KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer

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RAS genes (*HRAS*, *KRAS*, and *NRAS*) comprise the most frequently mutated oncogene family in human cancer. With the highest *RAS* mutation frequencies seen with the top three causes of cancer deaths in the United States (lung, colorectal, and pancreatic cancer), the development of anti-*RAS* therapies is a major priority for cancer research. Despite more than three decades of intense effort, no effective *RAS* inhibitors have yet to reach the cancer patient. With bitter lessons learned from past failures and with new ideas and strategies, there is renewed hope that undruggable *RAS* may finally be conquered. With the *KRAS* isoform mutated in 84% of all *RAS*-mutant cancers, we focus on *KRAS*. With a near 100% *KRAS* mutation frequency, pancreatic ductal adenocarcinoma (PDAC) is considered the most *RAS*-addicted of all cancers. We review the role of *KRAS* as a driver and therapeutic target in PDAC.

Mutationally activated *RAS* genes (*HRAS*, *KRAS*, and *NRAS*) comprise the most frequently mutated gene family in cancer (27%; Catalogue of Somatic Mutations in Cancer [COSMIC] v80). *KRAS* is the predominant isoform mutated in cancer and the isoform mutated exclusively in pancreatic ductal adenocarcinoma (PDAC). With *KRAS* mutations found in nearly all PDAC, this cancer type is arguably the most *RAS*-addicted cancer. With substantial experimental evidence that mutant *KRAS* is essential for PDAC growth, the National Cancer Institute identified targeting *KRAS* as one of four major priorities for pancreatic cancer research. The current standards of care for PDAC consist of conventional cytotoxic drugs (Wolfgang et al. 2013). Although effective targeted therapies are now available for lung and colorectal cancer, no

effective targeted therapies have been found for PDAC. With deaths caused by pancreatic cancer on the rise, the need for new therapies is now dire.

The *KRAS* small GTPase functions as a simple binary ON–OFF molecular switch, cycling between an active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound state (Vigil et al. 2010a). In normal quiescent cells, *RAS* is predominantly GDP-bound and inactive. Upon extracellular stimuli activation of receptor tyrosine kinases (RTKs) and other cell-surface receptors, there is rapid and transient formation of *RAS*-GTP, leading to engagement of effector proteins that then regulate a diversity of intracellular signaling networks (Cox and Der 2010) and thereby tightly control mitogenic processes. Cancer-associated *RAS* genes harbor missense mutations

Editors: Linda VanAelst, Julian Downward, and Frank McCormick

Additional Perspectives on Ras and Cancer in the 21st Century available at www.perspectivesinmedicine.org

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Cite this article as *Cold Spring Harb Perspect Med* 2018;8:a031435

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that encode single amino acid substitutions primarily (98%) at one of three mutational hot spots: glycine-12 (G12), glycine-13 (G13), or glutamine-61 (Q61). These mutations render RAS persistently GTP-bound and constitutively active independent of extracellular stimuli, resulting in overstimulation of effector signaling pathways to drive cancer growth. Thus, by analogy to the successful development of clinically effective adenosine triphosphate (ATP)-competitive inhibitors for protein kinases, small molecule GTP antagonists should provide a straightforward strategy to target mutant RAS. However, with picomolar affinity for GTP, and with millimolar GTP cellular concentrations, this approach has not been feasible. Furthermore, when the structure of RAS was determined, it did not reveal a surface topology amenable to the design of high-affinity small-molecule antagonists, deterring efforts to develop direct RAS inhibitors. Consequently, much of the past and current efforts have centered on indirect strategies. However, recent success in the identification of direct RAS-binding small molecules has fueled excitement that perhaps RAS is druggable after all. In this review, we first provide an overview of the role of KRAS in PDAC. We provide a snapshot of past and ongoing efforts and direct and indirect strategies to develop the long elusive anti-RAS drug for cancer treatment. We then focus on the development of inhibitors of KRAS effector signaling.

KRAS MUTATION AND PANCREATIC CANCER TUMORIGENESIS

The three RAS genes encode four 188–189 amino acid proteins that share 82%–90% amino acid sequence identity and share near-identical structural and biochemical properties (Fig. 1A). However, they are differentially expressed and mutated with different frequencies in cancer (Prior et al. 2012; Cox et al. 2014). KRAS is the predominant mutated RAS gene in cancers (84% of all RAS missense mutations), followed by NRAS (12%), with HRAS rarely mutated (4%) (COSMIC v80) (Fig. 1B).

Significant cancer type preferences exist among the RAS genes (Cox et al. 2014). KRAS

mutations predominate in lung, colorectal, and pancreatic cancer, whereas NRAS mutations predominate in cutaneous melanomas and acute myelogenous leukemia, and HRAS mutations are found in bladder and head and neck squamous cell carcinomas. Although already known in 1988 (Almoguera et al. 1988), subsequent comprehensive exome-wide deep sequencing verified that KRAS is mutationally activated in 94% of PDAC (Fig. 2A) (Jones et al. 2008; Biankin et al. 2012; Sausen et al. 2015; Waddell et al. 2015; Witkiewicz et al. 2015). These studies also verified an already well-established portrait of PDAC in which there are four major genetic alterations associated with the initiation and progression of PDAC, with the majority of gene alterations found in >10% of PDAC. In addition to KRAS, the TP53 (64%), SMAD4 (21%), and CDKN2A (17%) tumor suppressor genes are significantly mutated (Fig. 2A). Missense mutations and intragenic or homozygous deletion mutations and promoter methylation of CDKN2A (encoding p16INK4A and p19ARF), together with promoter silencing, result in a near universal loss of CDKN2A function in PDAC (Schutte et al. 1997).

