

# Therapeutic Strategies for Targeting Ras Proteins

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## Abstract

Ras genes are frequently activated in cancer. Attempts to develop drugs that target mutant Ras proteins have, so far, been unsuccessful. Tumors bearing these mutations, therefore, remain among the most difficult to treat. Most efforts to block activated Ras have focused on pathways downstream. Drugs that inhibit Raf kinase have shown clinical benefit in the treatment of malignant melanoma. However, these drugs have failed to show clinical benefit in Ras mutant tumors. It remains unclear to what extent Ras depends on Raf kinase for transforming activity, even though Raf proteins bind directly to Ras and are certainly major effectors of Ras action in normal cells and in development. Furthermore, Raf kinase inhibitors can lead to paradoxical activation of the MAPK pathway. MEK inhibitors block the Ras-MAPK pathway, but often activate the PI3'-kinase, and have shown little clinical benefit as single agents. This activation is mediated by EGF-R and other receptor tyrosine kinases through relief of a negative feedback loop from ERK. Drug combinations that target multiple points within the Ras signaling network are likely to be necessary to achieve substantial clinical benefit. Other effectors may also contribute to Ras signaling and provide a source of targets. In addition, unbiased screens for genes necessary for Ras transformation have revealed new potential targets and have added to our understanding of Ras cancer biology.

**Keywords:** Ras, targeted therapy, signal transduction, Raf, MAPK kinase

## Background

Ras genes are frequently mutated in human cancers, and the proteins they encode have been considered drug targets since they were first identified and characterized 30 years ago. Yet, in 2011, no drugs that target Ras proteins directly or act on Ras-driven human cancers have been developed successfully. Indeed, tumors harboring Ras mutations remain the most difficult to treat and are excluded from treatment with specific targeted therapies.

Ras proteins cycle between an inactive GDP-bound “off” state and an active GTP-bound “on” state and function as molecular switches that mediate signal transduction between cell surface growth factor receptors and intracellular signaling pathways.<sup>1</sup> The activation of Ras proteins, that is, the exchange of GDP with GTP, is an intrinsically slow process and is catalyzed by guanine nucleotide exchange factors (GEFs). However, this exchange is, in principle, reversible. The inactivation, that is, the hydrolysis of the  $\gamma$ -phosphate of GTP to GDP, is catalyzed by GTPase-activating proteins (GAPs) and is irreversible. Oncogenic mutations occur most frequently in codons 12, 13, and 61 and elsewhere. The resulting

oncogenic versions of Ras proteins are resistant to GAP-mediated GTP hydrolysis, which renders them constitutively active. Besides cycling between GDP- and GTP-bound states, Ras proteins undergo posttranslational processing.<sup>2–6</sup> These modifications attach the proteins to cellular membranes, which is essential for activity.

The catalytic domain, also referred to as the G domain, is highly homologous between the 3 Ras proteins, K-Ras, H-Ras, and N-Ras, which are activated in human cancer. The first 80 amino acids are identical, and the next 85 amino acids only differ by 5%. Analysis of the approximately 50 crystal structures of H-Ras and the recently solved x-ray structures of K- and N-Ras confirms the remarkable similarity of these proteins.<sup>7–9</sup> For this reason, it is obvious that the functional differences between the 3 proteins are not embedded in the G domain but in the C-terminal hypervariable region (HVR), which comprises the last 23 of 24 amino acids. The Ras proteins share only 15% homology in this region. The HVR can be subdivided in 2 parts: the presumed unstructured linker region (AA 166–179 in N- and H-Ras and AA 166–174 in K-Ras) and the membrane-interacting lipid anchor (AA 180–189 in N- and

H-Ras and AA 175–188 in K-Ras). Both parts are involved in high affinity interactions with lipid raft and nonraft plasma membrane microdomains.<sup>10,11</sup> Determination of x-ray structures of membrane-bound Ras has been impossible. Therefore, biophysical experiments and computer simulations have been carried out in order to study membrane-bound Ras. Computer simulations with full-length H-Ras suggested that the GTP-bound protein undergoes a major conformational change at the membrane, bringing the catalytic domain in contact with the lipid bilayer. Basic residues in  $\alpha 4$  have been implicated in these interactions.<sup>12</sup>

The basic function of nucleotide binding and hydrolysis is carried out by the approximately 20-kDa G domain. The G domain is classified as a  $\alpha/\beta$  protein, typical for nucleotide-binding proteins. The critical regions involve a conserved

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phosphate-binding loop (P-loop, residues 10-17) and 2 switch regions (switch I, AA 25-40; switch II, AA 57-75) that bind the nucleotide. It is these 3 regions that are affected by oncogenic mutations. The switch regions usually show a high degree of flexibility when analyzed by x-ray diffraction or by nuclear magnetic resonance and electron paramagnetic resonance. The canonical switch mechanism can be considered as a loaded-spring mechanism, where the release of the  $\gamma$ -phosphate after the GTP hydrolysis allows the 2 switch regions to relax into the GDP-specific conformation. Different members of the Ras superfamily show variations of this mechanism.<sup>4,13</sup>

The release of guanine nucleotides from Ras proteins is a slow process and is accelerated by GEFs, by several orders of magnitude. The catalytic mechanism involves a series of fast reactions, which lead from a binary Ras-nucleotide complex via a trimeric Ras-nucleotide-GEF complex to a binary nucleotide-free complex. These reactions are reversible: GEFs act as catalysts and increase the rate at which equilibrium is reached. The position of the equilibrium is determined by the high affinity of Ras proteins for GDP or GTP, the intracellular concentration of nucleotides, and the affinities and concentrations of effector proteins that shift the equilibrium towards the GTP-bound state. GEFs interact with switch I and II regions and insert residues into the P-loop and the  $Mg^{2+}$ -binding area. This perturbation is considered to be the main cause for the decreased affinity between Ras proteins and nucleotides.<sup>14,15</sup> Conversely, Ras-GAPs accelerate conversion of active Ras-GTP back to Ras-GDP dramatically. Ras-GAPs insert an arginine side chain into the catalytic site and thereby neutralize developing charges in the transition state. Ras-GAPs stabilize the switch II domain and allow the conserved glutamine 61 to participate in catalysis. Oncogenic mutations of glycine 12 and glutamine 61 cause a perturbation, which renders Ras catalytically insensitive to GAP activity.<sup>16</sup>

Effector proteins of Ras show an enhanced affinity for the GTP-bound state. Some of the effector-binding domains are preformed and undergo no conformational change upon binding. This means that the recruitment of effector proteins by Ras-GTP at the membrane is considered as the activation process. In those cases where a large conformational change takes place, it has been shown that allosteric regulations of the effector proteins are involved. One of the best-characterized Ras effector proteins is the Raf kinase. Its Ras-binding domain (RBD) is a small domain that contains an ubiquitin fold with an interprotein  $\beta$ -sheet that is Ras-GTP sensitive.<sup>17</sup> Other proteins like Ral-GDS have a similar domain and bind to Ras-GTP in a similar fashion. Both Raf and Ral-GDS bind to the switch I domain, as does the  $\gamma$ -subunit of PI3'-kinase. In the latter case, Ras uses also its switch II domain in order to bind the catalytic subunit. These interactions cause a structural change in PI3'-K $\gamma$  and affect the binding to phospholipids and the catalytic activity.<sup>18</sup> Ras proteins undergo a series of posttranslational processing steps in order to become attached to cellular membranes and become biologically functional,<sup>19</sup> as shown in Figure 1.

