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Review

Review

RAS signaling and anti-RAS therapy: lessons learned from genetically engineered mouse models, human cancer cells, and patient-related studies

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Abstract

Activating mutations of oncogenic *RAS* genes are frequently detected in human cancers. The studies in genetically engineered mouse models (GEMMs) reveal that *Kras*-activating mutations predispose mice to early onset tumors in the lung, pancreas, and gastrointestinal tract. Nevertheless, most of these tumors do not have metastatic phenotypes. Metastasis occurs when tumors acquire additional genetic changes in other cancer driver genes. Studies on clinical specimens also demonstrated that *KRAS* mutations are present in premalignant tissues and that most of *KRAS* mutant human cancers have co-mutations in other cancer driver genes, including *TP53*, *STK11*, *CDKN2A*, and *KMT2C* in lung cancer; *APC*, *TP53*, and *PIK3CA* in colon cancer; and *TP53*, *CDKN2A*, *SMAD4*, and *MED12* in pancreatic cancer. Extensive efforts have been devoted to develop therapeutic agents that target enzymes involved in RAS posttranslational modifications, that inhibit downstream effectors of RAS signaling pathways, and that kill *RAS* mutant cancer cells through synthetic lethality. Recent clinical studies have revealed that sorafenib, a pan-RAF and VEGFR inhibitor, has impressive benefits for *KRAS* mutant lung cancer patients. Combination therapy of MEK inhibitors with either docetaxel, AKT inhibitors, or PI3K inhibitors also led to improved clinical responses in some *KRAS* mutant cancer patients. This review discusses knowledge gained from GEMMs, human cancer cells, and patient-related studies on RAS-mediated tumorigenesis and anti-RAS therapy. Emerging evidence demonstrates that *RAS* mutant cancers are heterogeneous because of the presence of different mutant alleles and/or co-mutations in other cancer driver genes. Effective subclassifications of *RAS* mutant cancers may be necessary to improve patients' outcomes through personalized precision medicine.

Key words: *RAS* genes, neoplasms, adenocarcinoma, animal models, clinical trial, antineoplastic agents

Introduction

RAS proteins are small G proteins that cycle between active GTP-bound and inactive GDP-bound forms and function as molecular switches for signal transductions initiated in the cell membrane [1,2]. Synthesized in cytosol, RAS proteins are transferred to the inner leaflet of the plasma membrane, where they interact with diverse membrane receptors and execute signal transduction in a variety of signaling pathways that govern cell growth, proliferation, differentiation, and death. Activation of upstream growth factor receptors, such as epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor, and platelet-derived growth factor receptor (PDGFR), results in the assembly of adaptor proteins Grb2 and the Son of Sevenless (SOS) complex. SOS is one of the guanine nucleotide exchange factors (GEFs) that activate RAS by promoting binding of

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RAS with GTP via catalysis of the release of GDP from RAS [3,4]. Intrinsic GTPase activity enhanced by GTPase-activating proteins (GAPs) [5] converts GTP to GDP, leading to inactive GDP-bound RAS (**Fig. 1**). RAS mutations that diminish GTPase activity or decrease GDP-binding capacity render RAS in constitutively active GTP-bound status. In the absence of a RAS mutation, increased RAS activity in human cancer cells frequently results from *RAS* gene amplifications [6,7] and overexpression [8], an increase in activity of upstream signals from tyrosine kinase growth factor receptors such as HER2 and EGFR [4,9], or/and altered expression of microRNAs such as let-7 [10,11].

RAS activation leads to stimulation of a wide range of downstream signaling pathways, most notably the RAF/mitogen-activated protein kinase (MAPK) kinase (MEK)/ERK [12,13], phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), RalGEF/RAL [14,15], and Tiam1/RAC [16,17] (**Fig. 1**) (see details in other review articles [18,19]). GTP-RAS binds directly to and activates RAF [12,13,20], the catalytic subunit of PI3K p110 [21,22], Ral guanine nucleotide exchange factors (RalGEF) [23,24], and RAC GEFs such as Tiam1 and Vav [16,25]. The signaling cascades initiated by these RAS-interacting proteins form networks through crosstalk and feedback interactions, which have been shown to play critical roles in the initiation and progression of malignancies [14,26–28]. Because activating mutations in *RAS* genes are among the most frequently observed oncogenic mutations in human cancers, RAS signaling and anti-RAS therapeutic agents have been intensively investigated. However, RAS proteins are regarded as non-druggable with small molecule inhibitors because of their high affinity for GTP and their simple protein structures. Thus, extensive efforts have been made to develop therapeutic agents that modulate posttranscriptional modification and/or plasma membrane localization of RAS proteins [29,30], that intervene in downstream signal transductions, and that induce synthetic lethality in *RAS* mutant cancer cells [31]. Recently, small molecules have been reported to bind irreversibly to the mutant KRAS (G12C) protein [32], or to interfere with RAS/SOS [33,34] or RAS-effector protein interactions [35]. Nevertheless, effective anti-RAS treatment is not yet available clinically. This review discusses knowledge gained from genetically engineered mouse models (GEMMs), human cancer cell lines, clinical studies about RAS-mediated signaling in tumorigenesis, and the development of anti-RAS therapy. It is likely that *RAS* mutant cancers are heterogeneous and different therapeutic strategies may be required for different subclasses of *RAS* mutant cancers.

