, miR-200, Rb1, Bmi1, ZEB1, ZEB2, (…)	Cell culture/KRAS mice/NCBI database (GSE11969)	RT-PCR/WB/ISH/H& E/immunostaining/human lung adenocarcinoma microarray analysis	Rb1 pathway status regulates a ZEB1-miR-200 loop downstream of RAS to control expression of Bmi1. Rb1 and ZEB1-miR-200 link RAS to Bmi1 to regulate a cellular choice between oncogene-induced senescence and tumor progression in RAS mutated cells, also triggering EMT
Knudsen et al. (133)	Mir-200b, TB, E-cadherin, β-catenin, and laminin-5γ2	FFPE CRC	IHC/CISH/IF	MiR-200b is downregulated in the TB, but not statistically associated with the expression of the other markers. Loss of membranous E-cadherin and ↑ nuclear β-catenin in the TB (majority of the cases), while laminin-5γ2 expression is upregulated at the invasive front and in the TB (half the cases)
Jang et al. (134)	KRAS, NRAS, BRAF, PIK3CA, TP53, and POLE mutations, and clinicopathological correlations, TB	FFPE CRC	H&E/Sequenom MassARRAY/direct DNA sequencing of KRAS	21 of 34 tumors with high-grade TB had *KRAS mut*; *KRAS* G12D and PIK3CA exon 9 variants were significantly associated with high-grade TB; exons 3 and 4 *KRAS mut* tumors tend to have lymphovascular tumor emboli and perineural invasion
Chang et al. (135)	Clinicopathological features, TB, p16, E-cadherin, β-catenin, HPV-status, KRAS, BRAFV600E	FFPE CRC	H&E/IHC/PCR/HPV-ISH	Comparing early-onset (≤ 40 years of age) and control (> 40 years) CRC groups, no difference emerged in the occurrence of TB, as well as lymphatic invasion, mucinous histology, or tumor-infiltrating lymphocytes, neither in *KRAS* mutations occurrence
Graham et al. (136)	TB, KRAS, BRAF, MSI, CIMP, clinicopathological features	FFPE CRC	H&E/IHC	High TB (≥10 tumor buds in a 20× objective field) is present in 32% (179 of 553) of cases, and is associated with advanced pathologic stage, MSI, *KRAS* mutation and on multivariate analysis with a >2 times risk of cancer-specific death
Steinestel et al. (137)	KRAS, BRAF, MMR status, TB, clinicopathological features	FFPE CRC	H&E/IHC/DNA pyrosequencing	TB is associated with infiltrative growth, absence of peritumoral lymphocytic reaction, and blood/lymph vessel infiltration. Neither *KRAS* nor *BRAF* mutations are associated with a certain growth pattern or TB intensity
Zlobec et al. (138)	KRAS, BRAF, MGMT, CIMP, TB	FFPE CRC	H&E/IHC/molecular analysis*	TB is not associated with *KRAS*, *BRAF*, MGMT, or CIMP, but is correlated inversely with MSI-H. TB has an independent role of all these five molecular features and is predicted by MSI status

(Continued)

TABLE 1 | Continued

References	Markers	Materials	Methods	Results
Pai et al. (139)	TB, BRAFV600E, KRAS, MSI, CIMP	FFPE CRC	H&E/MSI PCR and IHC	In the adenocarcinomas of the proximal colon, no relationship between *KRAS* mutation and TB is identified
Pai et al. (140)	TB, molecular profiling, MSI, clinicopathological features	FFPE surgically resected pT1 CRC (western cohort)	H&E/NGS/MSI PCR and MMR IHC	High grade TB is significantly associated with lymph node metastasis on univariate and multivariate analysis [OR 4.3 (p = 0.004)]. No relation with *RAS* mutation is identified
Landau et al. (141)	KRAS, BRAF, MMR status, TB, clinicopathological features	FFPE CRC	H&E/IHC/PCR	Adenocarcinomas of the caecum display the highest frequency of *KRAS* mutations and high TB in the colon (compared to right (non-cecal proximal) and left (distal) adenocarcinomas). Cecal tumor site and high TB are also predictive of poor survival, particularly in stage III/IV of disease

When the definition of tumor budding differs from up to five cells at the invasive front, the definition applied is reported.
[a]*Defined in this study as 10 or fewer cells at the tumor front, counted on IHC (keratin positive cells or clusters) in 3 different tumor fields (400x magnification).*
[§]*KY1022 is a destabilizer of RAS protein and β-catenin.*
[*]*DNA bisulphite conversion, amplification of modified DNA, and pyrosequencing.*

CRC with high-grade tumor budding and *KRAS* mutations (130). Indeed, RAS signaling promotes TrkB-induced EMT, anoikis resistance, and metastasis through TWIST and SNAIL (129). Morphologically, treatment of *KRAS* mutated cell lines with a destabilizer of β-catenin and RAS proteins can prevent spindle cell morphology as well as E-cadherin loss and vimentin over-expression (124). A xenograft model of CRC was also studied, but *KRAS* mutational status did not correlate with TB or podia formation (127).

