Synthetic Vulnerabilities in the KRAS Pathway

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Mutations in *Kristen Rat Sarcoma* viral oncogene (*KRAS)* are among the most frequent gain-of-function genetic alterations in human cancer. Most *KRAS*-driven cancers depend on its sustained expression and signaling. Despite spectacular recent success in the development of inhibitors targeting specific *KRAS* alleles, the discovery and utilization of effective directed therapies for *KRAS*-mutant cancers remains a major unmet need.

Keywords: KRAS ; cancer

1. Direct *Kristen Rat Sarcoma* viral oncogene (*KRAS)* Inhibition

To date, numerous approaches to directly target KRAS [1][2][3][4] and its post-translational modifications, which promote the association of KRAS to the cell membrane, have been investigated [5][6][7]. The discovery in 2013 by Ostrem et al. of a covalent inhibitor to lock the GDP-inactive form of KRAS G12C marked the beginning of a new era in the development of KRAS inhibitors [3]. Subsequently, other research groups reported the discovery of small molecules with similar mechanisms but improved binding and pharmacologic properties. ARS-853 [1][4] was the first direct KRAS G12C inhibitor that proved both efficacious and selective in *KRAS*-dependent cells. In 2018, Janes et al. [8] reported a new and improved KRAS G12C inhibitor (ARS-1620) that overcame the limitation of the previous compounds regarding their use in in vivo models, demonstrating that targeting switch II of KRAS G12C is a viable and promising clinical therapeutic strategy. One of these covalent inhibitors of KRAS G12C (sotorasib) was clinically approved in 2021 for the treatment of advanced non-small cell lung cancer (NSCLC) patients carrying a G12C mutation in the *KRAS* oncogene [9][10][11][12]. Ongoing clinical trials are evaluating the toxicities and efficacies of monotherapies as well as therapeutic combinations in eligible patient populations [13][14]. A multicenter phase 1 trial of AMG 510 (sotorasib) was performed in patients with advanced solid tumors harboring the G12C mutation (n = 129; including NSCLC and colorectal cancer patients, among others). It was demonstrated that a durable clinical benefit from sotorasib with relatively low toxicity in a heavily pretreated patient cohort: most strikingly, 32.2% of non-small cell lung cancer (NSCLC) patients had a confirmed response and a majority (88.1%) stable disease. The results for non-lung cancer patients were less promising. Three of 42 patients with colorectal cancer (7.1%) showed a partial response and 66.7% disease control, reinforcing the contribution of disease heterogeneity to driver mutation susceptibility [15]. A remaining barrier is the genotype specificity of current clinically available inhibitors, as G12C mutations account for nearly half (46%) of mutations in NSCLC [16] but only 4–14% of KRAS mutations in all human cancers [17]. One of the unique features of the *KRAS* G12C mutant is its retention of near wild type intrinsic GTPase activity, allowing covalent inhibitors to selectively target the inactive GDP state and retain high efficacy [18]. In contrast, the *KRAS* G12D mutant demonstrates a high nucleotide exchange rate. Thus, new strategies are being explored to discover inhibitors of both the GDP and GTP-bound states, such as non-covalent inhibition of the switch II region outside of the nucleotide-binding site. Other approaches that may be more generalizable to multiple alleles include PROTAC protein degraders targeting KRAS [19] or its signaling partners, as well as steric targeting of effector engagement by Ras-GTP [20].

Clinical trials for NSCLC patients with *KRAS* non-G12C mutations have previously been reviewed [21]. The long-term efficacy of the current *KRAS* G12C inhibitors remains unclear, mostly due to toxicity and acquired resistance mechanisms [9][12][22][23]. Notably, nearly all patients included in early-phase, single agent clinical trials of both sotorasib and adagrasib (MRTX949) developed acquired resistance and demonstrated disease progression despite the initial response. Next-generation sequencing analysis of adagrasib-resistant tumors suggested that the majority (45%) of identifiable mechanisms occurred as either secondary alterations in either *KRAS* itself, including within the targeted switch II pocket, or in members of the receptor tyrosine kinase (RTK)-RAS-MAPK pathway, emphasizing its importance as a major mechanism of survival and proliferation in this tumor context [24]. This fact highlights the continued need to identify and develop combinatorial therapies [23][25][26] or explore alternative opportunities for targeting *KRAS*-driven cancer.