GEMMs with *RAS* Mutations

Mammalian cells have three *RAS* genes (*HRAS*, *KRAS*, and *NRAS*) that encode four highly homologous RAS proteins, because the *KRAS* gene encodes two splicing isoforms: a major KRAS-4B and a minor KRAS-4A. These proteins have highly identical sequences in the first 164 amino residues containing the G domain for GTP binding and hydrolysis. The remaining 24/25 C-terminal residues are highly variable among isoforms and critical for membrane localization (**Fig. 1**). KRAS-4B, HRAS, and NRAS are ubiquitously expressed, whereas KRAS-4A is expressed mainly in renal, hepatic, and gastrointestinal tissues [36,37]. Evidence has shown that these highly conserved RAS isoforms carry out similar but indispensable functions that govern cell growth, differentiation, proliferation, apoptosis, tumorigenesis, and tumor progression [38]. Gene knockout studies reveal that *Hras* or *Nras* knockout mice, and even the *Hras* and *Nras* double knockout mice, are viable and show no obvious abnormalities [39,40]. In contrast, knockout of the *Kras* gene is embryonically lethal [41]. Although some of the differences may be derived from differences in their expression patterns [42,43], it has been reported that different RAS isoforms have different biological functions and different effects on tumor progression [42–46].

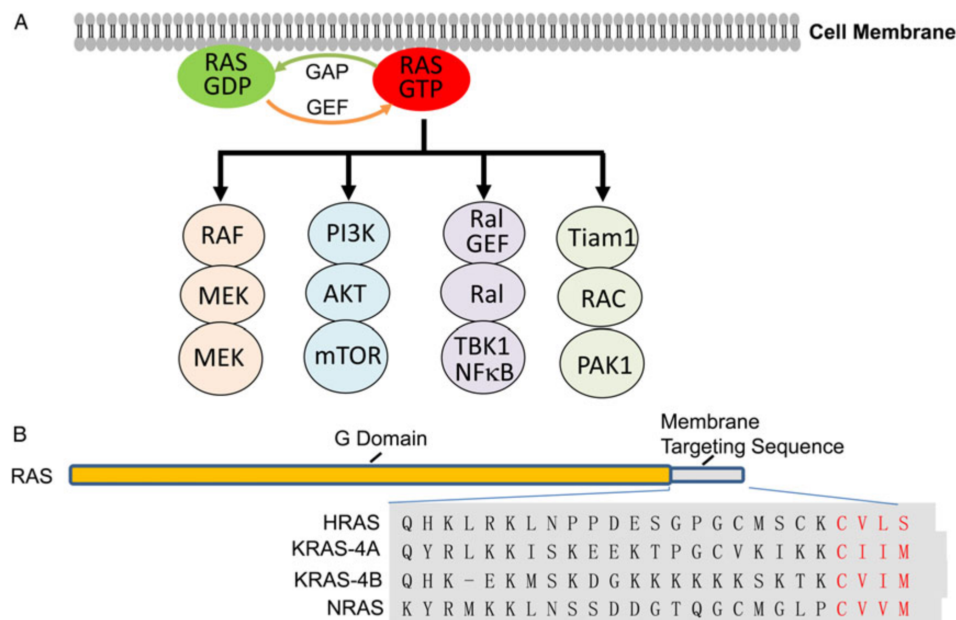

Figure 1. Diagrams of RAS proteins and RAS signaling pathways (A) Major RAS signaling pathways. RAS GEF activated by upstream growth factor receptors promotes binding of RAS with GTP via catalysis of the release of GDP from RAS, leading to the activation of downstream pathways (see details in other review articles [18,19]). Intrinsic GTPase activity enhanced by GAPs converts GTP to GDP, leading to inactive GDP-bound RAS. RAS mutations that cause the loss of GTPase activity render RAS in a persistent GTP-bound status. (B) Structures of RAS proteins. RAS proteins consist of G domain (amino acids 1–164) that has 93%–99% conserved sequences among RAS proteins and functions as GTPase, and membrane targeting sequences (amino acids 165–188/189) that is highly variable. The C-terminal CAAX motif required for farnesylation is marked red.

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Expression of mutant *Kras*^{12D} alone in murine embryonic fibroblasts (MEFs) induced enhanced proliferation and partial transformation accompanied with an elevation of CDK2 and CDK4. However, elevated ERK and AKT phosphorylation is not observed in MEFs expressing endogenous *Kras*^{12D} [47]. Moreover, expression of endogenous oncogenic *Kras*^{12D} during murine embryos results in widespread morphological aberrations and early embryonic lethality [47]. Expression of endogenous *Kras*^{12V} also frequently results in embryonic lethality, although some mice may reach adulthood and develop lung adenomas and adenocarcinomas [48]. Conditional expression of these mutant *Kras* alleles in the lung, pancreas, and gastrointestinal tract induces preneoplastic epithelial hyperplasias, adenomas, pancreatic intraepithelial neoplasia (PanIN), and adenocarcinomas [47–51]. The majority of these *Kras*-driven tumors do not have an invasive or metastatic phenotype, although progression to invasive and metastatic cancers is detected at low frequencies [51,52]. However, the progression to highly invasive and metastatic cancers is dramatically enhanced by the presence of mutations in other cancer driver genes, including defects in *Tp53* [53–55], *Stk11* [56–58], *Ink4a/Arf* [53,59], *Smad4* [60], *Pten* [61], *Tgfbr2* [62], and *Runx3* [63]; and activation of Wnt/beta-catenin signaling [64]. For example, *Kras*-activating mutations in lung epithelial cells predispose mice to early onset of lung adenocarcinoma [49,50,65]. Nevertheless, such tumors do not have an invasive or metastatic phenotype. Metastasis occurs when additional genetic changes, such as *Tp53* mutations or *Stk11* deletion, are introduced [54,57].