THE PROGNOSTIC RELEVANCE OF TUMOR BUDDING IN COLORECTAL CANCER

TB can be considered as a snapshot of the dynamic process of invasion and a surrogate morphological marker of EMT. The translation into the clinics of TB, for a long time believed as a sign of biological aggressiveness, fits with its demonstration as an adverse and independent prognostic marker in all stages of CRC (**Table 2**) (3, 90, 94–96, 122, 136, 141, 146, 148). Regardless of the assessing method, evidences suggest that TB has a prognostic effect independent of age, sex, and stage of disease (3, 90, 94–96, 122, 123, 135, 146, 148). TB is usually associated with high tumor grade, advanced stage, lymphovascular invasion, nodal and distant metastasis, locoregional and distant recurrence, and worse overall, disease free, and recurrence free survival (122). The clinical implications are not only prognostic but also therapeutic. In metastatic patients, the presence of high tumor budding can predict resistance to anti-EGFR therapies (149). Moreover, *KRAS* status assessment seems to be useful to identify possible non-responder patients in the metastatic setting (149). Recently, it has been demonstrated that intratumoral TB is related to nodal and distant metastasis in CRC (90, 150–152). Apparently, intratumoral TB has a prognostic effect assessed on a continuous scale (90), and similarly to peritumoral TB has been associated with higher stages, vascular invasion, infiltrative margin, poor survival, and to peritumoral TB itself (122, 147). To date, the prognostic impact of TB has been associated with three major clinical scenarios.

First, in CRC infiltrating the submucosa (categorized as pT1 according to the current staging system), TB is an accurate predictor of nodal metastasis (3, 88, 95, 153–155). A recent meta-analysis including over a thousand of patients with endoscopically removed pT1 CRCs has shown that tumors with TB are strongly associated with lymph node involvement (3). In a western cohort of 116 surgically resected pT1 CRCs, high grade TB has been significantly associated with lymph node metastasis on univariate and multivariate analysis (140). While the Japanese Society for Cancer of the Colo-Rectum has already incorporated TB among the mandatory prognostic variables for pT1 CRC reports, in Western countries this has not yet happened. However, the available evidences strongly support its incorporation also in Western guidelines to improve lymphadenectomy planning (3, 88, 89, 140).

Second, in stage II CRC (namely a tumor without nodal and distant metastasis) the presence of high-grade TB confers a more aggressive behavior similarly to stage III CRC (namely a tumor with nodal metastasis but without distant metastasis) (96, 109, 111, 113, 126, 144, 145, 156). A metanalysis including over a thousand and a half stage II CRC patients highlighted that tumors with high grade TB are associated with worse overall survival, with a difference of survival of about 25%, mostly in pT3N0M0 patients (96). The survival rate of stage II CRC patients stratified as low or high grade TB vs. stage III CRC ones has been directly studied showing significantly differences depending on TB level (111). In particular, the survival rates of stage II CRC patients with high grade TB resulted

TABLE 2 | Selected studies and reviews (^) which investigated tumor budding as a prognostic marker in colorectal cancer.

