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KRAS as a Therapeutic Target

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Abstract

KRAS proteins play a major role in human cancer, but have not yielded to therapeutic attack. New technologies in drug discovery and insights into signaling pathways that KRAS controls have promoted renewed efforts to develop therapies, either through direct targeting of KRAS itself, new ways of blocking KRAS processing, or by identifying targets that KRAS cancers depend on for survival. While drugs that block the well-established downstream pathways, RAF-MAPK and PI 3 kinase, are being tested in the clinic, new efforts are underway to exploit previously unrecognized vulnerabilities, such as altered metabolic networks, or novel pathways identified through synthetic lethal screens. Furthermore, new ways of suppressing KRAS gene expression and of harnessing the immune system offer further hope that new ways of treating KRAS are finally coming into view. These issues are discussed in this edition of *CCR Focus*.

Introduction

The pioneers of molecular oncology discovered a collection of mutant cellular proteins that are capable of initiating cancers in animal models with high efficiency. Later, many of these proteins were shown to be drivers of human cancer, and some, such as ERBB (EGFR), ABL, and RAF, have been successfully exploited as targets of therapeutic intervention. However, Ras proteins have not yielded to any type of therapeutic attack, and, indeed, have been dismissed as “undruggable” for many years. This is because the RAS proteins did not appear to present suitable pockets to which drugs could bind, except for the GDP/GTP binding site: unfortunately, Ras proteins bind very tightly to these nucleotides (picomolar affinities, and very slow off-rates) making the prospect of identifying competitive nucleotide analogs seem virtually impossible. As a substitute for direct attack on the RAS proteins themselves, much attention has been paid to pathways downstream of RAS, especially the RAF-MAPK pathway and the PI 3′ kinase pathways, in the hope that blocking these pathways will provide clinical benefit for patients suffering from Ras-cancers. These pathways are much more complicated than first imagined (Fig. 1) and, so far, these efforts have been generally disappointing: RAF kinase inhibitors paradoxically activate the RAF-MAPK pathway, and MEK inhibitors unleash upstream feedback mechanisms that render

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them relatively ineffective (1-3). Likewise, efforts to identify new targets that cancers expressing oncogenic RAS proteins depend on for their survival, on using synthetic lethal screens have not lived up to early expectations, for reasons that are discussed by Julian Downward (4) in this edition of *CCR Focus*. In addition to discussing synthetic lethal screens, the authors of this *CCR Focus* discuss progress in targeting the KRAS protein directly (5), targeting enzymes involved in KRAS processing and membrane localization (6) and a new aspect of KRAS biology: the effects of KRAS on metabolic pathways and opportunities for therapeutic attack based on these alterations (7).

The tremendous unmet clinical need, along with technical advances in drug discovery and a deeper understanding of signal transduction, have prompted renewed efforts to find therapies that target RAS-driven cancers, and RAS proteins directly. These include the National RAS Initiative (8), as well as many new efforts in academia and industry (9, 10). These efforts have, in turn, prompted some key questions. We will discuss these below and elsewhere in this *CCR Focus*, with special emphasis on KRAS, as this is by far, the major form of RAS that contributes to cancer, as shown in Table 1.

Are KRAS Cancers KRAS Dependent?

The most important question, from the perspective of drug development, is obviously whether KRAS cancers retain dependence on KRAS expression. While there are very few examples of oncogenes acting in a hit-and-run fashion (11), this possibility has to be considered seriously. Early studies showed that *RAS* oncogenes require co-operating oncogenes or loss of tumor suppressors to initiate transformation, but it has long been assumed that Ras-transformed cells retain dependence on Ras for their transformed phenotype. Early studies on Ras-transformed cell lines using temperature-sensitive Ras mutants, or injection of neutralizing antibodies showed this to be the case (reviewed in ref. 9). Later, when drugs were developed that inhibit oncogenic tyrosine kinases, the notion that tumor cells retain dependence on oncogenic drivers was re-enforced. Likewise, preclinical testing of farnesyl transferase inhibitors showed dramatic responses, at least in HRAS transformed cells, again suggesting that tumors expressing mutant *RAS* oncogenes retain dependence (see ref. 6 of this *CCR Focus*). More recent analysis of cell lines derived from tumors expressing oncogenic KRAS revealed a wide range of dependency on KRAS, at least for survival in 2D cultures (12). However, some cells that are oblivious to KRAS knock down in 2D cell culture, are highly dependent on KRAS in 3D cultures or in vivo (13). Likewise, ablation of KRAS from established tumors in mouse models results in dramatic tumor regressions, suggesting that KRAS is indeed likely to be as good a therapeutic target as other classical oncogenes (14-16).