Efforts were made to delineate the downstream effectors and pathways that are required for RAS-mediated tumorigenesis in GEMMs. Several genes or pathways have been identified as participants in *Kras*-driven lung and pancreatic cancers, because their ablation or deletion prevents or reduces mutant *Kras*-induced tumors. Among them are PI3K/PDK1 [26,66], PI3K/RAC1 [67–70], RAF/MEK/ERK [71,72], TBK1/IKK/NFκB [73–75], IL-6/STAT3 [76,77], YAP [78], Foxm1/NFκB [79], anabolic glucose metabolism [80], GM-CSF-mediated recruitment of myeloid cells [81], ERK/RHOA/focal adhesion kinase (FAK) network [82], fibroblast activating protein (FAP) [83], and Myc [84].

For example, PI3K signaling was found to be necessary in *Kras*-induced malignant transformations in pancreatic cancer [85] and lung cancer [66,86]. Pancreas-specific inactivation of *Pik3ca*, but not *Pik3cb*, prevented the occurrence of all types of malignant lesions induced by expression of mutant *Kras*, including PanIN and acinar-to-ductal metaplasia [69,85]. *Pik3ca* ablation and chronic inhibition led to up-regulation of AKT signaling, possibly resulting from compensatory activity from other PI3K isoforms. In contrast, *Pik3ca* ablation significantly diminished both *Rac1* activity and expression in *Kras* mutant pancreatic cells, accompanied by significant inhibition of Kras-activated Rac1 guanine exchange factors Tiam1 and Vav1 [69]. Pancreas-specific ablation of *Rac1* has the same phenotype as *Pik3ca* ablation in *Kras* mutant mice [68,69], indicating the PI3K/RAC axis plays an important role in *Kras*-driven pancreatic tumor development. Similarly, Stat3 phosphorylation is found to occur at multiple stages of *Kras*^{12D}-driven pancreatic tumorigenesis but not in normal pancreatic tissue [76,77]. Disruption of the *IL-6* gene and conditional inactivation of *Stat3* in the pancreas reduced PanIN and pancreatic ductal adenocarcinoma (PDAC) formation in mutant *Kras*^{12D} mice [76,77], indicating that Stat3 activity is required for the development of the early premalignant pancreatic lesions, acinar-to-ductal metaplasia, and PanIN, and for the progression from PanIN to invasive PDAC [77]. A recent study showed that TBK1/IKK regulates autocrine cytokines CCL5 and IL-6, which contributes to *Kras*-driven tumorigenesis [74].

Treatment Response in GEMMs with *RAS*-driven Tumors

In a doxycycline-inducible *Hras*^{12V}-driven mouse melanoma model with null Ink4a, withdrawal of doxycycline resulted in *Hras*^{12V} down-regulation, marked apoptosis in the tumor cells and host-derived endothelial cells, and histological regression of primary and explanted tumors [87], demonstrating that the mutant *Hras*^{12V} is required in both the initiation and maintenance of solid tumors. The same results were observed in lung tumors [88] and pancreatic tumors [89,90] induced by doxycycline-regulated *Kras*^{12D}. Withdrawal of doxycycline in transgenic mice expressing *Kras4b*^{12D} in type II pneumocytes, with or without deficiencies in either the *Tp53* gene or the Ink4a/Arf locus, led to rapid regression of *Kras4b*^{12D}-mediated tumors [88]. Although the *p53* gene or Ink4a/Arf deficiencies dramatically accelerate tumor initiation and progression, removal of doxycycline caused a rapid regression of tumor burdens, implying that continued production of mutant *Kras* is necessary to maintain the viability of tumor cells, regardless of the presence of other cancer drivers.

In *Kras*-driven mouse pancreatic cancer models, treatment with MEK inhibitors (AZD6244, GDC-0973) or PI3K inhibitors (BEZ235, GDC-0941, BKM120) (**Fig. 2**) alone induced only partial tumor growth suppression, which did not significantly prolong overall survival (OS). Treatment with a MEK1 inhibitor resulted in cytostatic effects accompanied by sustained activation of the PI3K/AKT/mTOR pathway and receptor tyrosine kinases EGFR, HER2, PDGFR, and AXL [90]. Similar results were observed in *Kras*^{12D}-driven lung cancer in mice [91]. Treatment with a dual pan-PI3K and mTOR inhibitor BEZ235 is able to substantially suppress the growth of *Pik3ca*^{1047R}-induced lung tumors but not *Kras*^{12D}-driven lung tumors [91], suggesting that PI3K may be required for *Kras*-induced tumorigenesis but less crucial for tumor maintenance. Nevertheless, simultaneously targeting both MEK and PI3K pathways led to marked synergy in shrinking these *Kras* mutant cancers, resulting in a significant survival advantage when compared with controls [90,92,93]. Partial inhibition of *Kras*^{12D}-driven lung tumorigenesis was also observed in treatment with the MEK inhibitor selumetinib (AZD6244) and the TBK1/IKK/JAK inhibitor CYT387 [74]. The synergistic combination's effects on *Kras*^{12D}-driven lung tumors were also reported for combination therapy of farnesyl and geranylgeranyl diphosphate synthases inhibitor lipophilic bisphosphonates plus mTOR inhibitor rapamycin [94]; or combination of MEK inhibitor selumetinib plus the BCL2/BCL-XL inhibitor ABT-263 (navitoclax) [95].

