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Molecular Pathways: Targeting the Dependence of Mutant *RAS* Cancers on the DNA Damage Response

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Abstract

Of the genes mutated in cancer, *RAS* remains the most elusive to target. Recent technological advances and discoveries have greatly expanded our knowledge of the biology of oncogenic Ras and its role in cancer. As such, it has become apparent that a property that intimately accompanies *RAS*-driven tumorigenesis is the dependence of *RAS* mutant cells on a number of non-oncogenic signaling pathways. These dependencies arise as a means of adaptation to Ras-driven intracellular stresses and represent unique vulnerabilities of mutant *RAS* cancers. A number of studies have highlighted the dependence of mutant *RAS* cancers on the DNA damage response and identified the molecular pathways that mediate this process including signaling from wild-type Ras isoforms, ATR/Chk1, and DNA damage repair pathways. Here we review these findings, and discuss the combinatorial use of DNA damaging chemotherapy with blockade of wild-type H- and N-Ras signaling by farnesyltransferase inhibitors, Chk1 inhibitors, or small molecule targeting DNA damage repair as potential strategies through which the dependence of *RAS* cancers on the DNA damage response can be harnessed for therapeutic intervention.

Background

Oncogenic mutations in *RAS* (*KRAS*, *HRAS*, and *NRAS*) are highly pervasive in human solid tumors with poor therapeutic outcomes, including carcinomas of the pancreas, colon, lung, and melanoma (1). Deterring the malignant properties bestowed by *RAS* mutations is an imperative necessity in cancer therapy and a major focus of new pharmacological intervention efforts. Targeting oncogenic Ras, however, is a challenging task due to the nature of the mutations in the *RAS* genes and inherent difficulties in disrupting the mechanistic switches that determine Ras activation status (2). Ras proteins cycle between active GTP-bound and inactive GDP-bound states. GTP binding is catalyzed by guanine nucleotide exchange factors (GEFs) upon their recruitment to the plasma membrane by activated growth factor receptors. Ras inactivation relies on an intrinsic GTPase activity to hydrolyze GTP to GDP and is accelerated by the binding of GTPase activating proteins

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(GAPs). The most frequent mutations in *RAS*, which occur in codons 12, 13 and 61, lead to amino acid substitutions that impair GTP hydrolysis, thereby locking Ras proteins in a constitutively active state. Efforts to develop small molecules to impede GTP binding or restore GTP hydrolysis have faced difficulties due to the picomolar affinity of Ras for GTP/GDP and the predicament of achieving specific targeting of mutant Ras without deregulating non-mutated Ras molecules. Given this challenging biochemistry, substantial drug-discovery efforts have focused on inhibiting the hyper-stimulated mutant Ras signaling output by blocking the functional prerequisite plasma membrane localization of Ras, or impeding the wide number of downstream effector pathways which include Raf/Mek/Erk, PI3K/Akt, RalGDS/Ral, and MEKK/SEK/JNK. Although significant progress has been made over the past few years in these directions, a therapeutically viable approach to targeting Ras mutant tumors remains elusive.

