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Review

The genetics and biology of *KRAS* in lung cancer

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Abstract

Mutational activation of *KRAS* is a common oncogenic event in lung cancer and other epithelial cancer types. Efforts to develop therapies that counteract the oncogenic effects of mutant *KRAS* have been largely unsuccessful, and cancers driven by mutant *KRAS* remain among the most refractory to available treatments. Studies undertaken over the past decades have produced a wealth of information regarding the clinical relevance of *KRAS* mutations in lung cancer. Mutant *Kras*-driven mouse models of cancer, together with cellular and molecular studies, have provided a deeper appreciation for the complex functions of *KRAS* in tumorigenesis. However, a much more thorough understanding of these complexities is needed before clinically effective therapies targeting mutant *KRAS*-driven cancers can be achieved.

Key words *KRAS*, *RAS*, oncogene, lung cancer

KRAS (chromosome 12p12.1) is a member of the canonical *RAS* family of genes that also includes *HRAS* (chromosome 11p15.5) and *NRAS* (chromosome 1p13.1). The importance of *RAS* in cancer pathogenesis was first recognized more than three decades ago when it was discovered that mutated versions of *KRAS* and *HRAS* were responsible for the transforming activities of sarcoma-inducing retroviruses in rats. We now know that somatic activating mutations in the cellular homologs of all three *RAS* family members occur in a wide spectrum of human cancers. These mutations predominantly occur at codons 12, 13, and 61, and result in constitutive activation of RAS. Overall, *RAS* mutations have been found in approximately 30% of all human cancers, with *KRAS* as the most commonly mutated family member[1].

The three *RAS* genes are highly conserved across different species and encode monomeric GTPases that cycle between active (GTP-bound) and inactive (GDP-bound) states in response to extracellular cues. Unlike *HRAS* and *NRAS*, *KRAS* undergoes alternative splicing, resulting in two proteins (KRAS4A and KRAS4B) that differ only at their carboxyl termini (Figure 1). RAS proteins are 188/189 amino acids in length, and the sequence of the first 165 amino acids is almost identical. This region contains highly conserved domains that are responsible for GTP binding and hydrolysis and functional interactions with regulators and downstream effectors (Figure 1). The hypervariable carboxyl domain is the most distinguishing feature among the RAS family members and contains sequences important for determining post-translational modification, including the terminal CAAX domain that is responsible for membrane targeting. The post-translational modification of RAS proteins is a complex multi-stage process that has been extensively reviewed elsewhere[2]. Briefly, all four RAS proteins are farnesylated, and with the exception of HRAS, they can also be geranylgeranylated. This modification is followed by proteolytic cleavage within the CAAX motif and carboxymethylation of the exposed cysteine residue. Finally, HRAS, NRAS, and KRAS4A undergo palmitoylation at cysteine residues located adjacent to the carboxyl end. While KRAS4B is not palmitoylated, it contains a polybasic lysine-rich sequence that enables association with the plasma membrane through electrostatic interactions. In this review, we provide a summary of the extensive body of knowledge of *KRAS* and the *RAS* gene family, emphasizing particular aspects of genetics and biology that are relevant to lung cancer.

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RAS Signaling

RAS signaling begins with the stimulation of a vast array of upstream receptors including receptor tyrosine