A study comparing treatment responses of *Kras*^{12D}-driven lung cancer with concomitant loss of either *Tp53* or *Stk11* revealed that loss of either gene markedly impaired the response of *Kras* mutant cancers to docetaxel monotherapy [96]. Nevertheless, *Kras/Tp53* mutant tumors, which had increased MEK/ERK signaling, were sensitive to the combination therapy of docetaxel plus selumetinib. In contrast, *Kras/Stk11* mutant tumors, which had activation of AKT and SRC, were resistant to this combination therapy [96]. Similarly, *Kras* and *Kras/Tp53* mutant lung tumors were found to be susceptible to Myc inhibition with dominant-negative Myc [84] or with the bromodomain and extra-terminal bromodomain inhibitor JQ1 [97], whereas *Kras/Stk11* mutant mouse cancer cells and human lung cancer cells were resistant to the JQ1 treatment [97]. Knockdown of *STK11* in human *KRAS* mutant lung cancer cells sensitive to JQ1 caused resistance to this inhibitor, indicating the causal relationship between *STK11* deficiency and JQ1 resistance. In contrast, *Kras/Stk11* mutant mouse tumors responded to the treatment with the drug phenformin which affects metabolisms [98], and were highly sensitive to the inhibition of deoxythymidylate kinase [99], suggesting that the presence of

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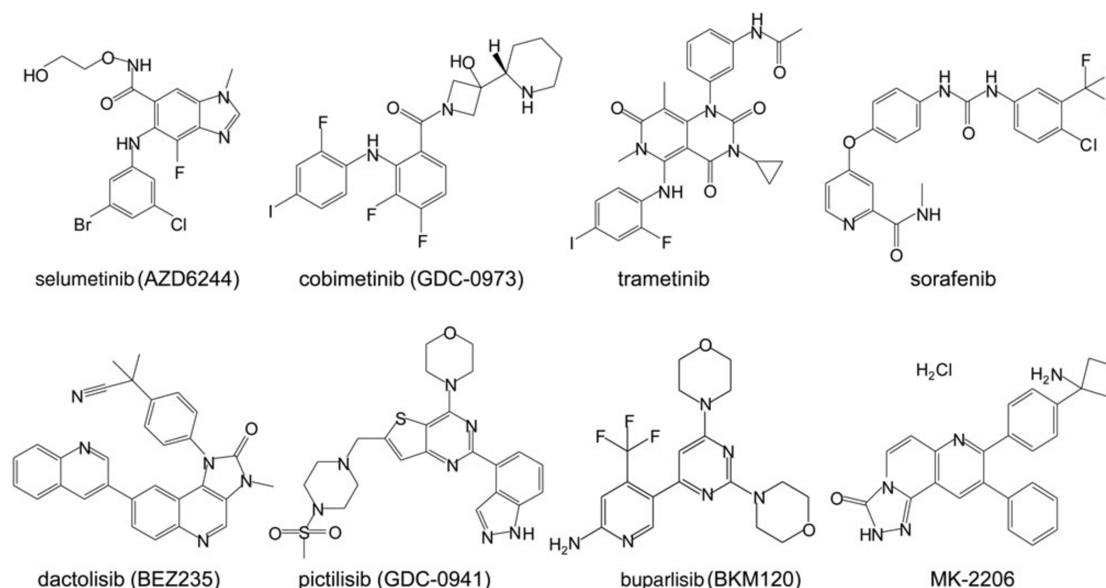

Figure 2. Chemical structures of small molecule inhibitors targeting RAS downstream effectors MEK inhibitors: selumetinib, cobimetinib, and trametinib; RAF inhibitor: sorafenib; PI3K inhibitors: dactolisib, pictilisib, and buparlisib; and AKT inhibitor: MK-2206.

co-mutations of another cancer driver gene may have a dramatic impact on responses to anticancer drugs.

Although both the ablation of oncogenic *Kras* expression and the combination of MEK and PI3K/mTOR inhibitors can induce complete regression of *Kras*-driven tumors, resistance to *Kras* ablation or to the combination therapy has been observed in relapsed tumors [100,101]. Some of these relapsed tumors had increased activity in mitochondria, autophagy, and lysosomes functions, had reduced glycolytic activity, and were sensitive to oxidative phosphorylation inhibitors such as oligomycin [100]. *Yap1* amplification is another mechanism of resistance identified in the relapsed tumors after $Kras^{12D}$ ablation, indicating that Yap1 activation may lead to a bypass of *Kras* dependency [101]. In a systemic screening with 15,294 open reading frames, YAP1's overexpression was identified in surviving cells after inducible shRNA-mediated *KRAS* knockdown in the human KRAS-dependent colon cancer cell line HCT116, and in acquired resistance to *Kras* suppression in a *Kras*-driven murine lung cancer model [102]. In addition, constitutive AKT activation also caused resistance to *Kras* ablation [90], indicating multiple mechanisms underlying resistance to anti-RAS therapy.

