1 Introduction

The 2021 FDA approval of Lumakras (sotorasib) [1] for lung cancer patients with the KRAS G12C mutation represents a major milestone in cancer therapeutics. This achievement, which comes approximately 40 years from the publication describing RAS as the first human oncogene to be discovered [2], may very well be the first of many more RAS-directed therapeutics to be approved for clinical use this upcoming decade.

The RAS GTPases (KRAS, NRAS, and HRAS) play major roles in human cancer (Figure 1). According to the National Cancer Institute (NCI) RAS Initiative, more than 30 percent of human cancers are driven by a mutation in one of these three RAS genes [3]. *KRAS* is the most commonly mutated of the three RAS genes. *KRAS* mutations can be detected in nearly all pancreatic adenocarcinomas [4, 5] and approximately 30 to 40 percent of lung adenocarcinomas and colorectal adenocarcinomas [6, 7]. *NRAS* mutations are observed in approximately 20 percent of malignant melanomas [8], and *HRAS* mutations are observed in head and neck cancers [9] as well as in bladder cancers [10] (Figure 2). These examples highlight some of the cancers in which the RAS genes are most commonly mutated; however, mutations to these genes are encountered to a lesser extent in a much wider variety of cancers.

RAS mutations have been linked to several important cancer phenotypes (Figure 3). Perhaps most importantly, RAS mutations are typically capable of conferring the "self-sufficiency in growth signals" cancer hallmark [11, 12]. This is critical for the dysregulated, elevated proliferation common to malignant tumors. Additionally, RAS mutations have been linked to metabolic phenotypes that appear critical to cancer [13–15] and to increased pro-survival signaling [16, 17].

2 RAS Biology

Several aspects of RAS targeting, including KRAS G12C targeting, are best understood after a review of the fundamental aspects of RAS biochemistry (Figure 4). To begin with, RAS has a nucleotide-binding pocket that can bind the guanosine nucleotides GTP and GDP. RAS proteins adopt different structural conformations on the basis of whether GTP or GDP is bound [18]. Downstream effector proteins bind specifically to the GTP-bound form of RAS (whether wild-type or mutant). The RAS-effector complex may be further modulated (such as by phosphorylation of the bound effector protein) as part of the transmission of RAS-GTP-dependent signals downstream from RAS through the effector [19]. RAS downstream effectors include the RAF kinases (BRAF, CRAF, and ARAF) as well as the phosphoinositide 3-kinases (*3*).

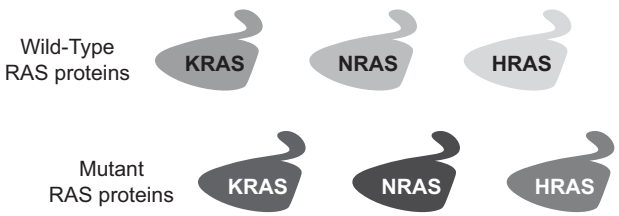

Figure 1 The RAS proteins.
The three RAS GTPases most associated with cancer are KRAS, NRAS, and HRAS. The wild-type RAS are cell signaling proteins that carry a variety of signals, including those for cellular proliferation. Mutant forms of RAS proteins are commonly found in cancer and are critical drivers of the cancer phenotype.

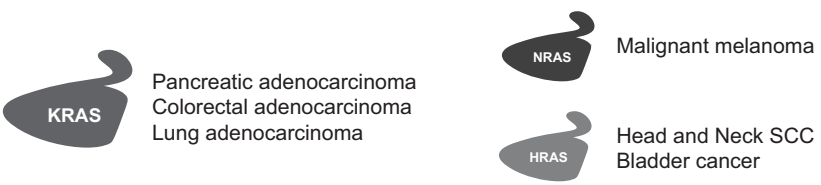

Figure 2 Mutant RAS proteins are common in many forms of cancer.
Mutant KRAS is found in a high proportion of pancreatic, colorectal, and lung adenocarcinoma patients. Mutant NRAS is common in malignant melanoma. Mutant HRAS is commonly observed in head and neck squamous cell carcinoma and bladder carcinoma.

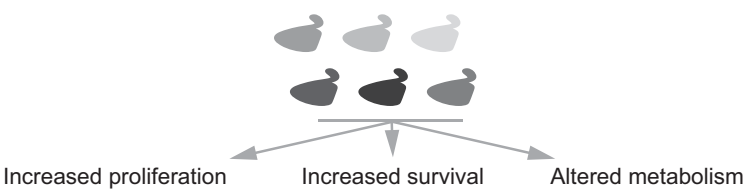

Figure 3 Mutant RAS phenotypes.
Mutant RAS proteins are associated with several important cancer phenotypes. These mutants are well-established drivers of increased cellular proliferation. RAS mutants have also been implicated with increased cellular survival signals and metabolic alterations that support increased cancer cell proliferation.