KRAS mutation is the initiating genetic event for PDAC. The progression of normal pancreatic tissue to PDAC involves a stepwise genetic transition projected to span 12 years (Iacobuzio-Donahue et al. 2012). Most commonly, pancreatic duct epithelium transitions to advancing stages of noninvasive microscopic ductal lesions, or pancreatic intraepithelial neoplasms (PanINs). In early-stage PanIN formation, flat pancreatic epithelial cells take on a cuboidal appearance, increase mucin production, and acquire atypical cytological and morphological features (Hruban et al. 2000, 2004; Distler et al. 2014). High-grade PanINs are usually characterized by a papillary morphology (Distler et al. 2014). Greater than 90% frequency of KRAS mutations are found in PanIN-1 lesions (Kanda et al. 2012).

Genetically engineered mouse models also support the initiating role of KRAS mutation in PDAC and additionally support the cooperating role of subsequent tumor suppressor loss in facilitating KRAS-driven progression to the malignant disease (Gopinathan et al. 2015; Lee

Figure 1. RAS proteins. (A) Sequence identity of human RAS proteins. Amino acid sequence identity was determined by CLUSTALW multiple sequence alignment. (B) RAS mutation frequencies. Data were compiled from Catalogue of Somatic Mutations in Cancer (COSMIC) v80. (C) RAS domains. The amino-terminal amino acids (1–164) comprise the G domain involved in guanosine triphosphate (GTP) binding and hydrolysis and interaction with guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and effectors. RAS protein structure changes in the Switch I (SI; amino acids 30–38) and II (SII; amino acids 60–76) regions during guanosine diphosphate (GDP)–GTP cycling, with the GTP-bound form having higher affinity for effectors. Circled cysteine amino acids indicate sites of covalent modification by addition of a palmitate fatty acid. The boxed serine residue is phosphorylated by protein kinase C. The underlined lysine residues promote membrane targeting. The italicized cysteines in the CAAX motif are sites of covalent modification by addition of a farnesyl isoprenoid and carboxylmethylation.

et al. 2016). *Kras*^{G12D} mutation alone resulted in PanIN formation and infrequent and protracted onset of PDAC (Aguirre et al. 2003; Hingorani et al. 2003). When coupled with mutational inactivation of *Tp53* (*Tp53*^{R175H}) (Hingorani et al. 2005), *Cdkn2a* (Aguirre et al. 2003; Bardeesy et al. 2006), or *Smad4* (Kojima et al. 2007), PanIN formation was accelerated, progressing to rapid and high-frequency occurrence of metastatic PDAC.

It is currently not clear why PDAC is associated exclusively with KRAS mutations. One explanation for the isoform specificity of RAS-driven cancers could be that certain tissues are exposed to different carcinogens and environmental insults that lead to mutations in specific RAS genes. An alternative and intriguing possibility is that each isoform has specific functional properties that lead to these tissue preferences. One proposed basis for why KRAS is the pre-

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Figure 2. *KRAS* mutations in pancreatic cancer. (A) Frequency of mutations in the four major genes in pancreatic cancer. (B) Genetic progression of pancreatic cancer. (C) *KRAS* amino acid substitutions in pancreatic cancer. (Compiled from data in Jones et al. 2008, Biankin et al. 2012, Sausen et al. 2015, Waddell et al. 2015, and Witkiewicz et al. 2015.)

ferred cancer isoform is protein expression levels. Although nearly identical on the amino acid level, *KRAS*, *NRAS*, and *HRAS* differ in the frequency of rare codon utilization, with the high frequency in *KRAS* contributing to a reduced rate of translation and lower RAS protein expression (Lampson et al. 2013). Elevated ectopic expression of mutant RAS in normal cells induces senescence rather than cell proliferation. Concurrent suppression of p53 function prevents senescence, allowing RAS-driven growth enhancement. With *KRAS* mutations, protein expression levels are hypothesized to be sufficiently high for tumor initiation but low enough to evade apoptosis and senescence. In contrast, mutant *HRAS* protein expression levels are high enough to elicit an apoptotic or senescent response. In support of this hypothesis, in vivo studies that show enriching the *KRAS* codons with more frequently translated *HRAS* codons, even though the amino acids are the same, resulted in reduced *Kras*-induced tumor formation in mice (Pershing et al. 2015).