References	Stage	ITB and/or PTB	Prognostic parameters associated with TB
Beaton et al. (95)^	Early CRC	n.a.	A total of 4510 patients from 23 cohort: TB is significantly associated with LN metastasis
Pai et al. (140)	pT1	PTB	High TB is significantly associated with LN metastasis on univariate and multivariate analysis
Cappellesso et al. (3)^	pT1	n.a.	A total of 10,137 patients from 41 studies (heterogeneous TB definition): strong association between the presence of TB and risk of nodal metastasis in pT1 CRC
Okuyama et al. (109, 144)*	II vs. III	PTB	TB-positive CRC have worse outcome and more frequently LVI and LN metastasis than TB-negative CRC. TB-positive stage II CRC have similar outcome as TB-negative stage III. TB is an independent prognostic factor in stage II and III CRC (multivariate analysis)
Nakamura et al. (111)	II vs. III	PTB	Significant correlation of TB and LN and distant metastasis, and survival. Similar survival rates between high TB stage II tumors and stage III disease
Wang et al. (145)	T3N0M0	PTB[#]	High-TB is associated with infiltrative growth pattern and LVI. 5-year cancer-specific survival is poorer in high vs. low TB. TB is an independently prognostic (multivariate analysis)
Petrelli et al. (96)^	II	n.a.	A total of 1,652 patients from 12 studies (heterogeneous TB definition): TB is associated with worse 5-y OS in stage II CRC, in particular in pT3N0M0 patients. High-grade TB is associated with an increased risk of death
Zlobec et al. (138)	I–IV	PTB	High grade TB is an independent prognostic factor even in presence of genetic and epigenetic aberrations (those investigated in this study). TB is predicted by MSI status
Steinestel et al. (137)	I–IV	PTB	TB is significantly associated with infiltrative growth, absence of peritumoral lymphocytic reaction, and blood and lymph vessel infiltration
Graham et al. (136)	I–IV	PTB	TB is associated with LVI, metastasis, MSI, *KRAS* mutation, 5-y survival. High TB is associated with 2.5 times increased risk for cancer-related death compared to no TB. More than 10 budding cells/×200 field is a good cut-off for high TB
Rogers et al. (94)^	I-IV	n.a.	A total of 7,821 patients from 34 papers (heterogeneous TB definition): TB in CRC is strongly predictive of lymph node metastases, recurrence, and cancer-related death at 5 years
Jang et al. (134)	I–IV	PTB	High-grade TB is significantly associated with conventional histological G, T, N, and M stages, LVI, infiltrative growth pattern, and *KRAS* mutations; patients with low-grade TB had high 4-years DFS and DSS rates, compared to those with high-grade TB
Landau et al. (141)	I–IV	PTB	Adenocarcinomas of the caecum display the highest frequency of *KRAS* mutations and high TB in the colon (compared to right [non-cecal] and left [distal] adenocarcinomas). High TB and cecal tumor site are predictive of poor survival, particularly in stage III/IV of disease
Oh et al. (146)	I-III	PTB	High TB is associated with adverse histologic features such as elevated levels of preoperative carcinoembryonic antigen, advanced stage, poor histology, and the presence of LVI/perineural invasion. High budding is an independent poor prognostic factor in DFS and OS, whereas tumor-budding positivity itself was not an independent prognostic factor (multivariate analysis)
Lugli et al. (147)	I–IV	ITB and PTB	ITB correlates with PTB and is independently associated with a shorter survival time. In MMR-proficient tumors: high-grade ITB is associated with right-sided location, advanced T and N stage, LVI, infiltrating tumor margin, and shorter survival time; MMR–deficient cancers: high ITB is linked to higher tumor G, vascular invasion, infiltrating tumor margin, and more unfavorable survival time
Trinh et al. (90)	I–IV	ITB and PTB	Adverse prognostic factor independent of age, stage, and sex. Independent in metastatic setting and in mixed stage cohort

When the definition of tumor budding differs from up to five cells, the paper is highlighted (*).

[#]Usually but not always at the invasive front.

CMS, consensus molecular subtype; CRC, colorectal cancer; ITB, intratumoral budding; LN, lymph node; LVI, lymphovascular invasion; MMR, mismatch repair protein; MSI, microsatellite instability; n.a., non-applicable, PTB, peritumoral budding; TB, tumor budding.

comparable to those of patients with metastatic disease. These findings raise the opportunity of offering adjuvant chemotherapy to these patients, since they are expected to have a more aggressive disease.

Third, pre-operative biopsies could benefit of intratumoral TB assessment. Indeed, in CRC surgical samples intratumoral and peritumoral TB are strongly related and associated with a shorter survival (147). Moreover, high-grade intratumoral TB correlates with higher tumor grade, more advanced primary tumor, lymphatic and vascular invasion, and nodal metastasis (147). Intratumoral TB could be used as predictive parameter in the selection of candidates for neo-adjuvant therapy (88, 90).

CONCLUSION REMARKS

The deepening of the knowledge on the molecular mechanisms linking common gene mutations, such as those affecting *RAS*, to specific gene-expression profiles, tumor cell characteristics, and biological behavior will disclose novel opportunities for the prevention, detection, and tailored treatment of CRC.

AUTHOR CONTRIBUTIONS

VM and LN reviewed the literature, wrote the first draft, and edited the manuscript. RC planned the article, coordinated and supervised the work, reviewed the literature, wrote the first draft, and edited the manuscript.

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

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