The level of GTP-RAS is therefore a critical variable that governs cellular phenotypes. The cellular level of GTP-RAS and GDP-RAS reflects a dynamic equilibrium between several processes. RAS itself is a GTPase, as it can catalyze the hydrolysis of GTP to GDP [20]. This reaction is generally slow, and GAP proteins (GTPase Activating Proteins) can expedite GTP hydrolysis [21]. RAS GTPases bind to guanosine nucleotides with very high affinity, and spontaneous dissociation of GTP and GDP by a free RAS protein is very slow [20]. GEF proteins (Guanine Nucleotide Exchange Factors) reduce the affinity

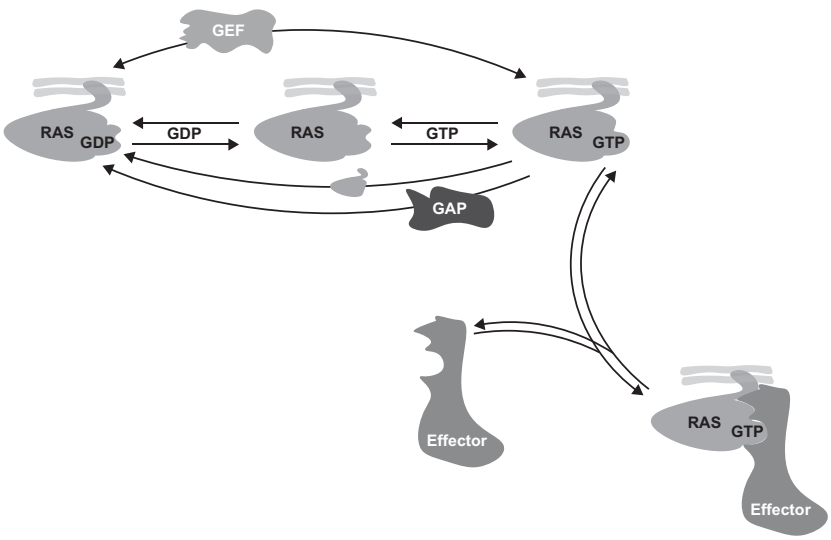

Figure 4 The RAS nucleotide exchange cycle.
Cellular levels of GDP-bound RAS (the inactive form of RAS) and GTP-bound RAS (the active form of RAS) are controlled by a variety of biochemical processes. RAS GEFs promote nucleotide dissociation and exchange, and tend to promote net conversion to GTP-bound RAS due to the cellular excess of GTP over GDP. Spontaneous nucleotide dissociation without a GEF can also occur at much slower rates for wild-type RAS. Some oncogenic RAS mutants, however, have significantly elevated spontaneous nucleotide dissociation (and activation). RAS-GTP may be converted to RAS-GDP directly through the GTPase activity of RAS. RAS-GAPs, however, are much stronger promoters of RAS-GTP-to-RAS-GDP conversion. Most oncogenic RAS proteins are completely to strongly resistant to the activity of GAP proteins. RAS effector proteins specifically bind to RAS-GTP.

of RAS for its nucleotides and increase the dissociation rate for nucleotides [22]. As there is an approximately 10:1 excess of GTP to GDP in the cell [23], nucleotide exchange tends to result in the conversion of GDP-bound RAS for GTP-bound RAS. Effector proteins also contribute to the dynamic equilibrium, as effectors, GAPs, and GEFs bind to RAS in an overlapping region that includes the nucleotide binding pocket. Thus, these reactions are competitive, and the binding of RAS-GTP to a GAP, GEF, or effector can effectively prevent the simultaneous binding of another RAS binding partner.

