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Metabolic Dependencies in *RAS*-Driven Cancers

Alec C. Kimmelman

Division of Genomic Stability and DNA Repair, Department of Radiation Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts

Abstract

The ability to inhibit the *RAS* oncogene has been the holy grail of oncology because of the critical role of this gene in a multitude of tumor types. Additionally, *RAS* mutant tumors are amongst the most aggressive and refractory to treatment. While directly targeting the *RAS* oncogene has proven challenging, an alternative approach for treating *RAS*-driven cancers is to inhibit critical downstream events that are required for tumor maintenance. Indeed, much focus has been put on inhibiting signaling cascades downstream of *RAS*. Recent studies have shown that oncogenic *RAS* promotes a metabolic reprogramming of tumor cells, shifting them towards an anabolic metabolism necessary to produce biomass to support unconstrained proliferation. These cancers also use a diverse set of fuel sources to meet their metabolic needs and have even developed a variety of mechanisms to act as metabolic scavengers to obtain necessary metabolic substrates from both extracellular and intracellular sources. Collectively, these adaptations can create “metabolic bottlenecks” whereby tumor cells rely on particular pathways or rate-limiting metabolites. In this regard, inhibiting individual or combinations of these metabolic pathways can attenuate growth in preclinical models. Because these dependencies are tumor selective and downstream of oncogenic *RAS*, there is the opportunity for therapeutic intervention. While targeting tumor metabolism is still in the early days of translation to patients, our continued advances in understanding critical metabolic adaptations in *RAS*-driven cancers as well as the ability to study this altered metabolism in relevant tumor models, will accelerate the development of new therapeutic approaches.

Introduction

The *RAS* oncogene has been the intense subject of study for the past few decades upon the identification of its potent transforming potential (reviewed in 1–3). Indeed, the three *RAS* genes (*HRAS*, *NRAS* and *KRAS*) are the most frequently mutated oncogenes in cancer, with *KRAS* being the most prevalent. *RAS* proteins serve as intracellular signaling molecules that transduce extracellular signals from receptor tyrosine kinases to downstream effector cascades. While much of the early work had focused on the signal transduction related to proliferation, it is now understood that oncogenic *RAS* has wide ranging effects on a cell

Corresponding Author: Alec C. Kimmelman, Dana-Farber Cancer Institute, 450 Brookline Avenue, HIM 221, Boston, MA 02215. alec_kimmelman@dfci.harvard.edu.

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that drives neoplastic transformation. Indeed, the oncogenic effects of the *RAS* oncogene are diverse and touch most, if not all, of the hallmarks of cancer (4). While there has been a resurgence in efforts to directly target mutant *RAS* genes (in particular, the highly prevalent *KRAS* oncogene), other approaches include targeting combinations of downstream effector pathways that are essential for tumor maintenance (5–10). While progress is being made on both fronts, a potent *RAS* inhibitor has proven difficult to develop and the approach of inhibiting downstream effectors while showing promise in preclinical studies, is hampered by the fact that there are multiple downstream signaling pathways that are activated which may change dynamically under various conditions as well as differ in the context of particular tumor types.

Recently, it has become apparent that the *RAS* oncogene has yet another crucial role in tumorigenesis in that it orchestrates a metabolic reprogramming of tumors. Since many of these metabolic changes are critical for tumor growth and are dependent on the presence of oncogenic *RAS*, there are opportunities for a favorable therapeutic index. Moreover, the catalytic nature of the enzymes involved in metabolic pathways can be amenable to targeting using small molecules.

The purpose of this review is to discuss the emerging role of the *RAS* oncogene as a driver of metabolic changes in tumors. It is not meant to be a comprehensive review of tumor metabolism. Rather, it will focus on recent work illustrating that oncogenic *RAS* can promote a variety of metabolic dependencies in tumor cells and highlight how some of these adaptations may be attractive therapeutic targets. In particular, the focus will be on *KRAS* as this is the most commonly mutated *RAS* gene in human cancer.