Treatment Response in *RAS* Mutant Human Cancer Cells

Studies in human cancer cell lines demonstrated that *KRAS* gene expression is required for survival of some *KRAS* mutant cancer cells. Disrupting *KRAS* by homologous recombination in *KRAS* mutant colon cancer cell lines DLD-1 and HCT116 resulted in inhibition of MYC expression and suppression of tumor cell growth both *in vitro* and *in vivo* in nude mice [103], implicating the expression of mutant *KRAS* is crucial for growth of these tumor cells. Knockdown of *KRAS* with shRNAs in a panel of *KRAS* mutant lung and pancreatic cancer cell lines has revealed that *KRAS* mutant cancer cell lines can be classified into *KRAS*-dependent and -independent groups, based on the requirement of *KRAS* expression for cell survivals [104]. Many of *KRAS*-dependent cells exhibit a classic epithelial morphology and gene expression signature, expressing prominent E-cadherin, whereas *KRAS*-independent cell lines have epithelial mesenchymal transition phenotype, expressing little or no E-cadherin, but expressing the mesenchymal marker vimentin. SYK, RON kinases, and integrin b6 were high in *KRAS*-dependent cell lines and were required for epithelial differentiation and cell survival in *KRAS*-dependent cells [104].

Synthetic lethality screening with siRNA libraries in cell lines with or without mutant *RAS* genes has revealed a number of genes whose knockdown may selectively induce lethality in cells with mutant *RAS* genes. Reported synthetic lethal partners for oncogenic *RAS* include *PLK1*, a serine/threonine protein kinase that regulates cell mitosis [105]; the transcription factor *WT1* [106]; *TBK1*, an IκB kinase that regulates the stability of IκB [107]; *SYK* [104]; and *CDK4* [108]. The synthetic lethality interactions of these targets with oncogenic *RAS* suggest that the inhibitors targeting to these molecules might selectively kill *RAS* mutant cancer cells. Indeed, small molecule inhibitors that disrupt mitosis, including paclitaxel and the PLK1 inhibitor BI-2536 [109], were found to be synthetic lethal in *RAS* mutant cells [105]. Synthetic lethality screening is also employed to identify combination therapy for investigational drugs targeting RAS downstream pathways. Using a pooled 5000 shRNA library targeting 1200 'druggable' genes, Bcl-XL was identified as a synthetic lethal partner for selumetinib in *KRAS* mutant colon cancer cell lines HCT116 and SW620 [95]. A combination of selumetinib and ABT-263 induced substantial apoptosis in ∼50% of *KRAS* mutant cancer cell lines derived from colon, lung, and pancreatic cancers, particularly in those with epithelial signature [95]. The synergistic effects of the combination are also demonstrated *in vivo* in xenograft tumors and in $Kras^{12D}$-driven mice lung tumors [95]. Of note, a study using shRNA library screening for genes that may have synthetic lethal interactions with the oncogenic *KRAS* gene in the colon cancer cell line DLD-1 has led to the identification of ∼370–1600 mutant *KRAS* synthetic lethal genes, depending on the stringency of statistical analyses [105], demonstrating the diversity of biological processes or pathways regulated by KRAS and the possible challenges that may be encountered by employing the synthetic lethality approach to identify therapeutic targets.

The synthetic lethality approach has also been investigated in the identification of anticancer agents for *RAS* mutant cancer cells by

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screening chemical libraries [110–112]. Several of such anticancer agents have been reported, including triphenyl tetrazolium and a sulfinyl cytidine derivative that showed ~6-fold selectivity for cell lines containing mutant *KRAS* [111]; erastin which exhibited lethal selectivity in human tumor cells harboring mutations in the *HRAS*, *KRAS*, or *BRAF* oncogene by acting on mitochondrial voltage-dependent anion channels and inducing oxidative cell death [112,113]; lanperisone [114]; and oncrasin-1 [115]. Erastin [113], lanperisone [114], and oncrasin compounds [116,117] all induced cell killing effects in *RAS* mutant tumor cells by inducing oxidative stress, although through different underlying mechanisms.

Through chemical library screening on cells with or without a mutant *KRAS* gene [115] and lead compound optimization [118–120], we recently developed a novel compound designated oncrasin-72 (NSC743380) that is highly active [median growth inhibitory concentration (IC_{50}) between 10 nM and 1 μM] *in vitro* in 30 of 102 cancer cell lines tested [118,121], including many *KRAS* mutant cancer cell lines [115,119,121]. Mechanistic characterization revealed that NSC743380 and its analogs induced apoptosis in sensitive cancer cells [115,118,119], inhibited phosphorylation of the C-terminal domain of RNA Pol II [120,122], induced sustained JNK activation by inhibiting its dephosphorylation [119], induced reactive oxygen species (ROS) accumulation [117], inhibited STAT3 phosphorylation, and suppressed cyclin D1 expression [118], suggesting that NSC743380 modulates multiple cancer-related targets. Blocking NSC743380-induced ROS generation with antioxidants dramatically abolished its apoptosis-inducing ability but had minimal effect on its inhibition of STAT3, suggesting that STAT3 inhibition is not caused by ROS production. In contrast, knockdown of STAT3 by siRNA induced ROS generation and suppressed tumor cell growth [121], suggesting that STAT3 inhibition might be upstream of ROS induction. Interestingly, the activation of RAS signaling pathways has been reported to up-regulate the overall cellular antioxidant capacity [123]. The interactions among RAS, STAT3, and redox pathways have been discussed in another review [124]. *In vivo* studies have shown that the intravenous or intraperitoneal administration of NSC743380 caused complete tumor regression or significant growth suppression in several xenograft tumor models [118,121], indicating that NSC743380 has promising *in vivo* activity. More recently, we improved NSC743380's stability and safety through the synthesis and evaluation of its prodrugs. Oncrasin-266 spontaneously releases NSC743380 in physiological solutions *in vitro* and *in vivo*, has improved stability and pharmacokinetics, and is better tolerated in mice at a higher dose level (150–300 mg/kg, i.p.) than NSC743380 [125], suggesting that the prodrug is a favorable candidate for further development.