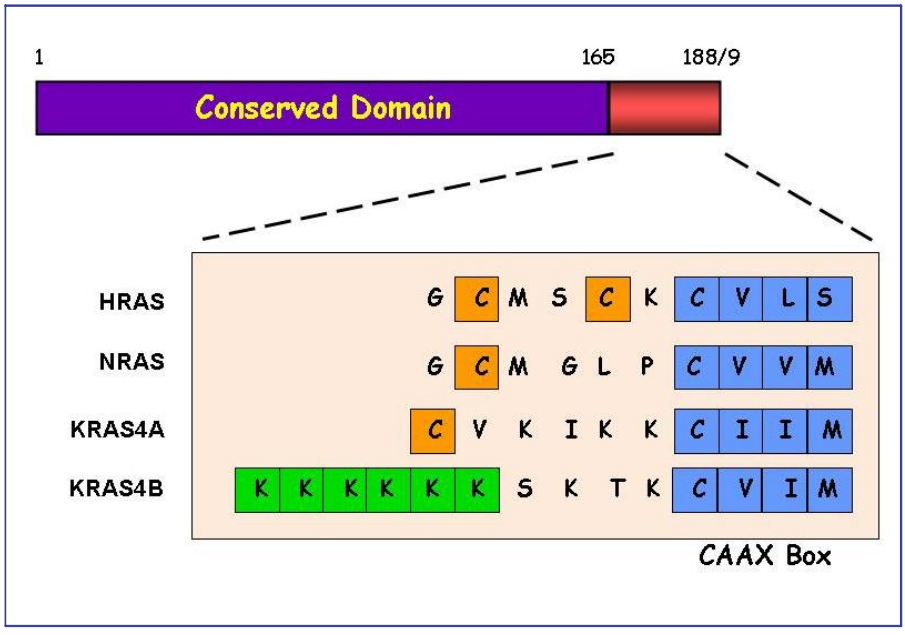

Figure 1. **Primary structures of RAS proteins.** The first 165 amino acids of RAS proteins are nearly identical and include motifs responsible for the binding and hydrolysis of GTP, binding of downstream effectors, and interactions with GAP and GEF. The hypervariable domain at the carboxy-terminus, including the CAAX motif, contains sequences important for the post-translational modification and membrane targeting of RAS proteins. The cysteine residue in the CAAX motif is a target for prenylation (i.e., farnesylation or geranylgeranylation). The cysteine residues (orange boxes) near the carboxy-termini of HRAS, NRAS, and KRAS4A are targets for palmitoylation. The poly-lysine track (green boxes) helps KRAS4B to associate with the membrane.

kinases (RTKs) of which the epidermal growth factor receptor (EGFR) is perhaps most relevant to lung cancer. Adaptor proteins (e.g., Grb2) interact with the intracellular domain of activated EGFR and in turn recruit guanine nucleotide exchange factors (GEFs) such as Son of Sevenless (SOS) to the cellular membrane where they can associate with RAS to promote the exchange of GDP for GTP (Figure 2). RAS signaling is terminated upon the hydrolysis of GTP to GDP by the intrinsic GTPase activity of RAS through the interaction with GTPase-activating proteins (GAPs). Cancer-causing mutations in RAS drastically impair the GTPase activity, resulting in RAS proteins that are locked in the active GTP-bound conformation, regardless of the upstream signal.

In their active, GTP-bound conformations, the four RAS proteins engage and activate a large number of downstream signaling pathways to varying degrees. Signaling through these downstream pathways regulates diverse cellular responses, including proliferation, survival, and differentiation (Figure 2). The canonical RAS/RAF/MEK/ERK pathway controls cellular proliferation by modulating the levels of many cell cycle regulators and is frequently hyperactivated in cancers [3]. RAS also promotes cell survival by activating PI3K/PDK1/AKT signaling, a pathway that is also commonly deregulated in many cancer types [4]. RALGDS and RALGDS-like proteins and tumor invasion and metastasis-inducing protein 1 (TIAM1) can also be activated by RAS to control vesicle trafficking and cytoskeletal organization, respectively [5,6]. Both *RalGDS* and *Tiam1* have been shown to be required for Ras-dependent tumor formation in a mouse skin cancer model[7,8]. Many of these downstream signaling pathways are involved in feedback regulation and crosstalk that together further contribute to the complexity of the RAS signaling network.

KRAS Mutations in Human Lung Cancer

Lung cancer is a common form of cancer in men and women, and it is responsible for the highest number of cancer-related deaths globally. Because of the high rate (>50%) of late diagnosis, the 5-year overall survival rate of patients with lung cancer has improved very little over the past 3 decades, hovering around the 13%–16% range[9]. Tobacco use is the most important risk factor for lung cancer, with ~80% of cases arising in smokers.