Tumor Metabolism

The altered metabolism of tumors has been appreciated for many decades (11,12). Indeed, it is clear that a cancer cell with the potential for unconstrained proliferation, has distinct metabolic requirements from its normal counterpart. One key difference is the need to increase biomass, which is critical for a tumor cell that undergoes rapid cell division. Thus, there is a concerted shift to anabolic metabolism to produce the building blocks required to accomplish this (13,14). Additionally, tumor cells use diverse fuel sources to produce ATP as well as maintain high levels of biosynthesis which can serve to promote survival in the potentially austere tumor microenvironment. This flexibility of fuel sources is yet another adaptation of tumor cells. One of the most well studied metabolic changes seen in many tumors is the high rate of aerobic glycolysis – also known as the Warburg effect (15). Glycolytic intermediates are shunted to anabolic pathways such as the pentose phosphate pathway. In addition, mitochondrial metabolism has been shown to be critical for the growth of many tumor types with the tricarboxylic acid (TCA) cycle used to produce precursors for biosynthetic reactions and oxidative phosphorylation responsible for the majority of ATP production (16,17). Because of the altered metabolism being shifted towards biosynthetic processes, certain metabolites become particularly critical and in some cases are rate-limiting for growth. One such example is NADPH, which is essential for reductive biosynthesis as well as for maintaining pools of reduced glutathione (GSH) to ensure proper redox homeostasis (18). These altered metabolic requirements of tumor cells make them

distinct from their normal counterparts and provide the possibility of a therapeutic index. However, a particular concern is that rapidly dividing cells may have overlapping metabolic requirements with tumor cells.

Ras and Metabolic Scavenging Pathways

Given the increased metabolic requirements of tumor cells, it is not surprising that certain tumor types have developed mechanisms to scavenge nutrients from both extracellular and intracellular sources. In particular, *RAS*-driven cancers have several different metabolic adaptations that allow them to recycle various metabolites. This serves two key purposes; 1) it provides metabolic flexibility and efficiency; and 2) it ensures adequate availability of biosynthetic precursors. Importantly, these scavenging pathways have become critical to the metabolism of these cancers and may provide therapeutic opportunities.

Macroautophagy (referred hereafter as autophagy) is a catabolic process whereby a cell can degrade its intracellular components to support survival under stressful conditions (reviewed in 19–23). This highly regulated process begins with cargo (damaged proteins, organelles, bulk cytoplasm) being sequestered in double-membraned vesicles called autophagosomes. These fuse to the lysosome, forming structures called autolysosomes, where the contents are degraded and the degraded products are recycled back into the cytosol where they can be used in a variety of bioenergetic and anabolic reactions (Fig. 1). While the process was initially thought to be a non-selective bulk degradation pathway, it has become apparent recently, that there are also various forms of selective autophagy whereby specific cargo are brought to the autophagosome via autophagy receptors (24–26). Because of the diverse nature of what is degraded, it is evident that autophagy can provide substrates for multiple aspects of cell metabolism. This includes nucleosides, fatty acids, amino acids, sugars, as well as critical elements such as iron (27,28).

While autophagy is present in all tissues at low levels for homeostatic functions, work from my lab as well as several groups identified that autophagy is elevated in *RAS*-driven cancers and is critical for tumor growth (29–32). Indeed, inhibition of autophagy either pharmacologically with chloroquine (CQ) or its derivative hydroxychloroquine (HCQ), as well as genetically, demonstrated responses in cancers using in vitro and in vivo systems. This includes *Kras*-driven autochthonous lung and pancreatic cancer models (29,33–37). One of the common observations in these studies is that autophagy loss results in a metabolic dysfunction. In particular, mitochondrial metabolism is significantly impacted upon autophagy inhibition. Because CQ and HCQ have been used in patients for decades for a variety of indications, testing this therapeutic approach in patients is feasible and already underway. These agents act on the lysosome by inhibiting lysosomal acidification (19) (Fig. 1). Therefore, they inhibit the late stages of autophagy at the level of autophagosome degradation. The inhibition of the lysosome likely has other cellular effects, some of which may also be anti-tumorigenic (see discussion of macropinocytosis below).