Another unresolved issue is the role of the remaining *KRAS* wild-type (WT) allele in *KRAS*-mutant PDAC. There is evidence that suggests that the WT allele serves a paradoxical role as a tumor suppressor, with loss of the WT allele facilitating PDAC progression (Qiu et al. 2011). When evaluated in a *Kras*^{G12D}-driven mouse model of PDAC, loss of the WT allele was associated with progression to metastatic disease. Loss of the *KRAS* WT allele is also seen in human PDAC. In contrast, a driver role for the remaining WT isoforms, *HRAS* and *NRAS*, has also been observed in *KRAS*-mutant pancreatic cell lines (Lim et al. 2008; Grabocka et al. 2014). However, whether their roles are simply quantitative or qualitatively distinct remains to be determined.

In addition to tissue-based isoform specificity, the frequency of different *KRAS* missense mutations can vary strikingly in different cancer types. For example, G12C mutations are rare in PDAC (1% of all *KRAS* mutations) but are the major *KRAS* mutation in lung adenocarcinoma

(LAC) (43% of all *KRAS* mutations) (Fig. 2C) (Cox et al. 2014; Stephen et al. 2014; Hobbs et al. 2016). In contrast, G12R mutations are infrequently seen overall in cancer (<3%), yet comprise 16% of all *KRAS* mutations in PDAC. It is likely these different frequencies in *KRAS* mutations reflect, in part, distinct roles in cancer development and growth. Until recently, it was assumed that the different mutations cause essentially identical consequences on RAS function; clearly this is not the case, as it was recently shown that there are altered nucleotide exchange kinetics and effector binding abilities of different mutant *KRAS* proteins (Smith et al. 2013; Hunter et al. 2015). Thus, a shift away from the long-standing “all RAS mutations are created equal” paradigm has emerged, in which the study of specific RAS mutants is being pursued in search of mutation-selective functional vulnerabilities that can be exploited by different therapeutic strategies. The impact of this shift is evidenced by the mutation-specific inhibitors that have been developed to target G12C-mutant *KRAS* in LAC (Ostrem et al. 2013; Lim et al. 2014). There is limited evidence that mutant specificity also plays an important role in pancreatic cancer prognosis. One study suggests that *KRAS* mutations are associated with more aggressive cancers and a shorter survival time (Bournet et al. 2016). Another found that *KRAS* Q61 mutations were associated with improved survival compared with *KRAS* G12 mutant patients (Witkiewicz et al. 2015).

PANCREATIC DUCTAL ADENOCARCINOMA CHEMOTHERAPY

In 2016, pancreatic cancer surpassed breast cancer and became the third leading cause of cancer deaths in the United States (Siegel et al. 2016). With a continued increase in incidence, pancreatic cancer is projected to become the second leading cause of cancer death by around 2020 (Rahib et al. 2014). With no biomarkers for early detection, the deadly nature of PDAC is largely the result of late onset of symptoms when the cancer has already reached the metastatic state. The 5-year survival rate of pancreatic cancer is 8% (Siegel et al. 2016), giving this cancer the

dubious distinction of being the deadliest cancer in the United States.

Surgery, followed by adjuvant chemotherapy, is the only potentially curative treatment for PDAC, but only 15%–20% of patients are candidates for surgery. Chemotherapy for PDAC involves conventional cytotoxic drug combinations. The only targeted therapy approved for the treatment of PDAC, the epidermal growth factor receptor (EGFR) inhibitor erlotinib, when used in combination with gemcitabine, extended life by a statistically significant but clinically unimpressive 12 days more than gemcitabine alone (Moore et al. 2007). Since 1997, gemcitabine was the standard of care for PDAC. In 2011, the FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin) chemotherapy regimen, an extremely toxic chemotherapy cocktail, was approved for PDAC. In 2013, the nab-paclitaxel (albumin-conjugated paclitaxel)-gemcitabine combination was found to increase life expectancy almost 2 months compared to gemcitabine and approved as a second standard of care for PDAC (Von Hoff et al. 2013). Therapeutic approaches as a whole have been largely unsuccessful in PDAC, with no treatment extending life longer than 1 year from diagnosis.

Although *KRAS* mutations are the initiating genetic step, continued mutant *KRAS* function is still required to maintain the growth of metastatic PDAC. RNA suppression studies in PDAC cell lines showed the strong requirement for tumorigenic growth (Brummelkamp et al. 2002; Lim and Counter 2005). *Kras* inactivation in *Kras*^{G12D}-driven PDAC, in the continued presence of a *Tp53* deficiency, showed rapid regression of primary (Collins et al. 2012a; Ying et al. 2012) and metastatic (Collins et al. 2012b) tumor growth. These studies support the significance of mutant *KRAS* as a therapeutic target in PDAC. Finally, although one study suggested that only a subset of *KRAS*-mutant PDAC cell lines were addicted to mutant *KRAS* (Singh et al. 2009), a subsequent study determined that all *KRAS*-mutant PDAC cell lines showed significant growth impairment on acute or sustained *KRAS* suppression (Hayes et al. 2016). The different conclusions from these two studies reflect the specific definition of “addiction” applied in each study.

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Thus, whereas there will likely exist intertumor variability in the specific mutant KRAS-driven cellular phenotypes, all KRAS-mutant PDAC will likely display KRAS addiction.