KRAS is the most commonly mutated member of the *RAS* family in lung cancer, although *HRAS* and *NRAS* mutations have also been reported in a few cases [10]. *KRAS* mutations predominantly occur in lung adenocarcinomas, the most common histological subtype of non–small cell lung cancer (NSCLC), with frequencies ranging from 16% to 40% of the samples analyzed [10-13]. *KRAS* mutations have also been observed at a low frequency in squamous cell carcinoma (another subtype of NSCLC), but never in small cell lung cancer (SCLC)[10,14]. Mutations predominantly occur at codon 12, occasionally at codon 13, and rarely at codon 61 of *KRAS*. Lung adenocarcinomas are usually associated with tobacco smoking[15], and *KRAS* mutations have been found to occur at a higher frequency in tumors in smokers compared to those in non-smokers[15]. In addition,

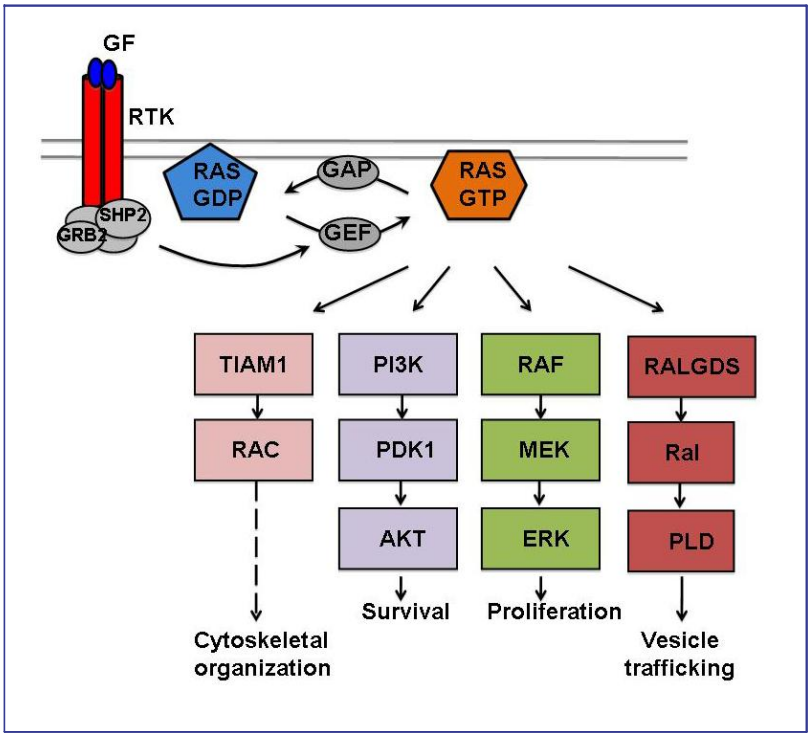

Figure 2. **The RAS signaling network.** Activation of RTKs by growth factors (GFs) creates intracellular docking sites for adaptor proteins (e.g., GRB2 and SHP2) that recruit GEF to the membrane to interact with RAS and promote the exchange of GDP for GTP. In the active GTP-bound conformation, RAS engages and activates an array of downstream effector pathways to regulate many cellular responses. The RAS signaling is terminated upon hydrolysis of the bound GTP by the intrinsic GTPase activity of RAS with the help of GAP. Oncogenic mutations in *RAS* lock the proteins in a constitutively active state, resulting in the deregulation of many cellular functions that together contribute to the cancer phenotype.

mutations involving nucleotide transversions (i.e., G/C or G/T), which are known to be associated with exposure to tobacco smoke, are more common among smokers than non-smokers. Indeed, the two most common *KRAS* mutations in NSCLC, G12C (~40%) and G12V (~22%), arise from G/T transversions[16,17].

Many studies have suggested that the presence of *KRAS* mutations in NSCLC is associated with shortened survival and time to relapse[18-20], although some studies have reported a lack of association[21]. The specific type of *KRAS* mutation may also provide information on disease aggressiveness or drug sensitivity. For example, the G12D mutation in NSCLC has been associated with better prognosis compared to the G12V or G12R mutations[22]. In contrast, the G12D and G13D mutations in human colorectal cancer (CRC) have been associated with a decreased response to chemotherapy compared to other mutations at these codons[23]. Further research is needed to conclusively establish the relationship between *KRAS* mutation specificity and prognosis in NSCLC. In contrast, there is compelling data demonstrating the usefulness of *KRAS* status as a marker for predicting therapeutic response. Adjuvant treatment with cisplatin and vinorelbine has been found to increase the survival of NSCLC patients with wild-type (WT) *KRAS* but not those with *KRAS* mutations in their lung tumors[24,25]. The presence of *KRAS* mutations is also associated with a lack of response to EGFR inhibitors[26,27] not only for NSCLC but also for human CRC where *KRAS* is also frequently mutated[28]. Given that KRAS is downstream of EGFR, it seems intuitive that EGFR inhibition would have no impact on the activity of mutant *KRAS*. However, it is surprising that concurrent treatment of *KRAS*-mutant NSCLCs with erlotinib and chemotherapy resulted in shortened overall survival and progression-free survival compared with chemotherapy alone[26]. These observations underscore the complexity of *KRAS* biology and further emphasize the advantage of having molecular information available (e.g., *KRAS* mutational status) when deciding the appropriate course of treatment.