There are currently more than 30 trials open using HCQ in various cancers (clinicaltrials.gov). Cancers with a high frequency of *KRAS* mutations such as pancreatic cancers (ductal adenocarcinoma) (>90%), lung (~30%) and colon adenocarcinomas (~40%)

(1) are included in these trials. There have been some early reports on the efficacy of HCQ in various cancer types with mixed results and several key questions remain (38–43). One issue with these results is that it is not clear how effectively HCQ is inhibiting autophagy in patient tumors. This is a complicated issue for several reasons. First, measuring autophagy is complex (44) and it is nearly impossible to make the dynamic measurements (autophagic flux) one would need to in resected tumor specimens. Measuring flux is not possible in fixed biopsy tissue and the surrogate markers used are static and may not accurately demonstrate autophagy inhibition. Additionally, the majority of trials use combinations of various chemotherapeutics and targeted agents with HCQ. While defining the most efficacious combinations will be critical, many of the other drugs activate autophagy as a reactive survival mechanism and therefore the measurements to determine inhibition may be difficult to interpret (19). It should be noted that the pharmacological properties of HCQ are not optimal and micromolar levels are required to inhibit autophagy and to see responses in model systems. While this may be possible to achieve in vivo, it is not ideal (45,46). There are multiple other potentially targetable proteins that are critical to autophagy (kinases such as ULK1 and ULK2; E1-like proteins such as ATG7; proteases such as ATG4b) (47) and many pharmaceutical companies are pursuing such targets. While having more potent and specific autophagy inhibitors will be exciting for the field, there are potential benefits to drugs targeting the lysosome. Indeed, in addition to the other pathways impacted by blocking lysosomal function, inhibiting the later stages of autophagy results in an accumulation of cargo filled autophagosomes. It is possible that this buildup may increase toxicity in cancer cells. Inhibition of proximal aspects of the autophagy pathway will abrogate autophagosome formation, which may have distinct cellular effects. The question still remains regarding how tolerable it will be to significantly inhibit autophagy in patients. As it is likely that HCQ is not completely abrogating autophagy, this question remains unanswered in patients. However, a recent study acutely deleted *Atg7* in adult mice and showed that despite a complete blockade of autophagy, mice were viable for several months before they succumbed to sequelae of autophagy loss (34). Thus intermittent dosing of effective autophagy inhibitors may be tolerated in patients.

In a related manner, *RAS*-driven cancers also have developed the ability to utilize extracellular fuel sources. Pioneering work in the 1980's established that expression of the *RAS* oncogene can promote a process called macropinocytosis (48). During this endocytic process, membrane protrusions allow the engulfment of large portions of the extracellular space into large vesicles called macropinosomes. These go through a maturation process and eventually fuse with lysosomes (reviewed in 49,50) (Fig 1). Recent work has shown that *RAS*-transformed cells as well as tumor cells with oncogenic *KRAS* require macropinocytosis for growth (51). Tumor cells can take up extracellular albumin via macropinocytosis and degrade it into amino acids which can then enter the TCA cycle. Although many of the proteins involved in the control of macropinocytosis have yet to be identified, this process can be inhibited by the drug EIPA (ethylisopropylamiloride) which blocks Na^+/H^+ exchangers (52). Treatment of *KRAS* mutant pancreatic cancer xenografts with EIPA decreased their growth, while *Kras* WT tumors were not impacted (51). In addition to albumin, *RAS*-transformed cells have been shown to take up exogenous lipids to provide cells with fatty acids, thereby decreasing the need for de novo synthesis (53).

Whether this uptake is occurring by macropinocytosis is still not yet clear. While specific and potent macropinocytosis inhibitors are not being utilized clinically, it is likely that lysosome inhibitors such as HCQ will inhibit both autophagy and macropinocytosis. Therefore, there is rationale that inhibiting scavenging at the level of the lysosome may have increased anti-tumor effects in *RAS*-driven cancers that rely both on autophagy and macropinocytosis to provide critical fuel sources. Another potential therapeutic approach is to actually harness macropinocytosis to deliver a therapeutic payload. Indeed, it is tempting to speculate that the recent clinical success seen with the drug nab-paclitaxel (paclitaxel conjugated to albumin) in pancreatic cancer patients (54) may be in part due to increased uptake of the albumin conjugate by macropinocytosis.