As mentioned earlier, the RAS GTPases are signaling proteins and they are critical to the transmission of signals from surface receptors. One significant surface receptor that signals through RAS is the Epidermal Growth Factor Receptor (EGFR). The activation of EGFR by one of its ligands leads to activation of the EGFR kinase domain, trans-autophosphorylation of the EGFR dimer [24], and the recruitment of SH2 and PTB domain containing

"reader" proteins that transmit signals downstream to many pathways [25], including the RAS pathway. Critical to EGFR activation of RAS are the adapter proteins SHC1 [26], which can bind directly to EGFR, and GRB2 [27], which can bind directly to EGFR as well as to phosphorylated SHC1. The RAS GEFs SOS1 and SOS2 can bind with GRB2, and the recruitment of GRB2/SOS is critical for bringing the SOS GEFs to the plasma membrane where they can activate RAS. By "activate RAS" we refer to a net increase in total RAS-GTP from a state where RAS-GDP had predominated. Downstream from RAS, RAS-GTP signals may spread through distinct signaling pathways, such as through the RAF kinases to the ERK/MAPK cascade and through the PI3-kinases to the AKT/mTOR survival pathway.

3 Oncogenic RAS

Within an oncogenic RAS mutant, the dynamic equilibrium between GTP-RAS and GDP-RAS is strongly perturbed such that a much higher fraction of RAS is bound to GTP (Figure 5). The term "constitutively active" is commonly used to describe this state of the dynamic equilibrium where GTP-bound levels are relatively high. Over the years, the term "constitutively active" has commonly been misconstrued to mean that the individual RAS proteins are each "locked" into a GTP-bound state. However, cellular measurements of mutant RAS-GTP and GDP suggest that oncogenic RAS is not all RAS-GTP bound [28, 29]. This point is important to consider for KRAS G12C inhibitors, which will be discussed in Section 8 of this Element.

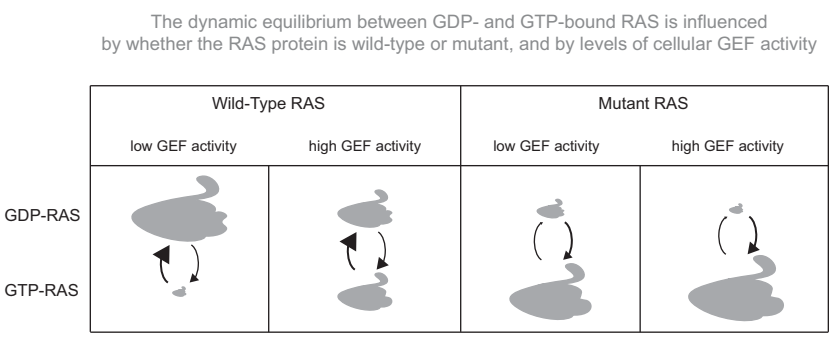

Figure 5 The RAS dynamic equilibrium in wild-type and mutant RAS. Levels of GDP- and GTP-bound wild-type RAS are dynamically controlled, predominantly by GEF and GAP activities. Wild-type RAS is predominantly GDP bound unless cellular GEF activity is elevated. Mutant RAS is predominantly GTP bound regardless of cellular GEF activity. Cellular GEF activity, though, may further increase cellular RAS-GTP levels.

There are many different RAS single nucleotide variants that may be observed in human cancer [20, 30]. Most of the oncogenic RAS alleles are constitutively active due to the presence of a point mutation that prevents RAS-GAPs from being able to promote the GTP-to-GDP conversion. This is usually thought of as an absolute elimination of GAP activity, although some RAS mutants may still display a very low level of sensitivity to GAP inactivation [31–33]. As GAP-mediated conversion of RAS-GTP to RAS-GDP is the dominant mechanism of wild-type (WT) RAS inactivation, an oncogenic RAS mutant's loss of sensitivity to GAP-mediated inactivation strongly shifts the dynamic equilibrium toward increased levels of GTP-bound RAS.

Despite an insensitivity to GAP-mediated GTP-to-GDP conversion, oncogenic RAS-GTP can still be converted to RAS-GDP through intrinsic GTPase activity (which is also typically partially impaired in oncogenic mutants). Additionally, nucleotide exchange should result in loading of oncogenic RAS with GDP rather than GTP approximately 10 percent of the time. These mechanisms of conversion of oncogenic RAS-GTP to oncogenic RAS-GDP are important to note because the KRAS G12C inhibitors that are currently in clinical trial bind specifically to the GDP-bound form of mutant RAS. Thus, mechanisms that result in conversion of GTP-mutant RAS to GDP-mutant RAS are of more than academic interest.

4 RAS Targeting

Oncogenic RAS has long been seen as a potentially valuable therapeutic target. That RAS could be a valuable target is supported by a range of data that suggest RAS cancers retain a dependency on the presence of the RAS mutation for ongoing survival [34, 35]. This ongoing dependence on the presence of the oncogenic mutation is known as "oncogene addiction," and it provides a philosophical basis for many targeted therapies [36].