The C-terminal membrane-interacting lipid anchor region of HVR contains distinct motifs that are subject to these modifications. In all 3 Ras proteins, the cysteine of the CAAX box (Cys185 in K-Ras 4A and 4B and Cys186 in N- and H-Ras) becomes farnesylated under normal conditions (Figure 2). This reaction is catalyzed by the enzyme farnesyl transferase (FTase).<sup>20</sup> The farnesyl 15 carbon chain becomes attached to the cysteine via a stable thioether linkage. Ras farnesylation is, therefore, an irreversible reaction. In the presence of farnesyl transferase inhibitors (FTIs) (see below), K-Ras and N-Ras become alternatively prenylated by the attachment of a 20-carbon geranylgeranyl chain through geranylgeranyl transferase I (GGTase I). This has been a major obstacle for the successful application of FTIs in K-Ras- or N-Ras-driven

tumors,<sup>21</sup> as discussed below. K-Ras 4A and N-Ras have 1 additional cysteine (Cys179 in K-Ras 4A and Cys181 in N-Ras), whereas H-Ras has 2 additional cysteines (Cys181 and Cys184) that become palmitoylated. Ras palmitoylation is reversible and is carried out by palmitoyl transferases (PTase).<sup>22</sup> The DHHC family of PTases has been characterized, and it has also been shown that Ras palmitoylation takes place at the endoplasmic reticulum (ER)/Golgi endomembrane system, whereas depalmitoylation, carried out by less-studied acyl protein thioesterases, takes place at the plasma membrane.<sup>23</sup>

N-Ras, H-Ras, and likely, K-Ras 4A undergo a palmitoylation/depalmitoylation cycle. This is an efficient way of shuttling these proteins from the ER/Golgi to the plasma membrane and reverse.<sup>24</sup> In contrast, K-Ras 4B contains a polybasic lysine stretch in the adjacent upstream region of the CAAX box. This sequence mediates the interaction of K-Ras 4B with acidic phospholipids in the plasma membrane. Furthermore, K-Ras 4B contains a serine residue (Ser181) that is subject to phosphorylation. Phosphorylation by members of the protein kinase C family had been proposed, leading to dissociation of K-Ras 4B from the plasma membrane.<sup>25,26</sup>

The last 3 amino acids of the CAAX box (-AAX) are subject to proteolytic processing. In humans, the reaction is carried out by a prenyl protein-specific endoprotease known as ras-converting enzyme 1 (RCE1). It is an integral membrane protein of the ER. *Rce1*-null mice die between embryonic day 15.5 and the first week of life. The reasons for this lethality are not clear.<sup>27,28</sup> The final CAAX processing step is carboxyl methylation. Isoprenylcysteine-carboxyl-methyltransferase (ICMT) is also an integral membrane protein of the ER, which is unusual for methyltransferases. After modification by ICMT, the fully processed Ras protein consists of a methyl-esterified farnesyl or geranylgeranyl cysteine. ICMT is also essential for mouse development, as mice lacking the gene die by embryonic day 11.5.<sup>29,30</sup>

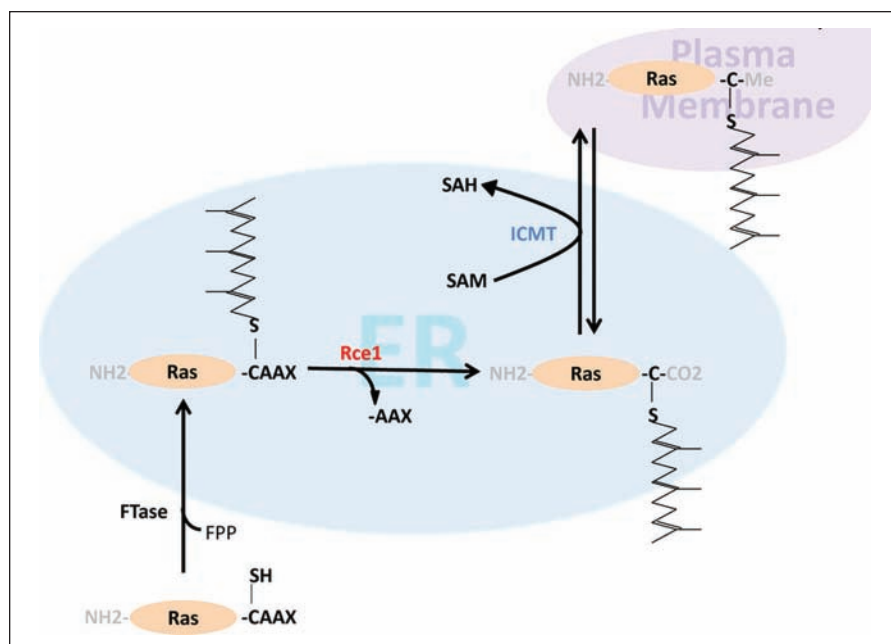


Figure 1. Ras processing.

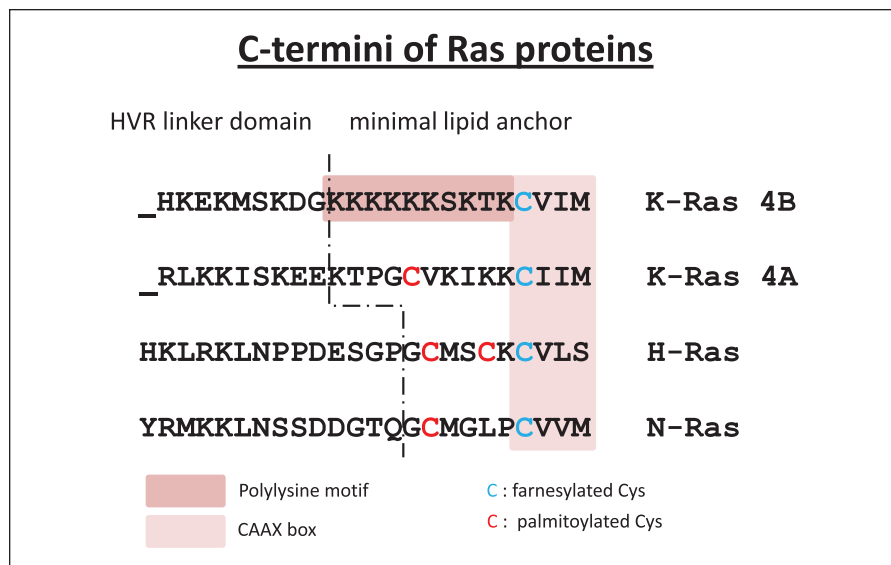


Figure 2. C-termini of Ras proteins.