Although clearly a validated therapeutic target, KRAS-targeted therapies will also be subject to the limitations seen with targeted and conventional chemotherapy: the development of cancer-cell resistance. One mechanism by which PDAC loses its addiction to KRAS is through activation and expression of the Hippo pathway component YAP1 (Kapoor et al. 2014; Shao et al. 2014).

TARGETING KRAS: DO EFFECTOR INHIBITORS HOLD THE GREATEST PROMISE?

The main past and current strategies for developing therapeutics to block mutant KRAS function have focused on indirect approaches: to target proteins that support KRAS function and promote KRAS-driven cancer growth (Papke and Der 2017). These approaches have centered on targeting (1) proteins that promote KRAS association with the plasma membrane, (2) KRAS effector signaling, (3) components that support KRAS-dependent metabolic processes, and (4) synthetic lethal interactors critical for the growth of mutant but not WT KRAS cancer cells. More recently, RAS-binding small molecules that disrupt KRAS function have been identified, raising hope that RAS can be targeted directly. An ambitious goal of pancreatic cancer research is to double the rate of survival by the year 2020. With this urgency at hand, of these approaches, arguably inhibitors of KRAS effector signaling hold the greatest promise for the most immediate transition to the PDAC patient within the next several years. Therefore, for this review, we have focused on the development of inhibitors of KRAS effector signaling.

KRAS-GTP binds preferentially to downstream effectors that regulate a complex diversity of cytoplasmic signaling networks. The majority of effectors are characterized by the presence of a RAS-binding domain (RBD) or RAS association (RA) domain (Fig. 3). There are at least 11 different effector families with distinct catalytic

functions. It is likely that KRAS drives tumorigenesis by the integrated result of multiple effector signaling pathways, with multiple KRAS effector pathways contributing to the tumor etiology. Thus, the concept of targeting KRAS effector signaling is not straightforward. Which effector pathway(s) are the best to target? Will concurrent inhibition of multiple effectors be required?

There is substantial experimental evidence from cell culture and mouse model studies that validate driver roles for components of four KRAS effector signaling networks in cancer (Cox et al. 2014). However, some cautious interpretation is important when assessing the therapeutic value of effectors ablated by pharmacologic inhibitors. First, a majority of studies use genetic approaches to ablate effector expression and loss of protein expression, which may not accurately model target inhibition by pharmacologic approaches. Second, genetically engineered mouse model studies typically involve ablation of effector expression concurrently with induced expression of mutant RAS. Hence, they assess the role of effectors in tumor initiation and progression, and not maintenance of an already established metastatic PDAC. With these caveats in mind, we summarize the experimental evidence validating the therapeutic potential of targeting different effector signaling components, with a focus on the RAF effector signaling network.

The RACGEF-RAC1 Small GTPase Effector Signaling Network

The least validated and therapeutically tractable KRAS effector pathway implicated in oncogenesis is the RACGEF-RAC1 pathway (Fig. 3). Early studies in HRAS-transformed rodent fibroblasts provided the first indication of the role of RAC1 in RAS oncogenic functions (Bar-Sagi and Feramisco 1986; Khosravi-Far et al. 1995; Qiu et al. 1995). The subsequent identification of RAC1 missense mutations (P29S), primarily in cutaneous melanomas, provided strong evidence for a driver role for RAC1 activation in cancer (Hodis et al. 2012; Krauthammer et al. 2012). Further, *Rac1*-deficient mice exhibited

Figure 3. KRAS guanosine diphosphate (GDP)–guanosine triphosphate (GTP) regulation and effector signaling. RAS-selective guanine nucleotide exchange factors (GEFs) and GTP-activating proteins (GAPs) regulate GDP–GTP cycling. Cancer associated mutations, found primarily (>99%) at residues G12, G13, or Q61 disrupt GDP–GTP cycling by impairing intrinsic and GAP-stimulated GTP hydrolysis and/or accelerating GDP–GTP exchange. KRAS effectors are characterized by a RAS-binding domain (RBD) or RAS association (RA) domains. AKT1 phosphorylates and inactivates GSK3 β , preventing MYC phosphorylation at T58 and FBW7-mediated polyubiquitination and degradation.

reduced Kras^{G12D}-driven pancreatic tumor formation and increased survival (Heid et al. 2011). However, constitutively active Rac1^{G12V} alone was not sufficient to induce PanIN and PDAC formation (Eser et al. 2013).

One RACGEF, TIAM1, has been identified as a RAS effector (Lambert et al. 2002). Like the majority of KRAS effectors, TIAM1 contains an RBD that facilitates association with RAS-GTP. The importance of TIAM1 in RAS-driven tumorigenesis was shown by the impaired onset of Hras-driven skin carcinoma formation in *Tiam1*-deficient mice (Malliri et al. 2002). Phosphoinositide 3-kinase (PI3K) activation can also activate RACGEFs (e.g., PREX1/2). Although

tool compound inhibitors of RACGEF activation of RAC1 have been described (e.g., NSC 23766), it has been difficult to advance clinically effective and selective inhibitors for cancer therapy.