Ras Promotes Shifts in Anabolic Metabolism

Recently, advances in tumor models have allowed for a detailed assessment of what factors are required for tumor maintenance. Indeed, inducible oncogenic *Kras* alleles have been used to explore these issues in pancreatic cancer (55,56). While there are certainly many facets that mutant *KRAS* contributes to in order to sustain tumor growth, my group and others have shown that one of its prominent roles is to reprogram aspects of central carbon metabolism (55,57–59). In pancreatic cancer cell lines and genetically engineered mouse models oncogenic *Kras* expression increases glycolysis, but it also causes the shunting of glycolytic intermediates to specific anabolic pathways (Fig. 2A). One of the major mechanisms by which mutant *Kras* rewires metabolism is through regulating the expression of rate limiting metabolic enzymes. In the case of glucose metabolism, it was demonstrated that *Kras* activates this transcriptional program through the activation of the Raf/Mek/Erk pathway leading to upregulation of the Myc transcription factor (55). One of the pathways that is upregulated by oncogenic *Kras* is the hexosamine biosynthesis pathway (HBP). The HBP produces precursors needed for glycosylation, a critical post-translational modification that has key roles in tumorigenesis (60). Inhibition of the *Kras*-regulated, rate limiting HBP enzyme Gfpt1 results in decreased overall cellular glycosylation levels in pancreatic cancer cells as well as attenuated growth in vitro and in vivo. Another key finding of the glucose rewiring was that oncogenic *Kras* shunted glucose-derived metabolites to the non-oxidative arm of the pentose phosphate pathway (PPP) to promote ribose biosynthesis, while not impacting flux to the oxidative arm (55). Thus, *Kras* decouples nucleotide biosynthesis using ribose produced via the non-oxidative arm from the NADPH production by the oxidative arm. Inhibition of *Rpia* or *Rpe*, the *Kras*-regulated PPP enzymes, decreased incorporation of glucose-derived ribose into DNA/RNA and decreased pancreatic cancer growth in vitro and in vivo.

Another key metabolic role of oncogenic *KRAS* is to maintain redox balance and there are several mechanisms through which it can do this. One such aspect is its role in NADPH biosynthesis. NADPH has multiple critical roles and is particularly relevant for tumor growth. It is important for reductive biosynthesis for the production of fatty acids as well as in maintaining reduced glutathione pools for redox homeostasis (18). Given the above findings where *Kras* shunted glucose flux through the non-oxidative PPP, this suggested that alternative carbon sources were utilized to produce NADPH. Indeed in PDAC cell lines, the amino acid glutamine was shown to be the critical carbon source that allows *KRAS*-driven

PDAC to produce NADPH and maintain redox balance (57,58). In this case, oncogenic KRAS regulates flux through a novel pathway whereby glutamine-derived aspartate produced by the mitochondrial aspartate aminotransferase (GOT2) is converted to oxaloacetate by the cytosolic aspartate aminotransferase (GOT1). This is then converted to malate by malate dehydrogenase (MDH1). Malic enzyme (ME1) then produces NADPH and pyruvate using this malate (57,58) (Fig. 2B). The NADPH produced by this pathway is essential for redox balance in these cells. In this case, KRAS increases the expression of *GOT1* and represses the expression of glutamate dehydrogenase (*GLUD1*), shunting the flux of glutamine away from the canonical *GLUD1* pathway. The downstream signaling pathways involved in these expression changes are still under investigation. Importantly, inhibition of this pathway at any level creates a redox imbalance as evidenced by an increase in reactive oxygen species (ROS) and causes a significant inhibition of growth in vitro and in vivo in xenografts. Growth can be restored simply by correcting the redox imbalance, suggesting that the critical role of the NADPH generated by this pathway is to maintain reduced glutathione pools (57,58). Another mechanism whereby KRAS can promote redox balance in addition to its role in glutamine metabolism, is through its ability to upregulate NRF2, a transcription factor that plays a critical role in the antioxidant response (61). NRF2 controls the expression of multiple genes that result in ROS detoxification, including *ME1* (62–64). The precise mechanism by which the NRF2 response is integrated in the control of redox homeostasis with other aspects, such as glutamine metabolism, remains to be fully elucidated.