Nevertheless, the correlations between NSC743380's anticancer activity and *KRAS* mutations in the NCI-60 cell lines and in the 50 lung cancer cell lines tested were not significant [118,121], because some *KRAS* mutant cancer cell lines were resistant while some *KRAS* wild-type cancer cell lines were sensitive to NSC743380. Our recent study revealed that the expression of a sulfotransferase (SULT), SULT1A1, in cancer cells is required for NSC743380's anticancer activity and that the expression of SULT1A1 is capable of predicting the responses to NSC743380 [126]. SULT1A1 is a biotransformation enzyme that bioactivates several pro-carcinogens [127–133] and some anticancer drugs, such as tamoxifen [134]. Identification of SULT1A1 as a predictive biomarker for NSC743380 sheds light on mechanisms of selectivity and the possible toxicity of this compound [135]. The process of identifying this predictive biomarker underscores the importance of activity characterization in a large set of molecularly annotated cancer cell lines and rigorous validation of causal relationships between the sensitivity and the biomarker.

Studies on Clinical Specimens and Clinical Trials

KRAS mutations are frequently found in human adenocarcinomas of the pancreas (70%–90%) [136–138], colon (50%) [139,140], and lung (35%) [141–143]. Based on human cancer gene mutation datasets retrieved from www.cBioPortal.org, *KRAS* mutations are also frequently detected in multiple myeloma (22%) and cancers of the ovary (15%), uterine (18%), and stomach (16%). *NRAS* mutations are frequently detected in melanoma (30%), multiple myeloma (18%), colorectal cancer (10%), and thyroid cancer (8%). In contrast, *HRAS* mutations occur in low frequencies (5% or less) in cancers of the bladder, head/neck, and uterine. The majority of genetic alterations in these oncogenic *RAS* genes are missense mutations in codons 12, 13, and 61. The most common mutant alleles for *KRAS* are 12D, 12C, 12V, 12R, and 13D [144,145]. Evidence has shown that different *KRAS* mutant alleles may have different clinical impacts on the prognosis of lung [146,147], colon [148–150], and pancreatic [151] cancers, although the alleles associated with poor clinical outcomes are not consistent in these studies. Molecular characterization of clinical specimens from patients who participated in prospective phase II biomarker-integrated approaches of targeted therapy for lung cancer elimination revealed that the expressions of cell cycle regulators PLK1, CCNB1, and CCNE1 were lower in $KRAS^{12C}$ and $KRAS^{12V}$ mutant tumors, but were higher in the remaining *KRAS* mutant tumors, when compared with *KRAS* wild-type cancer [146]. Analysis of NSCLC cell lines revealed that cancer cells with mutant $KRAS^{12D}$ had activated PI3K and MEK signaling, whereas those with mutant $KRAS^{12C}$ or $KRAS^{12V}$ had activated Ral signaling and decreased growth factor-dependent AKT activation. Moreover, ectopic expression of $KRAS^{12D}$ or $KRAS^{12C}$ in *TP53* knockdown human bronchial epithelial cells (HBECsiP53) had different effects on AKT activation and RalA/B expression [146], suggesting that different mutant alleles may have different preferences in activating downstream pathways.

Consistent with the roles of *Kras* mutations in tumor initiation in mouse models, analysis of clinical specimens revealed that *KRAS* mutations are frequently detected in human hyperplastic/metaplastic pancreatic acinar-ductal cells [152–154] and colorectal adenomas [155–157]. *KRAS* alterations may represent an early event in pancreatic ductal tumorigenesis, whereas *TP53* gene changes may represent an event required for the malignancy progression of ductal tumors from lower to higher grades [158]. Whole-genome analysis on pancreatic cancer revealed that, although *KRAS* is mutated in ~95% of PDACs, PDACs can be classified into four classes based on patterns of chromosomal rearrangements: stable, locally rearranged, scattered, and unstable [138]. These results demonstrated that mutations in the cooperative pathways or cancer drivers may further differentiate *KRAS* mutant cancers into subgroups.

The data retrieved from whole-genome sequencing analyses for lung, pancreatic, and colorectal cancers at The Cancer Genome Atlas (TCGA) databases (http://www.cbioportal.org) revealed that the majority of *KRAS* mutant cancers have co-mutations in other cancer driver genes (**Fig.** 3). The common co-mutations detected in lung cancer are *TP53*, *SKT11*, *CDKN2A*, and *KMT2C*; in colon cancer, *APC*, *TP53*, and *PIK3CA*; and in pancreatic cancer, *TP53*, *CDKN2A*, *SMAD4*, and *MED12*. A recent study using immunohistochemical analysis showed that LKB1 (encoded by *STK11*) is lost in 30% of *KRAS* mutant lung adenocarcinoma. *KRAS* mutant NSCLC patients with concurrent LKB1 loss had a high number of metastatic sites at the time of diagnosis,