Beyond targeting hyper-stimulated Ras signaling, there has been a growing awareness that *RAS*-driven malignancies are heavily dependent on cellular processes that are non-oncogenic per se, but rather are co-opted to bolster tumorigenic fitness. Although it is not fully understood how such non-oncogene dependencies arise, the current model holds that they are brought forth as a consequence of the inevitable stresses that oncogene expression imparts on cancer cells (eg. replicative, metabolic, oxidative stress) (3). Surviving such Ras-induced intracellular stresses demands adaptation through mechanisms that mitigate stress. As with all dependencies, the reliance of the evolving tumor on stress-coping mechanisms is also a weakness and offers a unique window of opportunity to specifically target cancer cells. Studies aimed at uncovering unique vulnerabilities of *RAS*-driven tumors have identified a number of genes that mediate mutant *RAS* cancer dependencies on stress mitigating mechanisms including proteotoxic stress (*GATA2*, *APC/C*), DNA damage (ATR, Chk1, CDC6, NEIL2, XRCC1, LIG3, Polb, CUX1, PARP1), mitotic stress (*CDK4*, *PLK1*, *APC/C*), energetic stress (*COPI*, *GLUT1*, *GOT1*, *GLUD1*, *HK1/2*, *PFK1*, *LDHA*,); and inflammation (*TBK1*, *TAK1*, *GATA2*) (4-14). Many of these genes are undruggable, however pharmacological inhibitors of TAK1, genes involved in mitigating DNA damage (Chk1), and mitotic stress (CDK4, PLK1) are available and at various stages of clinical testing. Approaches at targeting TAK1 and mitotic stress coping mechanisms are reviewed elsewhere (15). Here we describe the dependence of *RAS* cancers on pathways that mitigate DNA damage, and discuss pharmacological inhibitors of these pathways which are currently under development or in clinical testing, and could potentially be implemented for the treatment of *RAS* cancers.

The integrity of the DNA is continuously challenged by a number of factors including internal byproducts of cellular metabolism and environmental factors. Such factors can damage the DNA double helix backbone by causing single-strand breaks (SSBs) and double-strand breaks (DSBs) or introducing errors in DNA replication which if unrepaired or miss-repaired can induce mutations and rearrangements of the genome. In order to maintain genome integrity cells engage a collective of mechanisms known as DNA damage response (DDR), which includes DNA damage detection pathways, cell cycle checkpoints, and DNA damage repair mechanisms. Oncogenic Ras expression is frequently associated with an activation of the DDR as evidenced by elevated DNA damage upon acute oncogenic

Ras expression, activation of DNA damage checkpoints, and cell cycle arrest (1, 16). DDR activation is thought to arise as an intracellular reaction to genotoxic stress that is directly induced by oncogenic Ras. Several observations point to at least two routes through which oncogenic Ras can induce genotoxic stress. First, oncogenic Ras has been shown to induce production of reactive oxygen species (ROS) and increase oxidative damage to DNA (Fig. 1) (17-21). Secondly, oncogenic Ras expression induces DNA hyper-replication stress, as defined by aberrations in the number of active replicons and alterations in DNA replication fork progression, leading to DNA damage at sites of active DNA replication (Fig. 1). Excessive DNA damage triggers an anti-proliferative response, which is incompatible with cancer progression. As such, the continuous growth of cancer cells requires tempering DNA damage to a level that does not impair cancer cell proliferation. In coping with excessive DNA damage, mutant *RAS* cancers have been shown to modulate both the activation of the DNA damage checkpoints and function of DNA damage repair pathways (Fig. 1).

Recent studies have begun to identify the relevant pathways regulating oncogenic Ras-induced activation of DNA damage checkpoints and have pointed to the ATR protein kinase pathway as a key mediator of this response (10, 22). Oncogenic Ras expression led to an elevation in ATR activity and an enhanced dependence on ATR functionality for the maintenance of genomic stability on a per-cell-cycle basis (10). Recent work has demonstrated that oncogenic Ras can modulate the function of Chk1, the immediate effector of ATR. This study showed that oncogenic K-Ras engages the wild-type H- and N-Ras proteins to ensure optimal Chk1 activation (Fig. 1) (11). Suppression of wild type H/N-Ras activity led to impaired Chk1 function and checkpoint activation resulting in heightened DNA damage.