An important question also remains as to whether this novel glutamine pathway is active/required in all mutant *KRAS* tumors or is this specific to the context of *KRAS* mutations in pancreatic cancer. Recent unpublished data from my group suggests the latter whereby this may not be a global property of all tumor types that possess *KRAS* mutations. While more work needs to be done to assess the role of this pathway across a wide array of tumor types, this early work strongly supports the idea that metabolic dependencies are a product of both the genetic background (e.g., *KRAS* mutations) as well as the tissue of origin (A.C. Kimmelman; unpublished data).

Together, this work suggests several potential therapeutic approaches. While specific inhibitors are not available to the critical enzymes in the anabolic glucose metabolism discussed above, the fact that MEK/ERK pathway controls expression of several of these enzymes provides an opportunity to utilize MEK inhibitors to suppress key aspects of anabolic glucose metabolism (55). Indeed, these inhibitors are currently being tested in multiple clinical trials (65). Integrating these therapies in the context of their roles in decreasing nucleotide biosynthesis and production of glycosylation precursors, will require a more detailed understanding of these pathways in various *KRAS*-driven cancers and which combinations would be the most efficacious. Indeed, since decreased nucleotide pools would be expected to impact DNA repair, therapies that cause DNA damage such as radiation or various chemotherapies (cisplatin for example) would be predicted to work well in combination with MEK inhibitors. Moreover, given the critical role of both autophagy and macropinocytosis in providing fuel sources to the cell, particularly for the TCA cycle, a combination of a MEK inhibitor with HCQ would have strong scientific rationale.

In the area of glutamine metabolism, there are also possible therapeutic approaches that are suggested by the available data. As shown in pancreatic cancer, interruption of glutamine metabolism at any level of the KRAS-regulated pathway results in a growth inhibition due to a redox imbalance. Therefore, inhibiting glutamine metabolism in combination with therapies that increase ROS should be synergistic. This is supported by preclinical data in pancreatic cancer models showing a synergy with therapies that increase ROS and inhibitors of glutamine metabolism (57). While there are no selective inhibitors for GOT1, MDH1, or ME1, there are currently inhibitors to glutaminase (GLS1) that have just entered clinical trials (66). Moreover, therapies such as radiation treatment and various cytotoxic chemotherapies work in part via the creation of ROS and have the potential to be combined with inhibitors to aspects of glutamine metabolism. Whereas inhibition of GLS1, a very proximal enzyme in the utilization of the glutamine carbon skeleton, is currently feasible, it will be essential to determine how this may differ from inhibition of more distal aspects of glutamine metabolism, such as GOT1. This is particularly relevant as *KRAS*-driven tumor cells can potentially utilize autophagy and macropinocytosis to obtain glutamine, glutamate, and aspartate as well as other amino acids which can feed in further downstream in this glutamine pathway.

Conclusions/Critical Analysis

Because tumor cells have distinct metabolic requirements from their normal counterparts, they may be more reliant on specific pathways/fuel sources. This provides a unique opportunity for therapeutic targeting. Work in a variety of systems has demonstrated that the RAS oncogene plays a critical role in these metabolic shifts. There is still much work to be done to fully realize the potential of these approaches. First, it is likely that *RAS* mutations have tissue-specific effects on metabolism. This is due to the intrinsic metabolic wiring in the tissue of origin of a particular tumor and its interaction with oncogenic RAS. Together this may create distinct metabolic dependencies for *RAS* mutations in different tumor types and this needs to be explored in a systematic fashion. In a similar manner, *RAS* mutations act in the context of other alterations in tumors – namely other oncogenic events as well as deletion/mutation of a constellation of tumor suppressor genes. Indeed, the tumor suppressor p53 has been shown to have an impact on multiple facets of cellular metabolism (reviewed in 67–69). Therefore, it will be important to incorporate the tumor suppressor background when studying the impact of mutant RAS on metabolism. Another aspect that requires additional study will be how these RAS-dependent metabolic changes are altered *in vivo* in the tumor microenvironment. This includes areas of hypoxia, limited nutrients, as well as potential metabolic crosstalk between tumor and stromal cells. To understand these complex relationships will require the use of sophisticated autochthonous tumor models as well as the ability to perform metabolic tracing studies *in vivo*.