Targeting RAS directly at the nucleotide-binding pocket may seem like a valuable strategy that would be analogous to the targeting of protein kinases with small molecules that bind the ATP binding pocket. However, the RAS GTPases bind GTP and GDP with picomolar affinity, while kinases tend to bind ATP with millimolar affinity [3]. Thus, the million- to billionfold difference in affinity presents a very large obstacle for the development of RAS nucleotide-binding pocket inhibitors. Additionally, the high cellular levels of GTP and GDP [23] would further make it exceptionally hard for a small molecule to outcompete GTP and GDP to bind to an accessible pocket.

The pharmaceutical industry put a tremendous amount of effort into developing small molecules that prevent the proper membrane localization of RAS.

RAS is post-translationally modified on its C-terminus, where it may be farnesylated [37]. The farnesyl transferase inhibitors (FTIs) attracted the most attention in this category. Preclinical studies in mouse models suggested that FTI could be useful [38], although clinical trial results were much less promising [37]. One possible reason for the disappointing clinical trial studies could be off-target toxicity, as many proteins other than RAS are farnesylated. Additionally, studies suggested that KRAS and NRAS could obtain proper membrane localization through alternative post-translational lipid modification pathways [39, 40]. Surprisingly, although the field considered FTI to only potentially be useful for HRAS mutant cancers due to the alternative modification pathways for KRAS and NRAS, there does not seem to have been a significant effort to adapt FTI to HRAS mutant cancers at that time. Over the past decade, however, it seems that interest in considering FTI for HRAS mutant cancers has increased [41]. For example, Ho and colleagues recently evaluated tipifarnib (an FTI) for recurrent and metastatic head and neck squamous cell carcinoma with HRAS mutation, and they observed a 55 percent response rate (N = 20 evaluable patients) [42]. This renewed interest may reflect a trend where personalized cancer medicine is willing to consider smaller and smaller molecularly defined subsets of cancer as viable patient populations for targeting.

5 Downstream Targeting

Due to the challenges of targeting RAS directly, there has been much interest in targeting the signals that propagate from RAS through other proteins. The successful development of kinase inhibitors made kinases an appealing target, and the RAF and PI3 K proteins that are RAS effector proteins are kinases. At this time, however, no RAF or PI3 K inhibitor that has advanced to clinical trials has proven useful for RAS mutant cancers [30]. Consideration of inhibitors in the RAF/MEK/ERK pathway illuminates some of the complexity that comes with targeting downstream from RAS.

Although several RAF kinase inhibitors have been developed, they do not appear useful as a single agent against RAS mutant cancers. Indeed, RAF kinase inhibitors can actually increase the total level of oncogenic RAS signal through a process commonly referred to as “paradoxical activation” [43, 44]. For example, BRAF mutant melanoma patients treated with a BRAF inhibitor commonly develop cutaneous squamous cell carcinomas, and these squamous cell carcinomas commonly have an HRAS mutation whose signal is further potentiated (thereby driving increased proliferation) by the BRAF inhibitor [45]. Paradoxical activation is not completely understood, but it involves the

RAF-inhibitor-stabilizing RAF dimers that are critical for transmitting RAS signals. Ongoing work in BRAF inhibitor development aims to develop improved RAF inhibitors that may be less prone to paradoxical activation [46]. It is possible that BRAF inhibitors will someday be developed that prove useful for RAS mutant cancer patients.

MEK inhibitors would also be anticipated to be useful in RAS mutant cancers and should not display RAF paradoxical activation. In practice, however, MEK inhibitors have not proven useful in RAS mutant cancer [47, 48]. Intriguingly, MEK inhibitors recently demonstrated benefit in clinical trials for patients with neurofibromatosis [49]. Neurofibromatosis is a genetic disease that is caused by a germline mutation in the gene *NF1* that codes for the RAS-GAP neurofibromin. Increased levels of wild-type RAS-GTP result from the decrease in functional neurofibromin proteins that follows from such mutations, and increased RAS-GTP is believed to be critical to the development of the pleiotropic neurofibromatosis phenotypes. That MEK inhibitors offered benefit suggests that these MEK inhibitors can offer clinical benefit via inhibiting pathological RAS signals, and some personalized medicine clinical trials that use MEK inhibitors "off label" have reported overall positive trends [50, 51]. However, clinical trials that focus on the treatment of RAS mutant cancers with a MEK inhibitor have found little to no benefit [47, 52, 53]. One of the challenges with MEK inhibition could be co-genomic alteration that is associated with RAS alterations. We have previously reported that 95 percent of cancers with RAS mutations also harbor co-alterations (median of 3). The most common co-alterations that may explain limited efficacy with MEK inhibitors alone were found in the PI3 K pathway (31%), in cell cycle pathways (31%), in tyrosine kinases (22%), and in BRCA-associated genes (13%) [54]. Hence, targeting co-genomic alterations along with MEK inhibition may be necessary for RAS-altered cancers [55, 56].