Insights from Mouse Models of *Kras*-driven NSCLC

The mouse is an invaluable *in vivo* model system that has been widely used to study the importance of genes and pathways in different physiologic and pathologic contexts. The extensive sequence homology together with the broad overlapping pattern of expression in mice and humans suggests a high degree of functional redundancy among the *RAS* family members. However, studies using mice have shown that knockout of the *Kras* locus results in embryonic lethality, whereas knockout of either *Hras* or *Nras* has no effect on embryonic development and welfare of adult mice[29]. Furthermore, genetic disruption involving exon 4A of

Kras results in viable and healthy mice that express only *Kras4B*[30]. These observations indicate that *Kras*, namely *Kras4B*, is developmentally essential with unique functions that cannot be compensated by other *Ras* family members. Surprisingly, mice in which *Hras* is inserted into the *Kras* locus are viable despite completely lacking Kras proteins[31]. These data suggest that the role of *Kras* in development is not related to unique Kras protein functions, but rather involves regulatory properties that are specific to the *Kras* locus.

Several mouse models of mutant *Kras*–driven lung carcinogenesis have been widely used to study the role of *Kras* in NSCLC *in vivo*. One model involves chemical carcinogen exposure, resulting in lung tumors that almost invariably harbor activating mutations at codon 12 or 61 of *Kras* [32-35]. A second involves a transgenic strategy in which the mutant *Kras* allele is incorporated into the genome at random locations and expression is induced by treating mice with doxycycline [36]. The third employs a knock-in strategy that involves targeting the endogenous *Kras* locus to generate a mutationally activated *Kras* allele that is maintained in a latent state by inclusion of a STOP cassette flanked by loxP elements [37,38]. Removal of the STOP cassette, either by spontaneous recombination in the $Kras^{LA2}$ model [37] or by intranasal administration of an adenovirus containing Cre recombinase in the $Kras^{LSL\text{-}G12D}$ model [38], results in mutant *Kras* expression and the development of lung tumors. These mouse models have demonstrated that mutational activation of *Kras* alone is sufficient for lung tumor development, suggesting that it is an early event during lung carcinogenesis. The role of *KRAS* during the early stages of lung carcinogenesis is further supported by studies in human NSCLC showing that *KRAS* mutations can be detected in precancerous lesions [39]. In addition, lung tumors induced by both carcinogen and genetic approaches stain positively for SP-C and negatively for CCA/CC10[33,37,38], suggesting that they derive from the same cell lineage as human lung adenocarcinomas. However, the majority of mouse lung tumors are adenomas [33,37,38], which are thought to be adenocarcinoma precursors. Progression from adenomas to adenocarcinomas in these mutant *Kras*- driven mouse models of NSCLC can be accelerated by loss of the *p53* tumor suppressor gene[40]. Likewise, *KRAS* and *P53* are mutated at similar frequencies in human lung adenocarcinomas and occur concurrently in many cases[11].