In terms of therapeutic targeting of altered metabolism, there are still several obstacles that need to be overcome. Despite the fact that the metabolic dependencies are created by RAS, there may be physiologic situations where they are also required in normal tissues. Thus, it could be difficult to ensure a reasonable therapeutic index. This will require thorough preclinical studies using knockout mice for metabolic enzymes as well as tool compounds as they become available. Additionally, we must identify adaptive responses of *RAS*-driven

cancers to inhibition of particular metabolic pathways which could promote therapeutic resistance. Many of the pathways of interest discussed above do not have inhibitors available at this point. Inhibitors that are available such as HCQ (autophagy and macropinocytosis) may have potency issues. We are still in the early days of targeting aberrant tumor metabolism and anticipate that more inhibitors will become available that will enable us to target relevant pathways. Understanding how best to integrate inhibitors to metabolic pathways with existing chemotherapeutic agents as well as determining the appropriate combination of metabolic pathways to target will be critical and will require important pre-clinical development. Even if one were to develop a potent and effective KRAS inhibitor, an important consideration is the development of resistance. Recent evidence has shown that when *Kras* is genetically ablated in Kras-dependent pancreatic cancer cells, there is a small population of resistant cells that remains dormant and is likely responsible for the ultimate relapse. Interestingly, these cells display distinct metabolic properties and are highly reliant on oxidative phosphorylation (70). Inhibition of oxidative phosphorylation decreased survival of these cells and decreased tumor recurrence. These results highlight the potential for integrating metabolic targeted therapies with other approaches to target KRAS.

This is an exciting time in the cancer biology field and the pace of discovery is rapid. The renewed excitement in targeting the RAS oncogenes, coupled with improvements in technology, brings hope that we will make significant progress. There have also been advancements in our understanding of how tumors driven by oncogenic Ras have altered metabolic needs as well as reliance on particular metabolic pathways. By identifying and inhibiting these metabolic dependencies that are created in Ras-mutant tumors we have an opportunity to develop effective and well tolerated therapies.

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Figure 1.

Metabolic scavenging pathways have critical roles in Ras-driven cancers. Autophagy can degrade intracellular cargo by sequestering it in autophagosomes. These fuse with lysosomes where the cargo is degraded and subsequently recycled back into the cytosol. Macropinocytosis is associated with membrane ruffling that can lead to the internalization of extracellular material such as albumin with subsequent lysosomal degradation. Both pathways can supply metabolic substrates for various metabolic pathways. Chloroquine (CQ) and hydroxychloroquine (HCQ) inhibit lysosomal acidification and therefore block the degradation of cargo in both pathways. Ethylisopropylamiloride (EIPA) inhibits macropinocytosis.

Figure 2.

Oncogenic Kras (KRAS^{*}) can create metabolic alterations in pancreatic tumor cells. (A). Activation of the of the RAF/MEK/ERK cascade leads to increased MYC levels which promotes increased expression of various enzymes involved in glucose metabolism (representative enzymes that are increased are indicated). This promotes increased glycolysis as well as increased flux of glucose derived carbon through anabolic pathways such as the non-oxidative arm of the pentose phosphate pathway (PPP) to produce ribose for DNA/RNA biosynthesis and the hexosamine biosynthesis pathway (HBP) to produce glycosylation precursors. Kras regulated pathways are depicted in red and representative enzymes upregulated by oncogenic Kras are indicated for each pathway. The oxidative PPP (in purple) which can produce NADPH is not regulated by Kras. OAA (oxaloacetate); Asp (aspartate) (B). Kras causes a shift in glutamine metabolism in pancreatic cancers, through

an unknown downstream program, repressing GLUD1 and increasing GOT1 expression. This pathway is a major source of cytosolic NADPH in Kras mutant pancreatic cancer cells and is critical for redox balance. Both mitochondrial (the structure depicted is a mitochondria) and cytosolic enzymes are involved in this pathway.