These 2 postprenylation events render the C-termini of Ras proteins even more hydrophobic. Additionally, it has been speculated that carboxyl methylation is also required for the binding with interacting proteins. The carboxyl methylation reaction is reversible, and it is conceivable that this could represent another level of regulating Ras activity or subcellular localization.<sup>31</sup> However, a

Ras-specific methyltransferase has not yet been identified.

### Direct Therapeutic Attack on the G Domain

Ras proteins bind GTP with picomolar affinities. Furthermore, the concentration of GTP in cells approaches micromolar levels. Unlike protein kinases, in

which phosphoryl transfer from ATP to a substrate is a rapid, catalytic process, the role of GDP or GTP is to stabilize inactive or active states of the Ras protein. For these reasons, targeting mutant Ras with nucleotide analogs does not appear to be a promising approach. The search for small molecules that bind to the surface of Ras proteins has been almost as challenging, as Ras does not have an accessible active site or pocket to which molecules are likely to bind. Nevertheless, Taveras and coworkers at Schering-Plough (Kenilworth, NJ) were able to identify a small molecule that binds to pocket on Ras, without displacing bound nucleotide. The compound, SCH 54292, has not been developed clinically but demonstrates that novel chemical approaches may yet identify ways of targeting Ras directly.<sup>32</sup>

Another approach to inactivate oncogenic Ras could involve small molecules that restore GTP hydrolysis in mutant Ras. Analysis of the GTPase site suggests this will also be technically extremely challenging, as the GTPase site is occupied by guanine nucleotide, and there appears little room for a small molecule to bind. Ideally, such a compound would mimic the effects of GAP and insert an arginine-like residue that would facilitate GTP hydrolysis in the mutant protein. The capacity of mutant Ras to hydrolyze GTP was demonstrated by Ahmadian and coworkers, who constructed GTP analogs covalently attached to positively charged groups that facilitated GTP hydrolysis. While this approach appears encouraging, identifying a small molecule that can bind and interact with mutant Ras in cells remains a daunting challenge.<sup>33</sup>

### Strategies to Interfere with CAAX Processing

Ras prenylation, proteolytic processing, and carboxyl methylation are mechanisms that appear to represent reasonable targets for therapeutic intervention. Drugs that interfere with Ras prenylation have been developed over the last 2 decades. FTIs can be subdivided in 3 different classes: A) CAAX peptidomimetics that compete

with Ras-CAAX for FTase; B) nonpeptidomimetics: farnesylpyrophosphate (FPP) analogs that compete with FPP for binding to FTase; or C) bisubstrate inhibitors, which are combinations of A and B.<sup>34</sup> Attempts to covalently modify CAAX cysteines of Ras proteins have also been described with limited results so far.<sup>35</sup> The first inhibitor approaches focused on the development of competitors for the protein substrate. However, some of these peptides were not very cell permeable or became rapidly degraded in the cell. CAAX peptidomimetics in which the AA portion was replaced with benzodiazepine (C-BZA-M) or aminomethylbenzoic acid (C-AMBA-M) were good inhibitors of FTase and considerably more stable.<sup>36</sup> Later, high-throughput screening led to the identification of small molecule inhibitors. Two of those compounds made it into clinical evaluation: lonafarnib and tipifarnib. Lonafarnib (SCH66336) is a nonpeptidic CAAX-competitive inhibitor that is selective for FTase ( $IC_{50} = 1.9$  nM). Tipifarnib (R115777) is also selective for FTase with an  $IC_{50}$  of 7.9 nM.<sup>37,38</sup>

FTIs inhibit cell growth of a large variety of cancer cell lines *in vitro* and also *in vivo* as tumor xenografts.<sup>39</sup> In particular, FTIs were shown to prevent H-Ras farnesylation and reverse H-Ras-driven cell transformation.<sup>40</sup> FTIs induce tumor growth inhibition rather than regression when used as monotherapies. However, K-Ras, the major Ras oncogene in human cancers, and N-Ras are subject to alternative prenylation by GGTase I in FTI-treated cells.<sup>21</sup> This resulted in a persistent membrane localization of K-Ras and N-Ras and concomitant upregulation of downstream signaling. The fact that K-Ras and N-Ras became cross-prenylated had been often cited as the main reason why FTI monotherapy showed very poor efficacy in clinical trials. FTIs might be more effective in combination with cytotoxic, STI-571, or hormonal agents.<sup>39,41</sup>

Another problem hampering development of FTIs is the lack of reliable genetic markers of response. FTI activities do not

correlate with K- or N-Ras mutational status. Furthermore, FTIs clearly have targets other than Ras proteins that might be responsible for the effects seen in pre-clinical models. For example, the inhibition of RhoB farnesylation seems to be important for FTI antitumor activity. FTIs also inhibit farnesylation of Rheb1 and Rheb2 GTPases that play a role in tuberous sclerosis. Furthermore, some FTIs potently inhibit GGTase II (Rab GTPase) as well as farnesylation of other farnesylated proteins like lamins, the centromeric proteins CENPE and CENPF, PxF, and HDJ2.<sup>42,43</sup>

The alternative prenylation of K-Ras and N-Ras by GGTase I led to the development of GGTase I inhibitors (GGTIs). Because geranylgeranylated proteins are more numerous than farnesylated proteins, this strategy is likely to result in widespread toxicity. Indeed, GGTIs at doses sufficient to prevent K-Ras prenylation in the presence of FTIs were found to be lethal in a mouse model.<sup>44</sup> The development of dual prenylation inhibitors led to similar conclusions as with FTIs. Again, the observed antitumor activity did not correlate with the inhibition of K-Ras prenylation, which suggests that these inhibitors have a variety of targets in cells.<sup>45</sup>

Another strategy to interfere with Ras prenylation is to inhibit the formation of isoprenoids FPP and GGPP in the mevalonate pathway. Statins inhibit the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase. Biphosphonates inhibit 2 other critical enzymes in the pathway: isopentyl diphosphate (IPP) isomerase and FPP synthase that are required for FPP or GPP synthesis. Interference with the mevalonate pathway shows antitumor activity in some cancers, but here also, this effect cannot be solely attributed to the inhibition of Ras prenylation.<sup>46,47</sup>

The function of the postprenylation processing enzymes RCE1 and ICMT in tumorigenesis has been investigated in cells and mice in which the genes for these enzymes have been disrupted. The lack of RCE1 caused mislocalization of

Ras proteins. A conditional deletion of RCE1 in fibroblasts was shown to reduce Ras-induced transformation in cells.<sup>48</sup> However, interference with RCE1 function in tumor cells or cancer models has only shown modest effects. In hematopoietic cells of mice, simultaneous inactivation of RCE1 and activation of K-Ras led to acceleration of myeloproliferative disease development.<sup>49</sup>