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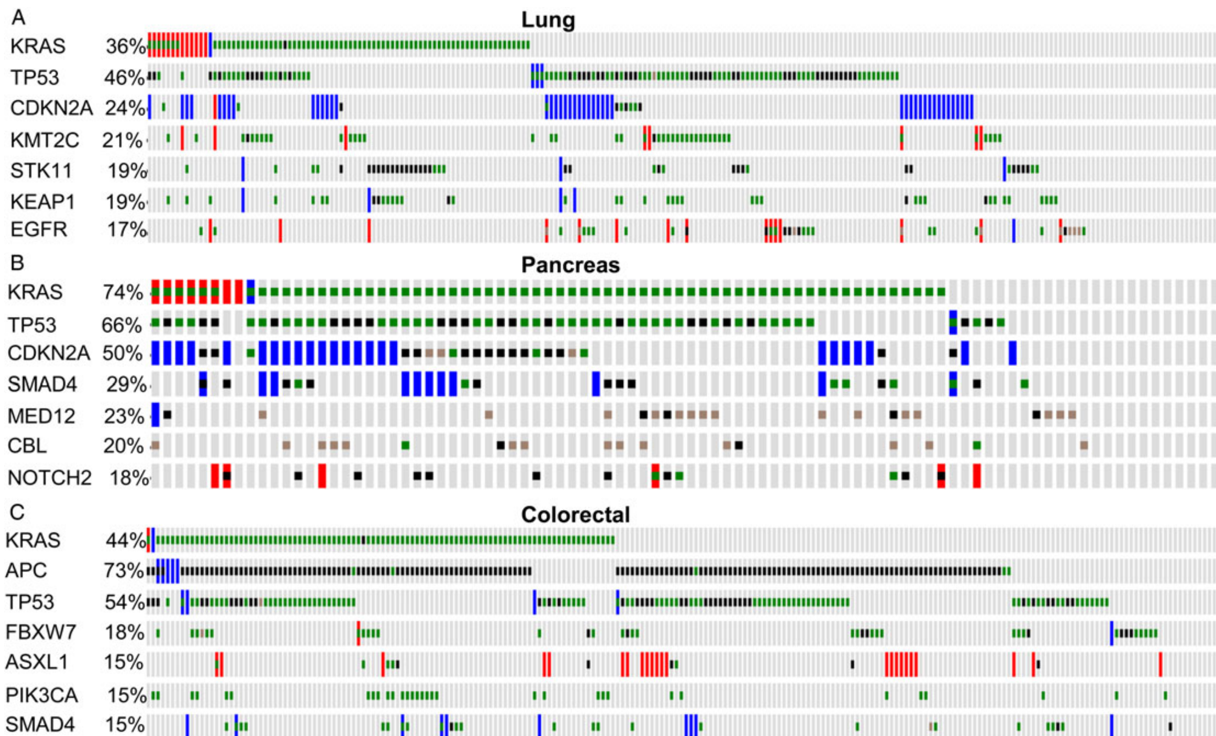

Figure 3. Molecular heterogeneity in KRAS mutant adenocarcinomas Status of KRAS mutations and co-mutations in other cancer driver genes in 230 lung adenocarcinomas (A); 90 pancreatic adenocarcinoma (B); and 220 colorectal adenocarcinoma (C) retrieved from The Cancer Genome Atlas (TCGA) databases at the website http://www.cbioportal.org. Each vertical line represents a tumor. The graph shows mutations in the top seven cancer driver genes in lung, pancreatic, and colorectal adenocarcinomas. Red, amplification; blue, homozygous deletion; green, missense mutation; black, truncating mutation; blown, inframe mutation. Note most of KRAS mutant cancers have co-mutations in other cancer driver genes.

and had a high incidence of extra-thoracic metastases [159]. Interestingly, concurrent mutations in *KRAS* and *STK11* in human cancer cells resulted in susceptibility to the depletion of the coatomer complex I subunits [160], which are required for lysosomal maturation and CDC42-mediated transformation. More recently, an integrative analysis of genomic, transcriptomic, and proteomic data from early stage and chemo-refractory lung adenocarcinoma demonstrated that *KRAS* mutant lung cancer can be classified into three subgroups by co-occurring genetic alterations in *STK11*, *TP53*, and *CDKN2A/B* [161]. These three groups have distinct clinical outcomes and treatment responses. *KRAS/STK11* cells showed increased vulnerability to HSP90 inhibitors. These results strongly indicate that concurrent mutations in *KRAS* and *STK11* genes may represent a subgroup of *KRAS* mutant tumors that differ from other *KRAS* mutant cancers in treatment responses.

Because posttranslational modifications of RAS proteins are required for them to be translocated to plasma membrane to execute their biologic functions, efforts have been made to target enzymes involved in these posttranslational modifications, including farnesylation at the cysteine residue of the C-terminal CAAX motif, removal of the AAX peptide, and methylation of farnesyl-cysteine at the C-terminal [162]. For HRAS and NRAS, palmitoylation on cysteine residues near the C-terminal is also required for RAS re-localization to the membrane. For KRAS-4B, a polybasic domain located at the C-terminal serves as the second signal for membrane localization [163,164] (**Fig. 1**). Because farnesylation of RAS is critical to its biologic function, farnesyltransferase inhibitors (FTIs) have been intensively investigated in clinical studies. Several phase II and phase III clinical trials showed that the FTIs tipifarnib and salirasib (**Fig. 4**), either alone or in combination therapy, did not have significant activity in lung, pancreatic, and colorectal cancers [165–168].