Adding to the modulation of DNA damage checkpoint activation, oncogenic Ras can also regulate DNA damage repair mechanisms (Fig. 1). Depending on the DNA lesion, cells engage different DNA repair mechanisms. Studies have linked activation of base excision repair (BER) and the alternative non-homologous end-joining (alt-NHEJ) pathways to oncogenic Ras. Several components of BER including DNA glycosylase *NEIL2*, X-ray repair cross-complementing protein 1 *XRCC1*, *Pol beta*, and *DNA ligase III* were identified as having synthetic lethal relationships to mutant *KRAS* by a genome wide RNAi screen (8). A recent study also showed that mutant Ras expression confers heightened DNA repair capability through the upregulation of *CUX1*, which functions in BER as an ancillary factor that stimulates the activity of the *OGG1* DNA glycosylase (23). Another study found that inhibition of the alt-NHEJ pathway selectively sensitized *KRAS* mutant leukemic cells to cytotoxic agents (24). Mutant K-Ras was shown to activate alt-NHEJ by upregulating DNA ligase III, poly (ADP-ribose) polymerase 1 (PARP1), and *XRCC1*. It remains to be elucidated whether additional DNA damage repair pathways are subject to regulation by oncogenic Ras.

Clinical-Translational Advances

The dependences of cancer cells on stress-coping mechanisms constitute vulnerabilities that can be exploited for therapeutic advantage. As these dependences are unique to cancer cells, exacerbating stress by either exogenous stress-inducing agents and/or inhibiting the stress

coping mechanisms could selectively lead to stress overload and cancer cell death. The body of work demonstrating the heightened DNA repair capabilities of mutant Ras cells predicts that interference with these capabilities would render Ras tumors particularly vulnerable to DNA damage-inducing agents. Strategies that exploit this concept are discussed below.

Combinatorial Chk1 inhibition and DNA-damaging chemotherapy

Due to the role of Chk1 in DDR activation, several Chk1 inhibitors have been developed for combinatorial use with DNA-damaging chemotherapeutic agents in order to potentiate their cytotoxic effects. Early Chk1 inhibitors lacked specificity and showed unfavorable pharmacokinetics and pharmacodynamics (25). However, several selective Chk1 inhibitors (MK-8776, CHIR-124, AZD7762, LY2603618, SAR-020106, GDC0425, and GDC0575) have been developed (26). In normal cells, or cancer cells with a functional *p53*, Chk1 inhibition can be partially compensated for by engagement of the ATM/Chk2/*p53* pathway (27). Therefore, mutations in *p53*, which occur in ~50% of all cancers, have been considered predictive of the efficacy of Chk1 inhibitors with DNA damaging agents. Pre-clinical work has provided evidence both in support of and against the usage of *p53* mutational status as a biomarker for sensitivity to combinatorial Chk1 inhibition and DNA-damaging agents (28, 29). We have found that in a panel of *p53* mutant cancer cell lines and tumors, the efficacy of Chk1 inhibitors at sensitizing to DNA-damaging agents is determined by the *KRAS* mutational status (11). A plausible explanation for this sensitization is that Chk1 is a critical component of the cellular adaptation to *RAS*-driven replicative stress. Consequently, the efficacy of combinatorial therapy consisting of Chk1 inhibition and DNA-damaging agents may be dictated by the *RAS* mutational status in combinations with defective compensatory mechanisms of DNA damage checkpoint activation (eg. *p53* mutations). In support of this idea, studies have reported that Chk1 inhibitors sensitize *KRAS* mutant cells to the cytotoxic effects of radiation therapy or gemcitabine (30, 31).

New selective Chk1 inhibitors were recently tested in combination with gemcitabine in Phase I clinical trials (NCT01359696 and NCT01564251); however, the results of these studies are, as of yet, unpublished. If capable of improving on toxicity and target engagement relative to the earlier Chk1 inhibitors, these new agents may prove successful for clinical use (26). It should be noted, that selective Chk1 inhibitors (MK-8776) do not sensitize to antimetabolites such as 5-FU and show higher sensitization to hydroxyurea (100-fold) versus gemcitabine (10-fold) (32-34). In addition whereas MK-8776 does not sensitize cells to SN-38, the active metabolite of irinotecan, AZD7762 and CHIR-124, enhance the effect of irinotecan in preclinical models (35, 36). It is not clear why different DNA-damaging agents would elicit such a different response when combined with Chk1 inhibitors, particularly so within the antimetabolite drugs which would have a similar mechanism of inducing DNA damage. Nonetheless, the design of clinical trials should consider such differences for optimal combinatorial regimens. Moreover, when considering combinatorial regimens with antimetabolites, it is important to note that Chk1 inhibitors sensitize to antimetabolites by destabilizing stalled replication forks, which become more dependent on Chk1 with time and as cells accumulate in S-phase. Hence, it has been suggested that Chk1 inhibitors would be most effective if administered following