Like KRAS, activated RAC1-GTP can interact with a spectrum of catalytically distinct effectors. Although the effectors critical to facilitate RAC1 as a cancer driver remain unclear, there is experimental support for the PAK1 serine/threonine kinase as an important effector in cancer (Baker et al. 2014). A *Pak1* genetic deficiency or treatment with a pharmacologic inhibitor of Pak1 impaired Kras^{G12D}-driven lung tumor formation (Chow et al. 2012). Another effector that will likely contribute to RAC1-de-

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pendent cancer growth is the p100 β catalytic subunit of PI3K (Fritsch et al. 2013). Inhibitors of PAK1 and PI3K β are under preclinical and clinical development.

The RALGEF-RAL Small GTPase Effector Signaling Network

The next-best-validated RAS effector family is a family of four RA domain-containing RALGEFs (RALGDS, RGL, RGL2, and RGL3) (Fig. 3). RALGEFs act as guanine nucleotide exchange factors and activate RALA and RALB RAS-like small GTPases. Mice deficient in *Ralgds* showed impaired incidence of chemical carcinogen-induced *Hras* mutation and formation of squamous cell skin carcinomas (Gonzalez-Garcia et al. 2005). *Rala/Ralb*-deficient mice showed impaired *Kras*^{G12D}-induced lung tumors (Peschard et al. 2012). Our studies found RGL2 overexpression in PDAC cell lines and patient tumors (Vigil et al. 2010b). Biochemical inhibition of RALGEF function as well as genetic suppression of RGL2 expression reduced PDAC cell line steady-state RAL activity, growth in soft agar, and Matrigel invasion. Interestingly, RGL2 exhibited both RAL-dependent and -independent roles in PDAC growth.

Like RAS, RAL proteins are activated by exchanging GDP in their active site for GTP. Although components of these effector pathways are not found mutated in cancer, sustained hyperactivation of this pathway and elevated levels of RAL-GTP were found more frequently in PDAC than the two more classical KRAS effector pathways, ERK mitogen-activated protein kinase (MAPK) and PI3K (Lim et al. 2006).

Interestingly, despite their strong sequence and biochemical identity, RALA and RALB have distinct roles in PDAC growth. RALA is necessary for PDAC anchorage-independent growth in vitro and tumorigenic growth (Lim et al. 2005). In contrast, RALB is dispensable during tumor initiation but important for PDAC invasion and metastases (Lim et al. 2006).

Active RAL-GTP can interact with a diversity of catalytically distinct effectors, several with demonstrated roles in supporting RAL-dependent cancer growth (Gentry et al. 2014). Of

these, the best-characterized effectors are the EXO84 and SEC5 subunits of the octomeric exocyst complex that regulates vesicular trafficking. SEC5 can also act as a RALB effector independent of exocyst function, regulating the activity of the TBK1 serine/threonine kinase. Therefore, it was very intriguing when TBK1 was identified as a synthetic lethal interactor of mutant KRAS (Barbie et al. 2009). However, subsequent studies found that TBK1 function was not tightly linked with RAS-mutant cancer (Muvaffak et al. 2014). Another effector implicated in RALB-dependent PDAC invasion is RALBP1/RLIP76 (Neel et al. 2012), a multidomain protein that can act as a GTP-activating protein (GAP) for the Cdc42 and RAC1 RHO family small GTPases.

The PI3K-AKT-Mechanistic Target of Rapamycin (mTOR) Effector Signaling Network

There is substantial experimental evidence supporting the critical role of the catalytic subunits of class I PI3K (p110 $\alpha/\delta/\gamma$) as critical effectors of mutant KRAS-driven oncogenesis (Fig. 3) (Castellano and Downward 2011). The p110 subunits contain amino-terminal RBDs and RAS-GTP binding promotes PI3K association with the cell membrane and additionally relieves autoinhibition. Mice harboring a *Pik3ca* locus that encoded a p110 α variant with RBD mutations that impaired RAS-GTP binding showed strikingly impaired incidence of mutant *Kras*^{G12D}-driven lung tumor or mutant *Hras*-induced skin tumor initiation and maintenance (Gupta et al. 2007; Castellano et al. 2013).

The important driver role for PI3K is supported by frequent activating *PIK3CA* mutations in cancer (10% all cancers; COSMIC v80). However, two studies reached different conclusions regarding the sufficiency of PI3K activation in driving PDAC development. One study found that the cancer-associated constitutively activated *Pik3ca*^{H1047R} mutant alone could not drive PanIN or PDAC development (Collisson et al. 2012). In contrast, a second study found that this mutant alone phenocopied *Kras*^{G12D} and induced metastatic PDAC (Eser et al. 2013). A

possible basis for these different findings may be the different pancreas cell types targeted.

PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) and stimulates formation of plasma membrane-associated phosphatidylinositol-3,4,5-bisphosphate (PIP3) (Castellano and Downward 2011). PIP3 can then activate a multitude of proteins that include RACGEFs, PDK1, and the AKT1/2/3 serine-threonine kinases. Activated AKT can phosphorylate many other proteins that can then promote cell growth. The PTEN lipid phosphatase and tumor suppressor negatively regulates the pathway by dephosphorylating PIP3 to PIP2 and is an important tumor suppressor in PDAC (Ying et al. 2016) and *PTEN* loss is found commonly in cancer. However, *PIK3CA* and *PTEN* mutations are rare in PDAC. Further, whole-exome deep-sequencing data of pancreatic cancer patients shows that 93% of the rare *PIK3CA* mutations co-occur with a *KRAS* mutation, suggesting activated *KRAS* alone is not sufficient to effectively activate PI3K (Jones et al. 2008; Biankin et al. 2012; Sausen et al. 2015; Waddell et al. 2015; Witkiewicz et al. 2015). Supporting a limited linkage between *KRAS* and PI3K, *KRAS* suppression did not alter AKT activation levels in a majority of *KRAS*-mutant PDAC cell lines (Hayes et al. 2016).

The RAF-MEK-ERK Signaling Network

The three RAF serine-threonine kinases (ARAF, BRAF, and CRAF/RAF1) are the most significant effectors of *KRAS*-driven PDAC (Fig. 4). The importance of the extracellular regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway in driving *KRAS*-dependent cancer growth is supported by the occurrence of *BRAF* mutations in cancer (18% all cancers; COSMIC v80). Although found infrequently in PDAC (<2%), *BRAF*-activating V600E mutations were mutually exclusive with *KRAS* mutations in PDAC (Witkiewicz et al. 2015). The widely studied BxPC-3 PDAC cell line, commonly used as a *KRAS* WT control in comparison with *KRAS*-mutant cell lines, harbors an in-frame genomic DNA deletion in *BRAF* that encodes a constitutively activated and transforming

BRAF protein with a five-amino-acid deletion (Chen et al. 2016; Foster et al. 2016). Database analyses found that similar *BRAF* deletions were found in 4%–5% of *KRAS* WT PDAC and were mutually exclusive of *BRAF* missense mutations. Hence, BxPC-3 cells are not bona fide *KRAS* WT PDAC cells in that a major *KRAS* effector pathway is chronically activated in this cell line. Unlike *BRAF*^{V600E}, *BRAF* deletion mutants were insensitive to the *BRAF*-selective inhibitor vemurafenib but sensitive to the pan-RAF inhibitor LY3009120.

Importantly, the predominant cancer-associated constitutive-activated *Braf*^{V600E} mutant phenocopied *Kras*^{G12D} and initiated PanIN formation in the pancreas of *KRAS* WT mice, and, when combined with a *Tp53* mutation, resulted in lethal PDAC (Collisson et al. 2012). Moreover, a genetic deficiency in both *Mek1* and *Mek2* or both *Erk1* and *Erk2* blocked *Kras*^{G12D}-driven mouse lung development (Blasco et al. 2011). Interestingly, genetic ablation of *Raf1/Craf* but not *Braf* also impaired *Kras*^{G12D}-driven mouse lung tumor development, suggesting that *RAF1* is the critical RAF isoform involved in *Kras*^{G12D}-induced lung tumor formation (Blasco et al. 2011; Karreth et al. 2011). However, surprisingly, a *Raf1* deficiency did not impair *Kras*^{G12D}-induced PDAC formation and progression and did not improve mouse survival, indicating tissue-specific utilization of effectors in *KRAS*-driven PDAC initiation (Eser et al. 2013). These observations argue that the RAF-MEK-ERK cascade is the key effector in supporting *KRAS*-dependent tumor initiation, progression, and maintenance.

GTP-bound RAS interacts with the amino-terminal RBD of RAF, increasing accumulation at the plasma membrane and leading to activating phosphorylation events, conformational changes and relief of autoinhibition, and RAF homo-/heterodimerization, promoting RAF kinase activation (Morrison 2012; Freeman et al. 2013). RAF subsequently phosphorylates and activates the MEK1 and MEK2 dual specificity kinases, which then phosphorylate and activate the ERK1 and ERK2 serine/threonine kinases. Activated ERK1/2 then phosphorylate >200 cytoplasmic and nuclear substrates, many of

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Figure 4. Components of the three-tiered extracellular regulated kinase (ERK) mitogen-activated protein kinase (MAPK) cascade. Each level of the ERK MAPK cascade is comprised of highly related isoforms. *BRAF* missense or deletion mutants (shown is the deletion in the BxPC-1 cell line) are found in pancreatic ductal adenocarcinoma (PDAC). *RAF* isoforms are regulated by positive (green) and negative (red) regulatory phosphorylation activities. Shown are the phosphorylation sites that activate MEK1/2 or ERK1/2. Domain topology was determined in simple modular architecture research tool (SMART) (smart.embl-heidelberg.de). Total protein and kinase domain sequence identities are indicated (%%) as determined by CLUSTALW multiple sequence alignment. *, point mutation; Δ, deletion mutation.

which are transcription factors that regulate the expression of genes involved in mitogenic signaling (McKay and Morrison 2007; Shaul and Seger 2007). Because the only well-established substrates of *RAF* are MEK1/2, and ERK1/2 are the only well-defined substrates of MEK1/2, the *RAF*-MEK-ERK protein kinase cascade is commonly depicted as a simple linear, unidirectional signaling pathway. However, with inputs and outputs at each level, with feedback regulatory mechanisms, and with a spectrum of scaffolds that regulate signaling input and output, this three-tier protein kinase cascade is the core of a complex signaling network.