The development of RCE1 inhibitors first concentrated on substrate analogs like modified CAAX peptides. A nitrophenyl modification of the second aliphatic amino acid in the CAAX peptide behaves as a competitive inhibitor. The activity of such compounds in cell-based assays has not been reported so far.<sup>50</sup> The yeast RCE1 enzyme Rce1p and the nonrelated CAAX-protease Ste24p can independently promote the proteolytic cleavage of -AAX of prenylated CAAX box proteins in yeast. Recently, a class of inhibitors was found that are dual-specificity inhibitors. Such peptidyl (acyloxy)methyl ketones (AOMKs) could be engineered so that they exhibit selectivity for either of these enzymes. The same group screened a small molecule library that yielded 9 compounds being able to inhibit Rce1p in the low micromolar range. Some of these inhibitors were effective in disrupting yeast Ras localization.<sup>51,52</sup>

The effects of interfering with RCE1 function were modest, but more striking effects were seen through blocking the activity of ICMT. Inactivation of ICMT inhibited cell growth and K-Ras-induced oncogenic transformation both in soft agar assays as well as in a nude mouse model. Cells had a strongly reduced level of RhoA and increased levels of p21. Interference with ICMT function inhibited transformation by B-Raf V600E, an event that is thought to be largely Ras independent.<sup>53</sup> Furthermore, disrupting ICMT ameliorated K-Ras-induced myeloproliferative disease.<sup>54</sup> The anticancer drug methotrexate induces higher levels of homocysteine that causes hypomethylation in cells: treatment with methotrexate reduced

Ras methylation by almost 90% and caused mislocalization and a decreased activity of ERK 1/2 and Akt.<sup>55</sup> More recently, synthetic as well as natural small molecule inhibitors were found that block ICMT, induce apoptosis, and reduce tumor growth in a variety of model systems.<sup>56</sup>

Taken together, strategies that compromise Ras-CAAX box processing have, for the most part, not been rewarding. However, the actual proof of concept that these drugs specifically interfere with Ras membrane interaction and could be clinically useful is lacking. Prenylation and postprenylation inhibitors generally lack the specificity to inhibit signaling of specific Ras proteins, especially K-Ras. However, new approaches to block Ras through provoking mislocalization are being investigated and may still hold promise for the future.

### Inhibition of Ras Expression

The idea of preventing Ras expression by antisense or RNA interference is promising, but the successful application of this technology is currently limited by lack of efficient delivery, uptake, and gene silencing. In cell lines, oncogenic mutation-specific, small interfering RNAs were able to silence K-Ras expression and inhibit tumor cell growth.<sup>57</sup> A more recent study using anti-K-Ras RNA interference demonstrated that tumor cell lines can be classified as K-Ras dependent or K-Ras independent. This dependency correlated with their state of epithelial-to-mesenchymal transition. Epithelial tumor cells were dependent on K-Ras, whereas cell lines expressing mesenchymal markers were relatively independent on the oncogene. This finding showed that it is important to know the biological phenotype of a tumor in order to apply the correct strategy for Ras interference.<sup>58</sup>

### Targeting Pathways Downstream of Ras

Developing therapeutic agents to directly block oncogenic Ras activity has thus

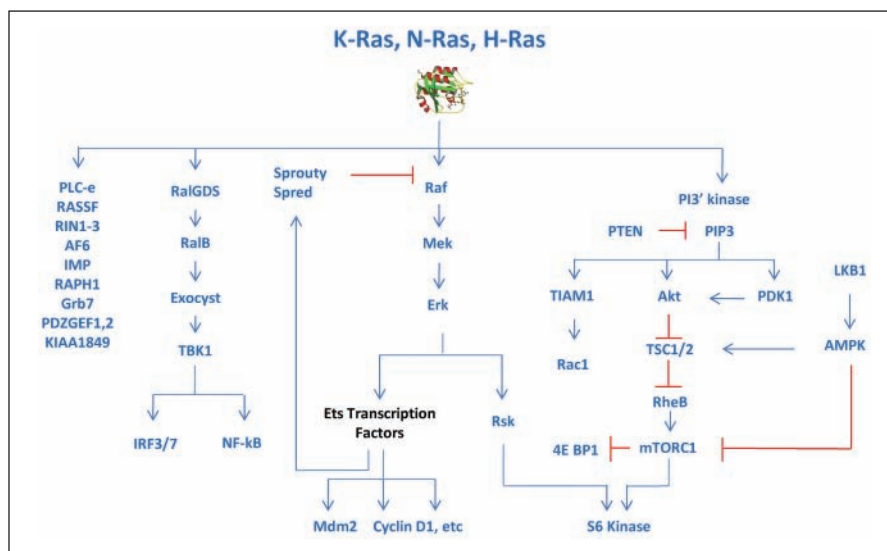


Figure 3. Pathways downstream of Ras.

far been a challenging and unsuccessful endeavor, for reasons discussed above. Therefore, a great deal of effort has been applied to developing therapies that target effector pathways downstream of Ras (Fig. 3). Constitutive activation of downstream effector pathways by oncogenic Ras results in the uncontrolled growth, proliferation, and survival of cancer cells. Understanding which effector pathways are required for Ras-driven oncogenesis is critical for determining which pathways should be targeted for therapeutic purposes. Many Ras effector pathways are comprised of kinase cascades, providing multiple nodes for potential therapeutic intervention. While several Ras effectors have been identified and comprehensively described,<sup>59-61</sup> below, we discuss 2 of the best-characterized Ras effector pathways: the Raf-MEK-ERK and PI3'K signaling pathways. Importantly, both pathways are integral to Ras-driven transformation, and small-molecule compounds targeting these pathways are currently under clinical investigation.<sup>62,63</sup>

### The Raf-MEK-ERK Pathway

The Raf-MEK-ERK signal transduction pathway, also known as the MAPK cascade, was the first Ras effector signaling pathway identified. Raf serine/threonine kinases (A-Raf, B-Raf, and C-Raf/Raf-1)

specifically interact with GTP-bound Ras, resulting in the activation of Raf protein kinase activity.<sup>64-67</sup> Upon activation by Ras, Raf phosphorylates and activates the serine/threonine kinase MEK, which in turn phosphorylates and activates the serine/threonine kinase ERK. This series of signaling events results in the activation of transcriptional regulators that promote a wide variety of cellular events, including cell cycle progression and cell proliferation.<sup>59,60,68</sup>

The requirement for Raf-MEK-ERK signaling in Ras-mediated transformation and tumorigenesis has been well established.<sup>60,69-72</sup> Dominant-negative mutants of Raf-1, MEK, and ERK inhibit Ras-driven transformation, highlighting the importance of this signaling cascade downstream of Ras.<sup>73-76</sup> In support of these findings, mutations in the effector loop of H-Ras V12 that abrogate its ability to bind Raf-1 eliminate its transforming potential in mammalian cells, demonstrating the requirement for Raf-1 activity downstream of activated Ras.<sup>69</sup> In addition, the growth inhibition induced by overexpression of dominant-negative Ras N17 can be overcome by expression of constitutively active Raf-1.<sup>77</sup> Finally, cells that lack Ras proteins altogether can be rescued from growth arrest by expression of activated alleles of Raf, MEK, or ERK proteins, again

showing that the Raf-MEK-ERK pathway is downstream from Ras in mammalian cells, as it is in *Caenorhabditis elegans* and *Drosophila melanogaster* and other model organisms.<sup>78</sup>