6 Upstream Targeting

The presence of a RAS mutation is widely believed to indicate resistance to EGFR inhibitors in both colorectal and lung cancers [57, 58]. However, erlotinib was FDA approved in pancreatic adenocarcinoma on the basis of a response duration of less than two weeks [59]. As KRAS mutations are almost universally present in pancreas cancer [4, 5], even a partial benefit is notable. More intriguingly, analyses of phase III clinical trials suggest that EGFR inhibitor cetuximab may offer benefit in KRAS G13D mutant colorectal cancer [60, 61], a topic that will be further addressed in Section 11 of this Element.

Perhaps less controversial will be the utilization of inhibitors with targets between the receptor tyrosine kinases and RAS. Inhibitors to SHP2 (*PTPN11*) and SOS1/2 fit this description. SOS1/2 are GEF proteins, and their activity on RAS is clear: they promote nucleotide exchange, and targeting SOS1/2 could potentially tip the dynamic equilibrium toward a state with less total RAS-GTP. Several groups have pursued SOS inhibitors to different extents [62–64], and one SOS inhibitor (BI 1701963, Boehringer Ingelheim) has advanced to clinical trial. SHP2 inhibitors are also in clinical trial, and these inhibitors are believed to result in reduced signaling within the RAS pathway. The mechanism by which the phosphatase SHP2 contributes to RAS pathway activation is not fully understood, but it appears that SHP2 phosphatase activity can counteract phosphorylation events that drive processes that negatively regulate RAS signals [65]. Thus, SHP2 activity promotes RAS pathway signaling, and SHP2 inhibition reduces RAS pathway signaling. Whether SHP2 inhibition offers benefit in RAS mutant cancers remains to be determined.

7 Targeting Specific RAS Mutants

The most commonly encountered oncogenic RAS alleles occur at codons 12, 13, or 61 [30]. Different specific amino acid substitution mutations may be observed at each of these hot spots. For example, G12D, G12V, and G12C mutations are the most common oncogenic variants observed at codon 12. Codon 13 is, coincidentally, also normally a glycine ("G"), and it is most commonly mutated to an aspartic acid ("D") in human cancer.

Biophysically, the major hot-spot oncogenic RAS mutants are impaired at GAP inactivation, and this defect accounts for the majority of their net shift to a GTP-bound state [66]. However, each of the amino acid substitutions introduces a slightly different collection of atoms into the RAS three-dimensional structure and also introduces different variations in the electrostatics of the region. Biophysically, the different substitution mutations also alter the chemical kinetics of all the other biochemical reactions that relate to RAS signaling, and the magnitude of the variations can vary between substituting mutations [20, 67]. Many of the exciting advances in RAS targeting come from a renewed interest in studying and exploiting these differences.

8 KRAS G12C Targeting

The major breakthrough that catalyzed a renewed interest in RAS therapeutics involved the development of covalent inhibitors that target the KRAS G12C mutation (Figure 6). This is the most common KRAS mutation in lung cancer [68]. This mutation is, however, uncommon in pancreatic cancer [4, 5] and in

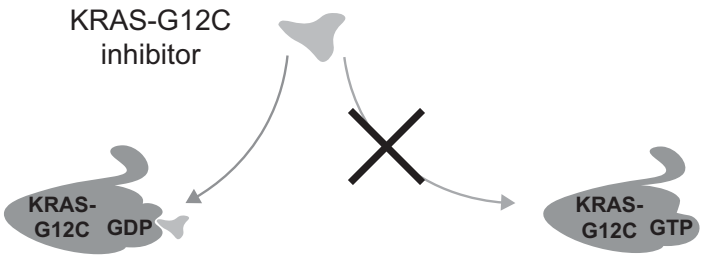

Figure 6 Small molecular targeting of KRAS G12C.
Small molecule inhibitors that specifically react with KRAS G12C and covalently bond with the cysteine residue at codon 12 have been described to exclusively to strongly favor the GDP-bound form of KRAS G12C.

colorectal cancer [7]. The field of RAS cancer therapeutics changed dramatically with the development and description of small molecules that specifically covalently react with the cysteine residue of KRAS G12C [69]. This demonstrated the value of this approach to the broader scientific and medical communities. In parallel and in follow-up studies, a variety of pharmaceutical and biotech companies have also advanced KRAS G12C inhibitor programs [1, 70–72].