The Tumor Suppressor Function of *Kras* and Implications for Lung Cancer Susceptibility

While mutant *Kras* is potently oncogenic, studies in the mouse have elegantly demonstrated that WT *Kras* functions as a suppressor of this activity [41]. Mice with only one copy of *Kras* were found to be more susceptible to carcinogen-induced lung carcinogenesis than mice with two copies of *Kras*[41]. These findings suggest that the remaining WT allele of *Kras* in mice of the latter genotype inhibits lung tumor development. This was further confirmed by *in vitro* studies in which ectopic expression of WT *Kras* attenuated the growth of cancer cells containing mutant *Kras*[41]. Analyses of lung tumors from mutant *Kras*–driven NSCLC models have shown that chromosomal duplication involving the *Kras* locus is the most common genetic event in these tumors that harbor *Kras* mutations [42,43]. Studies of human lung adenocarcinomas have also shown frequent *KRAS* copy number gains and corresponding increases in RNA levels of *KRAS* [11]. In addition, loss of heterozygosity spanning the *KRAS* locus has been observed in human lung tumors that relate with *KRAS* mutation and preferentially target the WT *KRAS* allele[44]. The imbalance in favor of mutant *KRAS* in human and mouse lung tumors is consistent with the requirement of tumor cells to overcome the tumor suppressor effect of WT *KRAS*.

Inbred strains of mice exhibit differential susceptibility to lung carcinogenesis, and genetic crosses between different mouse strains have led to the identification of pulmonary adenoma susceptibility 1 (*Pas1*) as a major locus regulating predisposition to mutant *Kras*–driven lung cancer[45]. Of the genes located within *Pas1*, *Kras* emerged as the most likely candidate; however, a lack of coding sequence variants in *Kras* among the different strains of mice called into question the mechanisms through which *Kras* would regulate lung cancer susceptibility. However, mice that are susceptible to lung carcinogenesis were found to express higher levels of *Kras* compared with mice classified as resistant [32]. We proposed that lung cancer susceptibility is regulated by the balance between the levels of mutant and WT *Kras*, taking into consideration the respective oncogenic and suppressor functions of these alleles [32]. Consistent with this notion, *Kras* mutations occur preferentially on the more highly expressed *Kras* allele inherited from the more susceptible parent[46]. In humans, a polymorphism in the 3'-untranslated region of *KRAS*, which results in increased *KRAS* expression via interference of binding by let-7 microRNA, is associated with an increased risk of developing NSCLC[47]. A number of additional genetic variants in the human *KRAS* gene have also been associated with the risk of developing lung adenocarcinomas [48,49], and in some cases the susceptible allele is found to be expressed at relatively higher levels in normal lung tissues[48]. However, many of these associations were not reproducible in later studies [50,51]. One possible explanation for the lack of consensus among human case-control studies may be the variations in frequency (16%–40%) of *KRAS* mutations in different cohorts of patients with NSCLC. Therefore, it may be necessary to consider the somatic events that

occur in tumors (i.e., *KRAS* mutational status) in association studies to identify lung cancer susceptibility genes.

Isoform-specific Functions of *Kras* in Lung Carcinogenesis

As a result of alternative splicing, the human and mouse *Kras* loci encode two highly similar proteins, Kras4A and Kras4B, that are jointly affected by activating mutations commonly found in cancer (Figure 1). While *Kras4B* is ubiquitously expressed, albeit at varying levels across tissues, *Kras4A* expression is tissue-specific and not essential for embryonic development, suggesting that *Kras4A* has a minor role in *Kras* biology. However, we have shown that mice lacking *Kras4A* are highly resistant to carcinogen-induced lung tumor development[33]. Similar findings have been reported using a different mouse model that also lacks *Kras4A*[52]. These studies suggest that *Kras4A* is essential for lung carcinogenesis. The requirement for *Kras4A* in carcinogenesis is compatible with the observation that *Kras4A* is expressed in the lung and a number of other tissues from which arising tumors frequently harbor *Kras* mutations, namely the colon and the pancreas[53,54]. In the lung, *Kras4A* is highly expressed in a subset of epithelial cells, which could potentially be the originating cells of NSCLC[33]. Studies of the cellular origin of NSCLC in the mouse have identified a number of candidates[55,56], but their relationship to *Kras4A* remains to be determined. Nevertheless, the identification of *Kras4A* as an essential component of mutant *Kras*–driven lung tumors may have important implications for the design and development of KRAS-targeted therapeutics.