Baccarini and colleagues recently demonstrated that Raf-1 is required for the initiation and maintenance of squamous cell carcinoma in 2 separate models of Ras-driven tumorigenesis.<sup>79</sup> In the first model, Ras activation is achieved through a classic chemical carcinogenesis protocol in which tumors are initiated through the topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA), which causes an activating mutation in codon 61 of H-Ras. Tumor development is then promoted through the topical application of 12-O-tetradecanoylphorbol 13-acetate (TPA). In the second model, activation of the Ras pathway is achieved by expression of a dominant-active form of Son of Sevenless (SOS), specifically in the epidermis. In both models, ablation of Raf-1 leads to the regression of established Ras-driven tumors, suggesting that Raf-1 might serve as an appropriate target of therapeutic intervention downstream of activated Ras. Interestingly, in these models, the ability of Raf-1 to promote and maintain skin tumors is dependent on the inhibition of the RhoGTPase target Rok- $\alpha$  rather than the activation of the canonical MEK/ERK signaling cascade.

More recently, activating mutations in various components of the MAPK signaling cascade have been identified in patients with related genetic developmental disorders.<sup>68,80</sup> For example, germline gain-of-function mutations in *KRAS*, *BRAF*, *MEK1*, and *MEK2* have been observed in patients with cardiofaciocutaneous (CFC) syndrome.<sup>81,82</sup> Activating mutations in Ras-Raf-MEK-ERK pathway components are present in patients with similar neurocardiofaciocutaneous syndromes, including Noonan, LEOPARD, and Costello syndromes.<sup>80,83,84</sup> These findings provide genetic evidence that the Raf-MEK-ERK pathway functions downstream of Ras.

The identification of activating *BRAF* mutations in cancer supports a role for

Raf-MEK-ERK signaling in oncogenesis.<sup>85,86</sup> Interestingly, in melanoma and colorectal cancer, a pattern of mutual exclusivity between *RAS* and *BRAF* mutation has emerged, suggesting that mutation of either gene may be functionally equivalent in the pathogenesis of these malignancies.<sup>87</sup> However, in the case of *BRAF* mutation, activation of additional oncogenic signaling pathways such as the PI3'K pathway may also be required.<sup>88</sup>

Attempts to target the Raf-MEK-ERK signaling pathway for therapeutic purposes have focused largely on the development of Raf and MEK kinase inhibitors. Sorafenib (Nexavar, Bayer, Leverkusen, Germany) was the first Raf kinase inhibitor to be tested in clinical trials and is now US Food and Drug Administration (FDA) approved for the treatment of renal cell carcinoma and hepatocellular carcinoma.<sup>89</sup> Although sorafenib was designed to inhibit Raf-1 kinase activity, it also has activity against additional cancer targets including VEGF-R2, PDGFR, Flt-3, c-kit, and FGFR-1.<sup>90</sup> In fact, the success of sorafenib as a cancer therapy has largely been attributed to its inhibitory effects on tumor angiogenesis,<sup>89</sup> particularly for renal cell carcinoma, which is largely driven by hyperactive VEGF-R signaling. In support of this, the VEGF-R2 inhibitor Sutent (Pfizer, New York City, NY) is equally effective in treating this disease. In contrast, Sutent (Pfizer) failed to show efficacy in hepatocellular carcinoma, suggesting that sorafenib's effects in this disease may indeed be mediated through inhibition of Raf kinase. Furthermore, clinical responses to sorafenib correlate well with levels of MAPK signaling in this disease.<sup>91</sup> Activation of this pathway in hepatocellular carcinoma is caused by loss of the negative regulatory proteins Spred and Sprouty<sup>92,93</sup> rather than by oncogenic Ras.

When B-Raf was identified as a major oncogene in human cancers,<sup>85</sup> sorafenib was tested for clinical efficacy in this disease. However, no clinical benefit was observed. This may be because sorafenib interacts with the inactive form of Raf kinase and is less

effective against B-Raf V600E than wild-type B-Raf. This prompted the development of second-generation Raf inhibitors, which demonstrate elevated specificity for B-Raf V600E.<sup>62,94</sup> While these inhibitors potently suppress Raf-MEK-ERK signaling and cell growth in cancer cells expressing B-Raf V600E, they paradoxically have the opposite effect in cancer cells with wild-type B-Raf, including those with oncogenic Ras mutations.<sup>95-98</sup> The promotion of Raf-MEK-ERK signaling in Ras mutant cancer cells by Raf inhibitors has been reviewed extensively<sup>62,98,99</sup> and precludes the use of these inhibitors for the treatment of Ras mutant cancers. Furthermore, although the use of the pan-RAF inhibitor PLX-4032 in melanoma patients harboring *BRAF* V600E mutations produced promising clinical results,<sup>100-102</sup> recent studies have identified multiple mechanisms of Raf inhibitor resistance, including enhanced receptor tyrosine kinase signaling as well as mutational activation of *NRAS*.<sup>103-105</sup> The finding that mutational activation of *NRAS* can bypass the effects of Raf inhibition suggests that targeting Raf in the context of an activating Ras mutation may not be beneficial, despite the evidence in cell culture and animal models that suggest otherwise.

Several small-molecule compounds have been developed to potently and selectively inhibit the activity of MEK. Studies in cancer cell lines and animal models demonstrate that B-Raf mutation predicts sensitivity to these agents, although a subset of Ras mutant cell lines displays sensitivity as well.<sup>106,107</sup> Despite these promising preclinical results, the outcome of early clinical trials was underwhelming in part because of the limited bioavailability and dose-limiting toxicity of the compounds.<sup>62,108</sup> Several compounds with improved pharmaceutical properties are currently under clinical investigation and hold promise for the treatment of Ras mutant tumors.<sup>108</sup> Defining the factors that underlie MEK inhibitor sensitivity and resistance in Ras mutant cancers is of great interest and will aid in determining which patients will benefit most from

therapy. Likewise, it will be critical to determine whether toxicities that limited dosing are on target or off target. In this respect, it is of interest that the MEK inhibitor PD0325901, which is considered a relatively specific compound (it acts through an allosteric mechanism rather than as an ATP competitor), was able to block growth of cells in culture that had been engineered to grow in the absence of Ras-Raf-MEK-ERK activity, strongly suggesting that it has off-target effects that could, in principle, have accounted for clinical toxicity.<sup>109</sup>