Importantly, this original study [69] also found that, after covalently reacting with the codon 12 cysteine, the small molecule inhibitor also nestled into a new, previously unknown pocket on the surface of RAS [69]. Thus, in addition to identifying a new strategy for targeting RAS, this work also suggested that there may be other unknown opportunities to target RAS.

One important consideration as these inhibitors move into the clinic is whether the benefit of the inhibitor can be increased by the addition of another targeted therapy. Notably, and perhaps surprisingly, EGFR inhibitors have been suggested to be a promising agent for combination with G12C inhibitors. This is supported by a variety of preclinical in vivo and in vitro studies [1, 70, 71, 73–78]. One mechanism that has been proposed to explain why EGFR inhibitors increase the benefit of G12C inhibitors is that the G12C inhibitors bind specifically to the inactive, GDP-bound form of KRAS G12C. This may at first be superficially surprising because it is common to think of oncogenic RAS as constitutively active and as GTP bound [73, 79]. However, a dynamic equilibrium exists, and oncogenic RAS may be found in both GDP- and GTP-bound forms, although the net balance within oncogenic RAS mutants tends to overwhelmingly favor the GTP-bound state (Figure 7). One reason that EGFR inhibition has been promoted to increase the effects of KRAS G12C inhibitors is that it may help suppress the conversion of GDP-bound KRAS G12C to GTP-bound KRAS G12C [73]. As EGFR activation can lead to RAS activation, and as EGFR activation plays an important role in

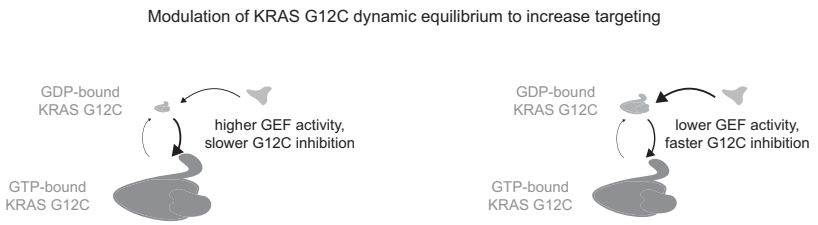

Figure 7 Modulation of the KRAS G12C GTP:GDP dynamic equilibrium may improve G12C targeting.

Many preclinical studies have found that EGFR inhibitors potentiate the ability of G12C inhibitors to bind to KRAS G12C. This is believed to in part follow from EGFR inhibition leading to reduced GEF activity, in turn shifting the dynamic equilibrium of KRAS G12C to a state with increased GDP-bound KRAS G12C that can then be targeted by the G12C inhibitor.

non–small-cell lung cancer, it seems reasonable to consider that there is some basal signaling from EGFR to RAS that may be partially suppressed with an EGFR inhibitor.

Another mechanism that has been argued to contribute to the value of EGFR inhibitors in combination with KRAS G12C inhibitors involves negative feedback [76]. Multiple negative feedback processes exist in the cell that combat aberrant activation of the RAS pathway. Thus, a KRAS G12C cancer cell typically has an elevated RAS pathway signal that promotes cellular proliferation despite stronger-than-normal inhibition of the EGFR/RAS/ERK pathway through activated negative feedback processes. Once RAS is inhibited, there is typically a very strong, initial, transient suppression, as the oncogene has been targeted and the strong negative feedback operates unopposed. The profound suppression of signal eliminates the ongoing induction of signals that promote negative feedback, and the negative feedback processes are reduced. The cell then "reactivates" to a new level that is typically assumed to be less than what was experienced before treatment was initiated, but that will be above the low level of signal that was caused by initial targeting. If this new level of reactivation (where the oncogene may only be partially inhibited, and where less negative feedback is present) is sufficient to drive proliferation, the treatment will not be fully effective. Targeting EGFR is believed to help block the reactivation of the EGFR/RAS/ERK pathway [80, 81]. This has perhaps best been described in *BRAF* mutant colorectal cancers, where a treatment regimen that combines an EGFR inhibitor along with a BRAF and MEK inhibitor has proven useful in phase three clinical trials, and where the believed mechanism is the blocking of feedback reactivation [53].