KRAS is a major driver in lung adenocarcinomas and in pancreatic cancers. This seems most likely because KRAS appears to be the initiating event in these tumors. In lung adenocarcinoma, for example, the common G12C mutation is a hallmark of exposure to tobacco smoke. In pancreatic cancer, KRAS mutations are detected in the earliest lesions and are retained in all metastases (17). On the other hand, KRAS mutations are probably not primary initiating events in colorectal cancers (18, 19), the vast majority of which are thought to be initiated by loss of APC or, in mis-match repair deficient tumors, by mutations

in β-catenin. KRAS mutations do occur as early events in about 50% of colorectal cancers, but the degree to which these tumors depend on KRAS is still being investigated.

How Specific Do KRAS Drugs Need to Be?

Drugs that block all four isoforms of Ras - HRAS, NRAS and KRAS4A and KRAS4B - are expected to be unacceptably toxic. Ablation of HRAS, NRAS and KRAS in fibroblasts in culture causes growth arrest and blocks cell movement (20), and failure to respond to growth factors. Likewise, ablation of all three *RAS* genes is embyonic lethal, even though loss of NRAS and HRAS can be tolerated, at least in mice (reviewed in ref. 18). The question remains as to whether drugs need to be selective for the mutant form of Ras, or whether sufficient potency and efficacy could be obtained by targeting KRAS, without discriminating between wild-type and mutant forms.

The possibility of identifying compounds that bind directly to KRAS and block its function has been a goal for many years. Several compounds that bind to Ras proteins have indeed been described, even though the surface of Ras proteins contain few, if any, binding pockets that would have been considered suitable for high affinity binding (21-24). The GDP/GTP binding pocket itself has been dismissed as a useful pocket, because the off-rate for bound nucleotide is slow, and cellular concentrations of GTP and GDP are very high. These issues are discussed in depth elsewhere in this issue (5). Identification of compounds that target the G12C allele of KRAS have encouraged the development of other compounds that target mutant alleles selectively (25, 26). Whether this is technically possible for mutants other than G12C remains to be seen. The G12D allele is the most attractive target, as it is the most common mutant form in human cancer, and the presence of the carboxylic acid side group might offer opportunities for specific chemical attack, though these opportunities are far less obvious than those aiming at the cysteine substitution. Indeed, drugs that covalently modify reactive cysteines, including dacomitinib and ibrutinib have already been approved for other cancer indications.

On the other hand, it is not clear that KRAS compounds need to be specific for mutant alleles, even though this would certainly be the most desirable outcome. A drug that targeted KRAS without distinguishing between wild type and mutant KRAS might be equally effective. In normal cells, HRAS, KRAS and NRAS are likely to perform similar, redundant functions, so that ablating KRAS may have little effect. This has been tested by Barbacid and coworkers, who showed that ablating KRAS in an adult mouse had no obvious effect, at least in the short term (M. Barbacid; unpublished data). However, targeting KRAS 4A and 4B without affecting HRAS or NRAS might be as challenging at targeting mutant KRAS proteins.

Do We Need to Target Both KRAS 4A and KRAS 4B?

KRAS 4B is the major isoform expressed in human tumors, as judged by mRNA expression analysis. Typically, KRAS 4B mRNA is about 5 times as abundant as KRAS 4A, according to TCGA data sets. KRAS 4A and 4B are widely expressed at the protein level (27). KRAS 4B localizes to the plasma membrane through a stretch of lysine residues (KKKKKKSKKC-farnesyl) while KRAS 4A, HRAS and NRAS utilize palmitoylation, in addition to

farnesylation of the C-terminal CAAX cysteine, an essential step for all Ras proteins (ref. 6 of this *CCR Focus*). KRAS 4B, but not 4A or HRAS or NRAS binds calmodulin (28). This interaction has been shown to affect the ability of KRAS 4B to interact with RAF kinase, with GAP and with the plasma membrane. In addition, we have found that binding of KRAS4B to calmodulin inhibits CaM kinase activity, with significant effects on non-canonical wnt signaling. While the full significance of this interaction is still being investigated, it is clear that the interaction is GTP-dependent and unique for KRAS 4B. As such it may present opportunities for therapeutic intervention in ways that target with some selectivity KRAS 4B, the major splice variant of KRAS (M. Wang and F. McCormick; submitted for publication).

Should We Revisit KRAS Processing as a Source of Targets?