antimetabolite treatment and when most cells have arrested in S-phase (37). Therefore, drug scheduling may also have a critical impact on Chk1 inhibitor efficacy.

Molecules downstream of Chk1 represent another opportunity for targeting the dependence of mutant *RAS* cancers on ATR/Chk1. Inhibitory phosphorylation of Cdk1-Tyr-15 is a critical determinant of Chk1-dependent cell cycle arrest and DNA repair. Chk1 promotes the inhibitory phosphorylation of Cdk1 by inactivating the phosphatase that removes the phosphate group (Cdc25). Addition of the phosphate group on the other hand is mediated by Wee1 kinase. Chk1 has also been reported to regulate Wee1 function. Consequently, Wee1 inhibitors should in principle mimic Chk1 inhibition and lead to impaired cell-cycle arrest and DNA repair and sensitize mutant *RAS* cancers to DNA damage (38).

Combinatorial DNA damage repair inhibitors and DNA-damaging chemotherapy

Another approach to enhancing the cytotoxicity of DNA-damaging agents is to impair the activity of pathways that repair damage. Although the mechanisms through which mutant *RAS* upregulates mediators of DNA damage repair pathways are not clear, pharmacological inhibition and RNAi-mediated approaches to suppress these pathways have selectively sensitized mutant *RAS* cells to DNA-damaging agents (8, 23, 24). Investigational compounds that target DNA damage repair proteins regulated by mutant *RAS* include inhibitors for PARP-1, as well as DNA ligase III and DNA glycosylase inhibitors. The latter are still in the preclinical phase whereas PARP-1 inhibitors are currently in clinical testing. These compounds represent potential combinatorial candidates to be utilized in conjunction with DNA-damaging agents (39, 40).

Combinatorial wild type H/N-Ras inhibition and DNA-damaging chemotherapy

Recent work has defined a functional dependence of *KRAS*-driven tumors on wild-type H- and N-Ras for the activation of the DNA damage checkpoint (11). Following previous work demonstrating that p90RSK and AKT kinases inactivate Chk1, this study showed that in the context of *KRAS* mutant tumors, wild type H- and N-Ras reinforce Chk1 activation by antagonizing p90RSK and AKT. Silencing wild type H- and N-Ras impaired Chk1 activity, and selectively sensitized *KRAS* mutant tumors to irinotecan suggesting that pharmacological inhibitors of wild type H- and N-Ras could sensitize *KRAS* mutant tumors to DNA damaging agents. Addition of a 15-carbon farnesyl moiety by the enzyme farnesyltransferase (FTase) is essential for Ras signaling due to the requirement of this post-translational modification for the plasma membrane localization of Ras molecules (41). As such, FTase inhibitors (FTIs) have been long recognized as a means to inhibit H-Ras and N-Ras signaling (42). K-Ras has proven resistant to inhibition by FTIs due to a higher affinity for FTase and alternate prenylation by GGTase-1. This resistance has been a significant causative factor to the moderate efficacy exhibited of FTI inhibitors in PhaseII/III clinical trials, which have in large part, targeted *KRAS* mutant tumors (43, 44). Nonetheless, as H- and N-Ras farnesylation and signaling is robustly impaired by FTIs, and with the increasing