Targeting the RAS downstream pathways, particularly the RAF/MEK/ERK and PI3K/AKT/mTOR pathways [18,169], has also been investigated for treatment of *RAS* mutant cancers in clinics. A clinical trial with biomarker-integrated targeted therapy for lung cancer has revealed that sorafenib, a pan-RAF and VEGFR inhibitor [170], has impressive benefits for *KRAS* mutant patients [171]. However, selective inhibition of BRAF with a dominant-negative construct [172] in mice or with BRAF-selective inhibitors such as vemurafenib in patients [173,174] promoted the development and/or progression of *RAS* mutant cancers, possibly because of the activation of other RAF isoforms, such as RAF1. MEK inhibitors selumetinib and trametinib have been investigated for treatment of *KRAS* mutant tumors in a few clinical trials. A biomarker-derived multi-arm phase II trial revealed that selumetinib monotherapy failed to achieve its primary end point, with a response rate of 11% [175]. Nevertheless, combination therapy of MEK inhibitors with either docetaxel [176], AKT inhibitors [177], or PI3K inhibitors [178] led to improved clinical responses. In a randomized multicenter phase II study, docetaxel plus selumetinib treatment for patients with advanced *KRAS* mutant NSCLC resulted in an objective response rate of 37%, with a median OS of 94 months and median progression-free survival (PFS) of 53 months, whereas the patients who received docetaxel alone had an OS and PFS of 52 and 21 months, respectively [176]. Combination of selumetinib with AKT inhibitor MK-2206 resulted in an objective response of 3/13 (23%) of *KRAS* mutant NSCLC patients [177], while combination of

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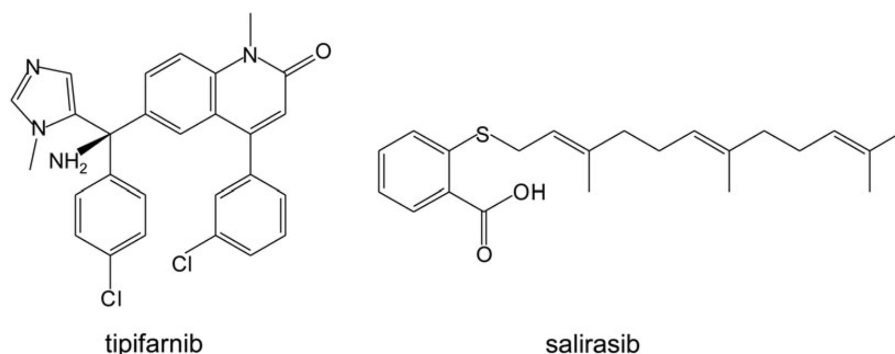

Figure 4. Chemical structures of therapeutic agents that modulate RAS subcellular localizations

trametinib with PI3K inhibitor buparlisib led to objective responses in *KRAS* mutant ovarian cancer and NSCLC [178]. However, a combination of trametinib with gemcitabine for treatment of metastatic pancreatic cancer [179], or selumetinib with irinotecan for treatment of *KRAS*-mutated colorectal cancer [180], did not improve PFS or overall response rate when compared with gemcitabine or irinotecan plus placebo. Together, these studies demonstrate that targeting multiple RAS signaling pathways may provide benefits to a subgroup of *RAS* mutant cancer patients, and that effective strategies to stratify patients for precision therapy will be required to improve efficacies.

Conclusions and Perspectives

Studies in GEMMs have demonstrated that *Ras* gene mutations are sufficient to initiate tumorigenesis, although the presence of additional genetic alterations in other cancer driver genes is often required for progression to invasive and metastatic cancers [51,52]. These studies have led to the identification of several genes whose abnormalities cooperatively promote the initiation and progression of *Ras* mutant tumors. Similarly, several genes whose ablations or inhibition prevent and/or reduce *Ras*-mediated tumorigenesis were reported, suggesting that they may serve as potential therapeutic targets for treating *RAS* mutant cancers. Studies in human cancers demonstrated that *RAS* gene mutations are frequently detected in premalignant tissue specimens and that most *KRAS* mutant cancers have co-mutations of other cancer driver genes. Moreover, emerging evidence has demonstrated that *KRAS* mutant cancers are heterogeneous in terms of signaling/metabolic aberrations and responses to treatments. The variations in co-mutations, mutant alleles of *RAS* genes, and origins of tumor cells can all cause such heterogeneity. Thus, effective classification of *RAS* mutant cancers will be required to improve anti-RAS therapy through personalized precision medicine. Because crosstalk and feedback activations are commonly observed in *RAS*-mediated signaling pathways, and because most solid tumors carry multiple concomitantly activated oncogenes or inactivated tumor suppressor genes [181], simultaneously targeting multiple cancer-associated pathways is likely required for effective anti-RAS therapy. Our experience with selective cell killing of NSC743380 in SULT1A1^{+} cells [126] indicates that even though some passenger mutations in tumor cells may not contribute to tumorigenesis, they may have an impact on treatment response because of altered drug metabolism. Therefore, they may be used as biomarkers to identify responders.

The initiation and progression of *KRAS* mutant tumors are also drastically affected by tumor microenvironments. Several tumor microenvironment factors have been identified as crucial in *Kras*-mediated tumor initiation and progressions in GEMMs. Among them are GM-CSF-mediated recruitment of myeloid cells [81], the IL-6/STAT3 signaling pathway [76,77], the ERK/RHOA/FAK network [82], and FAP [83]—a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers [182]. Genetic deletion and pharmacologic inhibition of FAP resulted in inhibition of *Kras*^{12D}-driven lung tumors in mice, possibly through indirect inhibition of tumor cell growth by modulating extracellular matrix/integrin-mediated singling [83]. These results demonstrate the feasibility of modulating the tumor microenvironment in the treatment of *RAS* mutant cancer, and highlight the necessity of incorporating factors of tumor microenvironment into the design of future anti-RAS therapies.

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