### The PI3'K Pathway

The phosphoinositide 3-kinase (PI3'K) pathway is another well-studied signaling cascade downstream of Ras. Class IA PI3'Ks are heterodimeric lipid kinases comprised of a p85 regulatory subunit and a p110 catalytic subunit. The p110 catalytic subunit of PI3'K was identified as a Ras effector when it was found to preferentially associate with GTP-bound Ras through its RBD.<sup>110,111</sup> Although PI3'K can be activated by upstream receptor tyrosine kinases in a Ras-independent manner, association with and activation by Ras-GTP have proven to be a principal mechanism of PI3'K regulation. PI3'K catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>) to the second-messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>). A primary downstream effector of PIP<sub>3</sub> is the serine/threonine kinase Akt, which activates a host of signaling programs to promote cell growth, survival, and migration.<sup>112,113</sup>

PI3'K signaling is often upregulated in tumor cells, indicating its importance in the pathology of cancer. Hyperactivation of the pathway can be achieved through a variety of mechanisms, including gain-of-function mutation in *PIK3CA*, which encodes the p110 $\alpha$  catalytic subunit of PI3'K.<sup>114-120</sup> PTEN is a lipid phosphatase that negatively regulates PI3'K signaling, and its expression is often lost in cancers, providing yet another method by which PI3'K signaling can be deregulated.<sup>121-123</sup> Additionally, increased activity of upstream regulators can also

activate the PI3'K signaling pathway, and this can be achieved through amplification or activation of upstream receptor tyrosine kinases or via oncogenic Ras mutation.<sup>113,115</sup> Interestingly, although Ras mutation drives PI3'K activity, oncogenic mutations in *RAS* and *PIK3CA* often coexist in colorectal cancers.<sup>113,124-126</sup> It is unclear whether these coexisting mutations cooperate to amplify common downstream pathways or function independently to activate nonoverlapping pathways.<sup>113</sup>

Several lines of experimental evidence suggest that Ras mutant tumors depend on the activation of the PI3'K pathway. For example, PI3'K activity is necessary for the transformation of mouse embryonic fibroblasts by Ras.<sup>127</sup> In addition, the interaction between Ras and PI3'K is essential in a mouse model of Ras-driven tumor formation.<sup>128</sup> Collectively, these studies demonstrate a requirement for PI3'K activity downstream of oncogenic Ras and suggest that targeting PI3'K in Ras mutant cancers may have important antitumor effects.

Although the aforementioned studies emphasize a role for PI3'K signaling in Ras-mediated tumorigenesis, preliminary data suggest that Ras mutant tumors are insensitive to single-agent PI3'K inhibitors. In fact, *in vitro* experiments have uncovered Ras mutation as a dominant predictor of resistance to PI3'K inhibitors.<sup>129-131</sup> In addition, murine lung cancers driven by oncogenic K-Ras do not respond to treatment with a single-agent dual PI3'K-mTOR inhibitor.<sup>132</sup> Therefore, while PI3'K activity is an important driver of Ras-mediated transformation and tumorigenesis in cell culture and animal models, inhibition of PI3'K pathway activity alone is likely insufficient for the treatment of established tumors harboring *RAS* mutations.<sup>63,133</sup>

### Dual Inhibition of the Raf-MEK-ERK and PI3'K Effector Pathways

The limited response of Ras mutant cancer cells to single-agent pathway inhibitors suggests that dual inhibition of Raf-MEK-ERK and PI3'K signaling

may be necessary to block the growth of Ras-driven tumors. The efficacy of a single-agent pathway inhibitor is often hindered by the release of negative feedback loops on the reciprocal pathway. For example, treatment of Ras mutant cancer cells with potent and specific MEK inhibitors results in increased phosphorylation of the PI3'K pathway effector Akt.<sup>107,134,135</sup> Upregulation of PI3'K signaling in response to MEK inhibition is due to the release of negative feedback from ERK to the EGF receptor.<sup>136</sup> In light of the crosstalk between the Raf-MEK-ERK and PI3'K signaling pathways, it has been proposed that dual inhibition of both pathways may be required to evade these feedback loops. In support of this hypothesis, combined inhibition of MEK and PI3'K signaling in Ras mutant cancer cells is superior to single-agent inhibition *in vitro* and *in vivo* and results in a synergistic decrease in cell viability and increase in apoptosis.<sup>107,134,135</sup> Furthermore, while dual pathway inhibition was also more effective than single-agent pathway inhibition in cancer cells driven by activated receptor tyrosine kinase signaling, the most pronounced synergistic effect was observed in cancer cells harboring oncogenic *RAS* mutations.<sup>107</sup>

Studies utilizing transgenic mouse models provide additional support for this therapeutic approach. In a mouse model of lung cancer driven by oncogenic K-Ras, single-agent treatment with a dual PI3'K-mTOR inhibitor had no effect on tumor growth. Additionally, single-agent treatment with a MEK inhibitor caused only modest tumor regression. However, combined treatment with both pathway inhibitors resulted in synergistic tumor regression.<sup>132</sup>

The necessity for dual pathway inhibition is most evident in cancers that harbor coexisting oncogenic mutations in *RAS* and *PIK3CA*. Reports indicate that mutational activation of *PIK3CA* in *KRAS* mutant cancer cells confers resistance to MEK inhibition.<sup>137,138</sup> Indeed, treatment with single-agent MEK or Akt inhibitors had no significant effect on tumor growth in a xenograft model with coexisting *KRAS* and *PIK3CA* mutations. However,

combined treatment with both inhibitors was effective at suppressing tumor growth.<sup>137</sup>

Collectively, these data indicate that dual inhibition of Raf-MEK-ERK and PI3'K signaling might be clinically beneficial in Ras mutant tumors and provide a rationale for the design of future clinical trials to test combinations of pathway inhibitors. Further, these studies emphasize that the efficacy of targeted therapeutics is genotype dependent and underscore the importance of stratifying patients by tumor genotype prior to therapy.

### Additional Ras Effectors as Potential Targets in Ras-Driven Tumors

While Raf-MEK-ERK and PI3'K represent the best-characterized effector pathways utilized during tumor development following activation of Ras oncogenes, several additional effectors have been implicated in Ras-driven tumorigenesis including RalGDS, Tiam1, and PLC $\epsilon$ . Each of these proteins has been shown to interact directly with Ras proteins, thus prompting the question of their role in Ras mutant tumors.<sup>60</sup>