Direct inhibition of Ras processing enzymes has a long history, but is now being revisited, as discussed by Cox and colleagues in this *CCR Focus* (6). Briefly, early efforts to block Ras activity by preventing farnesylation were thwarted by the presence of a back-up system that allows geranylgeranylation of unprocessed KRAS and NRAS following farnesyl transferase inhibition (6). This back-up system does not act on HRAS, suggesting that farnesyl transferase inhibitors may be useful in tumors driven by oncogenic HRAS, such as subsets of thyroid and bladder cancer. Furthermore, drugs that target palmitoylation of NRAS and KRAS 4A, or target other enzymes involved in Ras processing merit further consideration. Finally, efforts to target Ras chaperone proteins, such as PDE-delta have led to development of compounds that serve as proof-of-concept molecules that should encourage further attention to this newly recognized aspect of Ras signaling (29).

Can We Target KRAS with siRNA?

Recently, successful attempts at targeting KRAS in pre-clinical mouse models have been reported. For example, regression of KRAS driven tumors was observed following systemic administration of highly potent and specific siRNA targeting KRAS in a synthetic nanoparticles. Efficacy was improved by the addition of siRNA targeting PI 3′ kinase in the same particle (30). Using a different delivery system, Xue and coworkers showed that combinations of siRNA targeting miR-34A and KRAS prevented growth of KRAS driven lung tumors in vivo (31). Likewise, siRNAs directed against KRAS G12D were effective in treating pancreatic cancers in mouse models, when siRNA was delivered using miniature biodegradable polymeric matrices (32). These encouraging results suggest that clinical responses are indeed attainable using siRNA delivered by innovative targeted particles and we expect that several clinical studies will soon be initiated to test these possibilities. Furthermore, the capability of targeting KRAS isoforms specifically, and of targeting several genes and microRNAs simultaneously opens up multiple exciting possibilities for suppressing KRAS activity and preventing feedback and drug resistance.

Can We Harness the Immune System to Target RAS?

Equally exciting, cancer immunotherapy has recently made the transition from a powerful concept into clinical reality. We can now ask whether patients suffering from Ras cancers are likely to benefit from the new approaches. Anti-CTLA4, anti-PD-1, and anti-PDL-1

antibodies have shown remarkable clinical activity in patients suffering from malignant melanoma; the overall response rate is about 20-30% and these responses can be durable. Mutational load is the main predictor of clinical response, to date (33). While B-Raf is the most common oncogenic driver in melanoma, in 15 – 20% of melanomas, mutations in NRAS are observed. Differences in response to tumors driven by NRAS or V600E B-Raf mutations have not been reported. Therefore, in contrast to other therapies, KRAS cancers may not be refractory to this type of immune therapy, though this needs to be analyzed in more depth.

Mutant KRAS proteins themselves are not strongly antigenic, but efforts are underway to increase the ability of the immune system to recognize KRAS mutants as neoantigens. This concept has been tested for many years, and progress has indeed been made in generating T-cell responses against Ras-derived epitopes (see refs. 33 and 34, for example).

If We Target RAS Successfully, Will We Evoke Drug Resistance?

The simple answer to this question has to be yes, based on prior experience with targeted therapies. Pre-existing second-site mutations are often responsible for drug resistance to these therapies. These mutations exist at frequencies that are significant problems in the clinic because of the large number of cell divisions in a cancer cell's history (cancers can evolve over 20-30 year time spans), and the large number of cells in a tumor at clinical presentation. The mutation rate is often supposed to be higher in cancer cells than normal cells, but this is not necessary to generate enough mutants to be problematic. The second-site mutations must be neutral or have a gain-of-function to persist in the tumor cell population prior to selection by exposure to the targeted therapy. The frequency of such mutations clearly relates to the binding properties of the drug in question. For example, type I kinase inhibitors compete directly for binding with ATP. A second-site mutant must retain ATP binding and kinase activity, while preventing drug binding: the number of such mutations is small. In contrast, type II kinase inhibitors stabilize kinases in their inactive state. The number of mutations that can overcome this mode of inhibition is relatively high.

It is likely that second-site mutations that confer resistance to Ras-binding drugs will indeed present a challenge to the effectiveness of drugs that bind to Ras directly, though the extent of this challenge is not yet known. The G-domains of Ras proteins are remarkably well conserved, suggesting that most amino acids have an important role in the protein's function. Thus, second site mutations that are neutral may be rare, but this needs to be tested. The identification of such mutants is now feasible, since Ras-binding compounds have now been described.