With a diversity of substrates, the key substrates important for ERK-dependent cancer growth remain poorly defined. An important growth-promoting ERK substrate is the tran-

scription factor MYC (Farrell and Sears 2014), an oncoprotein required for maintenance of RAS-driven tumors (Soucek et al. 2008). MYC protein levels are elevated in mouse and human PanIN and tumor tissue (Lin et al. 2013). *Myc* overexpression alone has been shown to drive PanIN and metastatic PDAC development. MYC protein is normally unstable with a short half-life (~15 min). ERK stabilizes MYC by phosphorylation at residue S62, preventing FBXW7 E3 ligase-mediated protein degradation driven by GSK3β phosphorylation of MYC at T58 (Fig. 3) (Farrell and Sears 2014), and MYC protein half-life is prolonged in PDAC cell lines (Farrell et al. 2014; Hayes et al. 2016). Sensitivity to pharmacologic inhibitors of ERK was associated with MYC protein loss in growth-inhibited but not resistant PDAC cell lines (Hayes et al.

2016). ERK MAPK-directed regulation of MYC also drives the expression of genes that support the elevated glucose metabolism in *Kras*-mutant mouse PDAC cells in vivo (Ying et al. 2012).

Excessive ERK activation can drive senescence and growth cessation. To maintain a mitogenic level of ERK activity, the ERK MAPK cascade is also dynamically regulated by negative feedback signaling mechanisms that modulate the level of ERK activity (Ryan et al. 2015). Multiple ERK substrates can negatively regulate upstream signaling components that dampen the signaling strength of the cascade at different levels. For example, ERK phosphorylates CRAF at multiple negative regulatory sites that decrease RAS-driven RAF dimerization (McKay and Morrison 2007).

Substantial cross talk between KRAS effector pathways exists. This is particularly evident with the RAF and PI3K effector signaling networks. Inhibition of ERK signaling can cause compensatory activation of PI3K-AKT-mTORC1 signaling (Collisson et al. 2012). ERK inhibitor treatment stimulated increased AKT activation in sensitive but not resistant PDAC cell lines, and concurrent inhibition of PI3K synergistically enhanced ERK inhibitor growth suppression. Conversely, inhibition of PI3K signaling increased ERK signaling (Soares et al. 2015). The compensatory interrelationship between RAF and PI3K signaling contributes to the synergistic growth suppression seen in *Kras*-driven cancer models with concurrent pharmacologic inhibition of these two effector pathways. However, largely as a result of toxicity issues, such combinations have not shown the same promising antitumor activities in the clinical evaluation of *KRAS*-mutant cancers.

THERAPEUTICALLY TARGETING THE RAF EFFECTOR PATHWAYS

Because the ERK MAPK pathway is hyperactivated through mutations in *RAS*, *BRAF*, *EGFR*, *NF1*, and other critical cancer drivers, there has been intensive effort by the pharmaceutical industry to develop inhibitors of ERK MAPK signaling. Currently, there are more than 30 inhibitors under clinical evaluation, with two BRAF-

selective (vemurafenib and dabrafenib) and two MEK1/2-selective inhibitors (trametinib and cobimetinib) approved for use in *BRAF*-mutant melanoma (Fig. 5). In this section, we summarize the current status of inhibitors of each level of the cascade for the treatment of *KRAS*-mutant PDAC.

RAF Inhibitors

The first-generation RAF inhibitors (vemurafenib and dabrafenib) are BRAF-selective and have been effective in a subset of *BRAF*-mutant cancers, in particular, melanoma. They have not been effective with *BRAF*-mutant colorectal carcinoma due to up-regulation of EGFR signaling (Corcoran et al. 2012). Although there are cancer-type differences in the sensitivity to BRAF-selective inhibitors, the infrequent *BRAF*^{V600F} mutant PDACs were responsive in vitro (Witkiewicz et al. 2015). However, PDAC with the atypical BRAF deletion mutants were surprisingly not responsive to vemurafenib (Peng et al. 2015).

In *RAS*-mutant cells, the BRAF-selective inhibitors cause paradoxical activation of ERK signaling (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010). This occurs through a mechanism involving RAS-GTP-mediated RAF dimerization, with the predominant dimer BRAF-CRAF heterodimers. BRAF but not CRAF is inhibited in the dimer, with the inactivated BRAF causing allosteric activation of the associated CRAF protein, leading to MEK-ERK activation. This reactivation of ERK MAPK signaling is associated with increased proliferation in cell culture and tumor formation in patients (Oberholzer et al. 2012; Su et al. 2012). Because *KRAS* mutation frequencies are found in PanIN-1 lesions (Witkiewicz et al. 2015), an obvious concern is that treatment with BRAF-selective inhibitors may accelerate PDAC development in *BRAF*-mutant cancer patients.