awareness that the wild type H- and N-Ras isoforms play a pro-tumorigenic role in mutant *KRAS* tumors, a rational combination of FTIs with other therapies may improve their clinical utility. Specifically, the dependence of mutant *KRAS* cancers on wild type H-Ras and N-Ras for Chk1 activation suggests that combinatorial strategies composed of FTIs and irinotecan or other DNA damaging agents, could prove therapeutically efficacious in the clinic. In support of this idea FTase inhibition selectively radiosensitized *KRAS* mutant cancer cell-lines and tumors (45, 46). It is important to note that timing of dosing has been shown to be critical for the efficacy of combinatorial treatments with FTIs. As such, combination of the FTI SCH66336 with cisplatin only showed a supra-additive effect when SCH66336 administration preceded cisplatin treatment whereas co-administration of SCH66336 with cisplatin, or gemcitabine was strictly additive or less than additive (47). Moreover, co-administration of gemcitabine and the FTI R115777 showed no statistically significant difference in median overall survival or progression-free survival compared to gemcitabine alone or placebo in a Phase III clinical trial in patients with advanced pancreatic adenocarcinoma (44). Another important factor to consider in combining FTIs with DNA damaging agents are the functional properties of the latter. FTI treatment of *KRAS* mutant tumors would in principle suppress wild type H/N-Ras function and lead to impaired Chk1 activity, and as previously discussed in this review, the sensitization capabilities of Chk1 inhibition can vary with different DNA damaging agents. Therefore, it might be necessary to pre-clinically define a combination schedule that will allow optimal therapeutic efficacy. As a cautionary note however, despite the very low toxicity of FTIs to normal cells, several farnesylated proteins in addition to Ras have been shown to play a role in tumorigenesis including RhoB, the phosphatases PRL-1, 2, and 3, and CENP-E and CENP-F centromeric proteins. Hence, in the context of cancer cells, the effects of FTIs may be complex and may be affected by the complement of mutations present in the tumor (48). Rational design of combination therapies with FTIs will require a comprehensive understanding of the status and contribution of alternative FTI targets in *KRAS*-driven tumors.

Concluding Remarks

In sum, the dependence of *RAS* mutant cancers on processes that mitigate DNA damage occurs as a means of adaptation to Ras-induced genotoxic stress. The molecular pathways through which mutant Ras regulates these processes offer a number of potential drug targets and combinatorial therapies. Novel clinical approaches could consist of combinatorial regimens using existing therapeutic agents, such as Chk1/Wee1, FTI, or PARP-1 inhibitors, together with DNA-damaging agents. Alternatively, drug development approaches could be centered on Ras-targets within the DNA damage repair pathway. Overall, the clinical challenges currently being faced in treating *RAS*-driven cancers clearly demonstrate a need for novel effective therapies that take advantage of the multifaceted biological reliances that are characteristic of these tumors.

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Figure 1.

Oncogenic Ras mitigates DNA damage. Oncogenic Ras (KRas is provided as an example) expression lead to DNA damage through induction of reactive oxygen species (ROS) and replicative stress. To counteract the antiproliferative effect of heightened DNA damage oncogenic Ras modulates the activation of the ATR/Chk1 DNA damage checkpoint and upregulates mediators of DNA damage repair mechanisms such as base excision repair (BER) and alternative non-homologous end joining (alt-NHEJ) pathways. Wild type H- and N-Ras crosstalk with oncogenic K-Ras to regulate Chk1 function. Checkpoint activation leads to cell cycle arrest to allow time for DNA repair and promotes activation of DNA repair pathways. The molecular mediators through which oncogenic K-Ras upregulates NEIL2, XRCC1, LIG3, POL β , CUX1, and PARP1 are unknown. Pharmacological agents that can potentially be used in combination with DNA damaging chemotherapy for the treatment of *RAS* cancers include FTase inhibitors, Chk1 inhibitors, Wee1 inhibitors, and agents targeting components of DNA damage repair pathways. Asterisk marks oncogenic form of Ras. Dashed lines represent unknown molecular interactions.