The discovery that Ral guanine nucleotide dissociation stimulator (RalGDS) was able to interact with activated Ras proteins motivated the initial investigation of this effector arm of Ras signaling during tumorigenesis.<sup>139-143</sup> RalGDS is a GEF, stimulating the dissociation of GDP from its target Ral proteins and allowing for binding of GTP and subsequent activation.<sup>142</sup> In rodent cells in culture, RalGDS or the downstream Ral proteins cooperated with Ras oncogenes to induce transformation but were unable to do so on their own.<sup>61,144,145</sup> Subsequent studies in human cells utilizing Ras effector-binding mutants demonstrated that activation of the RalGDS pathway was sufficient to transform human epithelial kidney cells.<sup>146</sup> This pathway is thought to play a role in mediating proliferation and cell survival downstream of an activated Ras oncogene, as depletion of RalA

impairs anchorage-independent proliferation of Ras mutant pancreatic tumor cells, while RalB was found to be required for survival in a number of tumor cell lines.<sup>142,147,148</sup> Recently, Ral proteins have been implicated in the development of melanoma and myeloid malignancies, 2 cancers known to harbor frequent mutations in Ras oncogenes. RalA is activated in several melanoma cell lines with oncogenic N-Ras mutations, and RalA knockdown inhibited the tumorigenicity of these cell lines. Furthermore, studies in immortalized melanocytes showed that RalGEF was able to recapitulate several tumorigenic traits seen after transformation with N-Ras, including anchorage-independent growth, consistent with previous studies of this pathway.<sup>149,150</sup> Expression of a Ras effector-binding mutant only able to activate RalGDS in hematopoietic cells demonstrated that activation of this pathway alone was able to inhibit neutrophil differentiation and prolong proliferative potential.<sup>151</sup> Finally, *in vivo* evidence for RalGDS as a relevant Ras effector comes from data generated using a multistage model of skin carcinogenesis, leading to the development of squamous cell tumors harboring H-Ras mutations. Using this protocol in mice with homozygous deletion of RalGDS resulted in reduced tumor incidence, size, and progression to malignancy compared to wild-type mice.<sup>61,152</sup> Together, these data support a role for RalGDS both *in vitro* and *in vivo* as an important effector pathway utilized by oncogenic Ras to drive tumorigenesis that could potentially be exploited for therapeutic intervention, although the absence of somatic mutations in this effector pathway makes its precise role less clear than the Raf-MEK-ERK and PI3'-kinase pathways.

Activation of Ras signaling has also been linked to breakdown of phosphoinositides through its ability to bind and activate phospholipase C $\epsilon$ .<sup>153,154</sup> Activated PLC $\epsilon$  catalyzes cleavage of PI(4,5)P<sub>2</sub> into inositol-1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), which subsequently promote the release of Ca<sup>2+</sup> and

the activation of protein kinase C (PKC), respectively. Although studies have shown conflicting results *in vitro*,<sup>155</sup> the importance of this Ras effector pathway during tumorigenesis is supported *in vivo* by data generated again using the multistage mouse model of skin carcinogenesis. In these conditions, PLC $\epsilon$ -null mice showed delayed onset of the characteristic squamous tumors resulting from this protocol as well as markedly reduced tumor incidence.<sup>156</sup> Furthermore, tumors that did form in mice lacking PLC $\epsilon$  also failed to undergo malignant progression to carcinomas. While the understanding of the relationship between Ras oncogenes and the requirement for PLC $\epsilon$  is incomplete, these data suggest it may be an avenue to pursue to expand the list of potential drug targets in Ras mutant cancers.

Finally, Tiam1, a GEF that stimulates the activation of Rac, has also been implicated in tumorigenesis as an important Ras effector using the DMBA/TPA skin carcinogenesis model.<sup>157</sup> Previously, Tiam1 had been shown to bind directly to active Ras, leading to its activation and subsequent stimulation of Rac activity.<sup>158-160</sup> Tiam1-deficient mice are resistant to the development of DMBA/TPA-induced, Ras-driven skin tumors. Furthermore, the small number of tumors produced using this protocol grew much slower than the tumors formed in wild-type mice.<sup>157</sup> Interestingly, however, the few tumors that grow in Tiam1-null mice show increased invasiveness, suggesting there may be different roles for Tiam1 at different stages of tumor progression. Taken together, each of these effectors represents a potential drug target for Ras-driven tumors, given that deletion of each leads to impaired tumorigenesis in models of Ras-driven cancers. The true promise of each of these effectors, however, will have to be further explored using additional models of Ras-driven tumorigenesis to define the extent to which generalizations can be made about the usage and requirements of these noncanonical effector pathways.



## Candidate Synthetic Lethal Targets in Cells with Mutant Ras

While rationally targeting Ras itself or specific Ras effector pathways provides one therapeutic strategy, an alternative is to exploit vulnerabilities created specifically by the presence of a mutant Ras oncogene. Several potential targets have been identified in both cell culture systems and mouse models that are required for the initiation or maintenance of Ras mutant tumor cells. Considering that Tiam1 has been implicated in Ras-driven tumorigenesis, it is perhaps not unexpected that a role for the GTPase for which Tiam1 serves as a GEF, Rac1, has also been identified in Ras mutant tumors. The relevance of Rac1 in Ras transformation was initially described in cell culture systems in which dominant-negative Rac1 was able to inhibit focus formation by Ras oncogenes, while activated Rac1 was able to enhance Ras transformation in addition to growth in soft agar and motility.<sup>71,161,162</sup> More recently, mouse models have provided additional support for the role of Rac1 in Ras-driven tumors *in vivo*. Deletion of Rac1 in keratinocytes and subsequent treatment with the DMBA/TPA skin tumorigenesis protocol demonstrated a role for this GTPase in the development of these H-Ras-driven cancers based on an observed decrease in hyperproliferation of keratinocytes in cells lacking Rac1.<sup>163</sup> Furthermore, conditional deletion of Rac1 in combination with activation of K-Ras in a mouse model of lung cancer led to a dramatic reduction in cell proliferation with a reduction in the number of tumors.<sup>164</sup> Additionally, loss of Rac1 alone is dispensable for proliferation but is required in the context of activated *Ras* defining a synthetic lethal interaction in these cells. While Rac1 may be important for the growth of Ras-driven tumors, the exact role and mechanism of activation in these cells remain to be fully elucidated, as several pathways can lead to Rac activity, which in turn can drive a number of downstream cellular behaviors. These data support a role for Rac1 in

Ras-driven tumorigenesis, providing another member of a growing list of potential sites for therapeutic intervention and warranting further investigation of this pathway in Ras mutant cells.

Recently, a synthetic lethal interaction has been defined between the presence of a K-Ras oncogene and genetic deletion of Cdk4. Cdk4 has been implicated previously in models of breast tumorigenesis driven by alternate oncogenes<sup>165,166</sup>; however, Puyol *et al.* recently demonstrated that genetic or conditional deletion of Cdk4 led to a senescent response specifically in lung cells expressing an activated K-Ras oncogene.<sup>167</sup> Additionally, treatment with a pharmacological inhibitor of Cdk4 showed a reduction in the growth of K-Ras-driven tumors, further supporting a role for Cdk4 as a therapeutic target in this disease. Furthermore, a similar requirement for Cdk4 was identified in a mouse model of H-Ras-driven breast tumorigenesis.<sup>168</sup> Conversely, coexpression of Cdk4 and oncogenic Ras in normal epidermal cells leads to the development of squamous cell carcinoma-like invasive neoplasia, and Cdk4 expression was found to circumvent Ras-induced growth arrest in primary human keratinocytes, suggesting a requirement for Cdk4 during Ras-driven tumorigenesis in this system as well.<sup>169</sup> Finally, in mouse models, expressing activated H-Ras in melanocytes showed increased incidence of spontaneous cutaneous melanoma when crossed onto a Cdk4(R24C) background, where Cdk4 is insensitive to inhibition by p15INK4B and p16INK4A.<sup>170</sup> Furthermore, treatment of these mice with DMBA/TPA led to an increase in the number of nevi and melanomas compared to Cdk4 wild-type mice, suggesting a cooperative interaction between oncogenic Ras and Cdk4 during tumor development. Data from these different systems implicate Cdk4 as a promising therapeutic target in Ras-driven cancers.