More recently developed second-generation RAF inhibitors are not limited by paradoxical activation. Pan-RAF inhibitors can overcome this paradoxical activation by blocking WT A-, B-, and CRAF in addition to BRAF V600E. Although dimer formation is induced with pan-RAF inhibitors, MEK activation is abrogated

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Figure 5. Clinical candidate inhibitors of the RAF-MEK-extracellular regulated kinase (ERK) mitogen-activated protein kinase (MAPK) cascade. (Compiled from data in www.clinicaltrials.gov.)

because each dimer molecule is inhibitor-bound and lacks kinase activity (Henry et al. 2015; Peng et al. 2015). The pan-RAF inhibitor LY3009120 was found to be active in *RAS*-mutant cancer cell lines, including *KRAS*-mutant PDAC. Several pan-RAF inhibitors are currently in phase I clinical trials. Another second-generation RAF inhibitor is the “paradox breaker” PLX8394 that also effectively inhibits CRAF and BRAF (Basile et al. 2014; Choi et al. 2014; Zhang et al. 2015). PLX8394 effectively blocked ERK activation and growth of *RAS*-mutant, vemurafenib-resistant melanoma cells.

MEK Inhibitors

MEK1/2-selective inhibitors have not shown potent activity in *RAS*-mutant cancers. This reduced potency is the result, in part, of vertical compensation mechanisms that lead to reactivation of ERK through up-regulation of upstream pathway components such as RTKs, BRAF, or *KRAS* (Little et al. 2011; Ryan et al. 2015). An additional basis of resistance involves MEK

inhibitor treatment-induced activation of the PI3K-AKT-mTOR pathway. Further, reactivation of phospho-ERK on MEK inhibition with trametinib has been reported owing to compensatory mechanisms that involve kinome reprogramming of parallel pathways (Ryan et al. 2015), but these resistance mechanisms are still poorly understood (Samatar and Poulikakos 2014). Cell-culture analyses found that only a small subset of *KRAS*-mutant PDAC cell lines are sensitive to MEK inhibitor treatment (Witkiewicz et al. 2015; Hayes et al. 2016). Trametinib did not show efficacy in clinical evaluation with *KRAS*-mutant PDAC (Infante et al. 2014; Kasuga et al. 2015; Tolcher et al. 2015).

ERK Inhibitors

Because both RAF- and MEK-selective inhibitors are limited, in part, by mechanisms that cause reactivation of ERK downstream from the inhibitor block point, a logical approach to overcome this involve inhibitors that directly target ERK. Analyses with the ERK1/2-selective

inhibitors SCH772984 or ulixertinib/BVD-523 found that ~50% of PDAC cell lines were sensitive (Morris et al. 2013; Ryan et al. 2015; Hayes et al. 2016). ERK inhibitor treatment also blocked tumorigenic growth in PDAC cell lines and PDX tumor mouse models. BVD-523 (NCT02608229) and LY3214996 (NCT02857270) are currently being evaluated in phase I clinical trials in PDAC in combination with nab-paclitaxel and gemcitabine.

Treatment-induced loss of MYC protein was seen in sensitive but not resistant cell lines, suggesting that ERK-independent mechanisms that prevent MYC degradation may be a mechanism of de novo resistance.

Concurrent Inhibition of the RAF and PI3K Effector Signaling Networks

Pharmacological inhibition of the PI3K pathway by using AKT inhibitors with single-agent strategies has produced negative results in vitro and in vivo, and activated PI3K cannot phenocopy mutant KRAS in PDAC mouse models. However, MEK inhibition leads to increased AKT signaling in vivo and combined inhibition of MEK and AKT resulted in robust synergistic inhibition of PDAC growth in vitro and in vivo (Collisson et al. 2012). Similarly, ERK inhibitor treatment also increased AKT activity in sensitive but not resistant PDAC cell lines, and cotreatment with a PI3K inhibitor synergistically enhanced ERK inhibitor-mediated growth suppression and caused apoptotic cell death (Hayes et al. 2016). Concurrent treatment with a PI3K inhibitor did not, however, overcome de novo resistance. Several completed or ongoing clinical trials have evaluated or are evaluating combinations of inhibitors of specific components of the RAF and PI3K effector pathways (www.clinicaltrials.gov).

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the failures in developing anti-KRAS therapies, targeting KRAS remains one of the most promising directions in pancreatic cancer research. Although multiple directions continue

to be pursued, with inhibitors already under clinical evaluation, targeting the key KRAS effector pathway—the RAF-MEK-ERK protein kinase cascade—arguably remains the most promising for short-term clinical success. However, the essential role of this pathway as a driver of KRAS-dependent PDAC growth has ensured that cancer cells have compensatory mechanisms that overcome the effectiveness of inhibitors when used as monotherapy. Instead, combination approaches will be needed to achieve a prolonged antitumor response that is less vulnerable to mechanisms of acquired resistance.

ACKNOWLEDGMENTS

We apologize to all colleagues whose work could not be cited because of space restrictions. Our research is funded by grants from the National Cancer Institute (CA042978, CA175747, CA199235, CA203657, and CA196510), the Department of Defense, the Pancreatic Cancer Action Network/American Association for Cancer Research, and the Lustgarten Foundation. The authors declare no financial interest.

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Cold Spring Harb Perspect Med 2018; doi: 10.1101/cshperspect.a031435 originally published online December 11, 2017

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