The Challenges and Future of Therapeutics for *KRAS*-mutant NSCLC

The development of KRAS-targeting cancer therapy has proven to be a challenging endeavor. Because cancer-causing mutations render KRAS oncogenic by impairing its GTPase activity, the KRAS oncoprotein has been generally deemed "undruggable" by conventional chemical approaches. In contrast, a number of drugs have been designed to inhibit the post-translational modification of the RAS proteins to prevent proper localization and function. However, farnesyl transferase inhibitors (FTIs) failed to inhibit KRAS due to alternative prenylation by geranylgeranyl transferase (GGTase)[57], and combined treatment with FTIs and the more recently developed GGTase inhibitors have displayed unacceptable levels of off-target effects and toxicity[58,59]. An alternative strategy involving the use of RNA interference (RNAi) to deplete KRAS in cells has been shown to be effective in some human *KRAS*-mutant NSCLC cell lines[60], demonstrating that some tumors harboring *KRAS* mutations remain highly dependent on oncogenic KRAS for survival. While siRNA knockdown of gene expression is a promising strategy to treat such *KRAS* "addicted" tumors in the clinic, the lack of effective methods to deliver siRNA to tumors has precluded development of such therapeutics. However, recent advances in the use of nanoparticles for systemic siRNA delivery in humans may potentially help overcome this limitation[61].

A great deal of effort has been placed on developing inhibitors of effector pathways downstream of RAS with the understanding that inhibition of these pathways could counteract the activity of oncogenic RAS. The RAF/MEK/ERK pathway was the first RAS effector pathway identified and the best characterized. Activating mutations in *BRAF* have been identified in different human cancers, including lung cancer, but generally never together with *RAS* mutations[62]. BRAF-selective inhibitors that effectively block proliferation of *BRAF*-mutant cell lines have been developed but are surprisingly ineffective against *RAS*-mutant cells[63-65]. In fact, these inhibitors paradoxically potentiate RAF/MEK/ERK signaling in *RAS*-mutant cells[63-65], and may cause severe adverse effects when given to patients with *RAS*-mutant cancers. Indeed, a subset of patients with melanoma treated with BRAF inhibitors developed cutaneous squamous cell carcinomas that contained *RAS* mutations[66]. The mechanistic explanation for the paradoxical activation of RAF/MEK/ERK in *RAS*-mutant cells treated with BRAF inhibitors involves CRAF activation[63-65], suggesting that CRAF-selective inhibitors could potentially be effective against cancers driven by mutant *RAS*. In support of this notion, studies in the mouse have shown that *CRaf*, rather than *BRaf*, is essential for the development of mutant *Kras*-driven NSCLC[67].

Recently, there has been growing interest in exploiting the concept of synthetic lethality to target *KRAS*-mutant cancer cells[68]. This approach involves the selective killing of *KRAS*-mutant cancer cells through inhibition of a second protein. Studies in the mouse identified a synthetic lethal interaction between mutant *Kras* and *Cdk4*, where *Cdk4* ablation caused lung cells expressing mutant *Kras* to undergo senescence and prevented tumor growth [69]. A number of RNAi-based synthetic lethal screens in cell lines have identified many potential synthetic lethal therapeutic targets that preferentially cause death of *KRAS*-mutant cells[70-72]. Some of these targets, including *CDK4*, *STK33*, *TBK1*, and *PLK1*, encode protein kinases and thus may be tractable to inhibition by selective small molecular inhibitors, such as those that have demonstrated success against mutant *EGFR* and *BRAF*.

Conclusions

Much progress has been made over the years in our understanding of *RAS* genes and the critical role they play in tumorigenesis, yet we have been unable to exploit this knowledge to significantly improve the outcome of cancers driven by mutant *KRAS*. While recognizing that NSCLC and CRC with *KRAS* mutations are not likely to be responsive to EGFR inhibitors is an important step forward in improving patient treatment, there remains a pressing need for the development of effective KRAS-directed cancer therapies. Although efforts to develop KRAS-targeting therapies have so far been met with disappointment, we have gained important insights into the complex biochemistry of KRAS proteins and KRAS signaling networks. At the same time, *in vivo* studies in mice have and will continue to make important contributions to our understanding of the underlying biology of KRAS proteins and their roles in cancer. Going forward, it will be critical to continue interrogating the role of *KRAS* in cancer through mouse and molecular studies and to bridge this knowledge with clinical studies to facilitate the development of truly effective therapies against mutant *KRAS*-driven cancers.

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