Several other candidates have emerged as important mediators of the transforming effects of oncogenic Ras, including NF- $\kappa$ B, cyclin D1, and *myc*. NF- $\kappa$ B has been recently reported to be required for the development of tumors in mouse

models of lung tumorigenesis.<sup>171,172</sup> Meylan *et al.* demonstrated that inhibition of NF- $\kappa$ B signaling led to an apoptotic response in p53-null lung cancer cell lines, while inhibition of the pathway *in vivo* in the context of K-RasG12D-driven lung tumorigenesis showed reduced tumor development both at the time of tumor initiation or after tumor progression.<sup>172</sup> Additionally, Basseres *et al.* showed that deletion of NF- $\kappa$ B subunit p65/RelA reduced the number of K-Ras-induced lung tumors both in the presence and absence of p53, and tumors that emerged in the absence of p65/RelA showed a higher number of apoptotic cells, reduced spread, and showed lower grade.<sup>171</sup> The requirement for cyclin D1 has also been suggested in Ras mutant tumors. Initially, observations that activated Ras led to overexpression of cyclin D1 motivated investigation of the dependence of Ras on cyclin D1.<sup>173,174</sup> Subsequently, deficiency in cyclin D1 was shown to decrease tumor development in several different systems of Ras-driven tumorigenesis, including grafting of retroviral Ras-transduced keratinocytes and phorbol ester treatment of Ras transgenic mice, and in a 2-stage model of skin carcinogenesis.<sup>174</sup> Finally, a dependence on *myc* in Ras mutant tumors has been suggested based on data that in a mouse model of Ras-induced lung adenocarcinoma, expression of a dominant-negative *myc* mutant led to rapid regression of both incipient and established tumors, suggesting a requirement for *myc* in the development of Ras-driven tumor cells.<sup>175</sup> Each of the examples discussed above provides the basis for exploring new avenues of intervention in cells harboring activated *Ras* alleles that may demonstrate therapeutic efficacy specifically in these tumor cells.

## High-Density RNAi Screens for Synthetic Lethality in Ras Mutant Cells

Numerous studies have sought to define the molecular requirements that underlie Ras-driven tumorigenesis in order to inform potential sites of intervention.

Recent high-throughput approaches have provided an expanded list of potential therapeutic targets for Ras-driven tumors. Using loss-of-function RNAi screens, several groups have identified proteins that, when lost, elicit a synthetic lethal response with mutant Ras oncogenes while leaving cells with wild-type Ras proteins unaffected. Scholl *et al.* utilized a subset of the RNAi Consortium Lentiviral shRNA Library targeting 1,011 human genes to identify STK33, a member of the calcium/calmodulin-dependent protein kinase subfamily of serine/threonine protein kinases, as a target that is selectively required for viability and proliferation in the context of mutant *KRAS*.<sup>176</sup> They found that suppression of STK33 across a panel of K-Ras mutant versus wild-type cell lines demonstrated synthetic lethality independently of tissue origin. Furthermore, introduction of exogenous mutant *KRAS* resulted in newly acquired dependence on STK33. Having never been implicated in cancer previously and being insufficient for tumor initiation and maintenance alone, STK33 represents an example of a component of a signaling pathway that becomes aberrantly required in the presence of *KRAS* mutations. STK33 may promote viability in *KRAS*-dependent cells through regulation of S6K1-induced inactivation of the proapoptotic protein BAD.

Using a similar lentiviral shRNA screening strategy in a panel of cancer cell lines, Barbie *et al.* identified suppression of TBK1, a noncanonical I $\kappa$ B kinase as a second synthetic lethal interaction with K-Ras oncogenes. Viability in cell lines with endogenous mutations in *KRAS* was selectively reduced following TBK1 knockdown. Furthermore, growth of tumor xenografts was inhibited in *KRAS* mutant cells expressing TBK1 shRNAs, while TBK1 shRNA showed little to no effect on the tumor-forming ability of cells with wild-type *KRAS*.<sup>177</sup> Introduction of oncogenic *KRAS* in immortalized human lung epithelial cells led to acquired sensitivity to the knockdown of TBK1. Activation of TBK1 may be linked to NF- $\kappa$ B-driven

survival signals downstream of oncogenic K-Ras, consistent with the work discussed previously implicating NF- $\kappa$ B as playing an essential role in K-Ras mutant cells.

Finally, using a retroviral shRNA library, a comparable K-Ras synthetic lethal screen defined several mitotic genes that were selectively required in cells harboring mutations in K-Ras. These included several components of the anaphase-promoting complex, proteasome and polo-like kinase 1 (PLK1), expanding the list of strategies for therapeutic intervention in these cells.<sup>178</sup> The authors suggest that based on these findings, Ras oncogenes may lead to increased dependence on key mitotic proteins for survival when compared to non-Ras transformed cells, although the precise mechanism underlying this phenomenon remains to be completely understood. Recently, similar approaches have also identified WT1 and Snail2 as candidate proteins required in Ras mutant cells.<sup>179,180</sup> These high-throughput genome-wide screening strategies hold great value in providing rapid exploration of specific requirements of cells for growth and survival and may help to uncover previously unappreciated drug targets in tumors with various genetic lesions.

### Future Prospects

Efforts to attack Ras proteins directly are ongoing, based on new ways of developing compounds based on structural considerations and on a better understanding of Ras processing and membrane localization. These efforts are still in early exploratory phases of drug discovery. Targeting downstream pathways, in contrast, is now a major focus of clinical research, as a rich pipeline of drug candidates that target proteins within with MAPK and PI3<sup>γ</sup>-kinase pathways undergoes clinical evaluation. In parallel, new ways of identifying proteins that Ras depends on for malignant transformation are being evaluated, based on recent advances in RNA interference technology. Indeed, siRNA is being developed for systemic therapy

and may eventually enter the mainstream of clinical research opportunities. Successful targeting of other major oncogenic drivers, such as EGF-R and BCR-ABL, only serves to underscore the importance of targeting Ras and encourages these exploratory and clinical efforts. Thirty years after Ras genes were identified, the urgency of devising strategies for treating Ras-driven cancers has only increased.

### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. McCormick is a consultant to ONYX, and this review discusses the use of sorafenib. Drs. Gysin, Salt, and Young declare no conflicts of interest.

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