Recently, two groups identified a possible alternative pathway that could bypass loss of KRAS. Over-expression of YAP1 (YES-Associated Protein 1) rescued KRAS cells in which KRAS had been knocked down (34), in established tumors in which KRAS had been ablated (35) or in tumor cells that escape from MEK or BRAF inhibition (36). While the precise mechanism by which YAP1 rescues Ras or BRAF ablation is not yet clear, the possibility that this pathway could off-set therapeutic effects of Ras therapeutics must be considered.

In addition to mutations and by-pass mechanisms, we expect that inhibition of KRAS will lead to de-repression of upstream signaling proteins through relief of feedback inhibition. Indeed, knock-down of oncogenic Ras mutants have already been shown to lead to de-repression of upstream signaling pathways, by mechanisms that resemble those seen following inhibition of MEK (37)and with Raf inhibitors (1), such as activation of upstream tyrosine kinases (38, 39) or loss of NF1 (40).

At first sight, another obvious mechanism might be emergence of a different mutant in KRAS or in HRAS or NRAS. The former seems unlikely: KRAS cancers are generally clonal with respect to the KRAS mutation, so a second, pre-existing independent clone driven by a different Ras mutation would be very rare indeed. Obviously, a de novo mutation could emerge during treatment, however, and the frequency of this needs to be considered.

In conclusion, we are at the beginning of a new series of attempts to attack KRAS cancers, by direct attack on the protein, or by indirect approaches such as siRNA or harnessing the immune system. There no longer appear to insurmountable obstacles to targeting KRAS, and we may soon stop thinking of KRAS as un-druggable. The biggest question in the future will be which of these new approaches will be most effective and provide hope for the hundreds of thousands of patients currently suffering from KRAS driven cancers.

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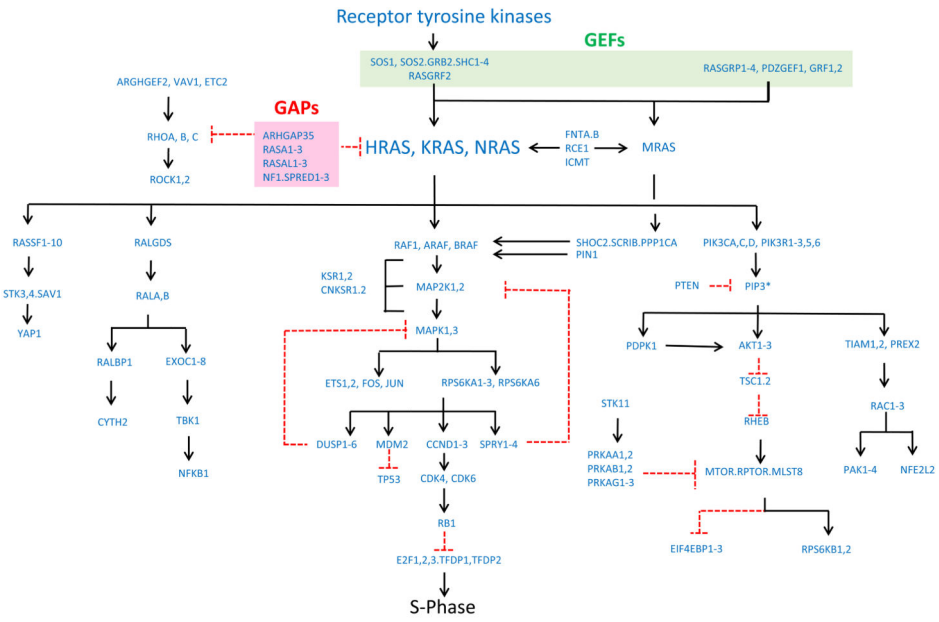

Figure 1.

The Ras pathway. RAS proteins are activated by GEFs (guanine nucleotide exchange factors), boxed in green, and inactivated by GAPs (GTPase activating proteins), boxed in red. Protein names correcond to gene names from TCGA (see ref 8. for conversion to protein names). Pathways downstream of Ras include RASSF proteins, RalGDS, the Raf kinases, and PI 3 kinases.

Table 1

Frequency of Ras isoform mutations in human cancers. Data were compiled from the TumorPortal (from TCGA datasets; ref. 41) and from Prior and colleagues (17).

Primary tissue	*KRAS* %	*HRAS* %	*NRAS* %	Total %
Pancreas	61	0	2	63
Colon	43	1	9	42
Endometrium	21	<1	3	22
Lung adenocarcinoma	26	<1	1	27
Multiple myeloma	<1	<1	17	19
Skin	3	6	18	27
AML	4	9	7	19