2. Indirect KRAS Inhibition

Indirect strategies for targeting the KRAS pathway can be classified in two main groups: (1) inhibition of upstream KRAS activators and (2) inhibition of downstream KRAS canonical effectors.

With regards to upstream KRAS activation, most studies have focused on blocking KRAS upstream RTK signaling through the epidermal growth factor receptor (EGFR) and other RTKs. However, clinical studies have indicated that patients harboring *KRAS*-mutant tumors are not sensitive to EGFR tyrosine kinase inhibitors (TKIs). In fact, KRAS activation is one of the signaling pathways conferring resistance to EGFR TKIs [27][28]. Although it has been demonstrated that the deletion of *EGFR* transiently reduces *KRAS*-mutant tumor growth, EGFR therapies trigger tumor escape mechanisms involving non-EGFR ERBB family members [29]. During the last few years, significant interest has focused on the protein tyrosine phosphatase SHP2. SHP2 acts downstream of many RTKs and mediates cellular signaling through the RAS/MAP kinase pathway. Several studies have provided evidence for a critical dependence of mutant *KRAS* on SHP2 and have shown the potential clinical use of combined SHP2/MEK inhibition for *KRAS*-driven tumors [30][31]. Two promising inhibitors of SHP2 (RMC-4630 and TNO155) are currently undergoing clinical trials [32][33]. Other approaches rely on blocking SOS1, a relevant GEF for KRAS, and suggest efficiency in combination with MEK inhibition [34][35] in the context of *KRAS* mutations.

While many KRAS effector pathways have been described, the most well-studied is the MAPK cascade, which regulates tumor cell proliferation and survival [36][37]. A number of inhibitors targeting the MAPK pathway have been developed and tested as single agents or in combination with chemotherapy in different *KRAS*-driven cancers in the clinic [38][39][40]. The limited efficacy of these inhibitors is likely explained by the rapid development of multiple feedback mechanisms that are able to re-activate the MAPK pathway at different signaling levels [41][42]. The phosphatidyl-inositol 3-kinase (PI3K) pathway is also critical in KRAS signaling, and inhibitors against its effectors are currently under clinical evaluation. However, mutant oncogenic *RAS* has been described as a dominant determinant of resistance to PI3K inhibitors even in tumors with coexisting mutations in *PI3K*, with *c-MYC* and *CYCLIN B* acting as potential mediators of such resistance [43]. Studies targeting the nuclear factor kappa B (NF-kB) pathway, activated by RAL, have demonstrated that *KRAS*-mutant tumor cells require *NF-kB* for viability [44] and inhibitors targeting this effector are also being tested in clinical trials [45]. A less characterized effector of RAL is phospholipase D (PLD), which is associated with the generation of lipid second messengers such as phosphatidic acid, lysophosphatidic acid and diacylglycerol. The activation of PLD does not depend on GDP/GTP exchange, but it needs the additional association with the GTPase ARF [46]. It has been demonstrated in the preclinical setting that targeting PLD survival signals in human cancer cells with *RAS* mutations could be an effective strategy to induce apoptosis. This node of RAS signaling portrays an opportunity for the development of novel anticancer drugs [47][48]. The therapeutic value of less studied KRAS canonical effectors, such as RIN, TIAM1 or MKK4/7, remains unknown.

In addition to emergent resistance, effector targeting is further complicated by heterogeneity in both mutation-specific affinities as well as heterogeneity of effector dependencies [16][49]. One example of this is that cell lines harboring a *KRAS* G12D mutation revealed increased sensitivity to MEK and RAF combination therapy relative to non-G12D *KRAS* mutations. This observation led the authors to hypothesize that, in the presence of MEK inhibition, mutant *KRAS* alleles with high intrinsic nucleotide exchange are dependent on RAF dimerization to maintain a GTP-bound state [50]. Currently, significant clinical barriers to complete MAPK blockade are dose-limiting toxicities as observed both in cell lines [51] and patients, and most clearly evidenced in clinical trials testing BRAFi in combination with trametinib (MEKi) in melanoma patients [52]. However, preclinical studies indicate that KRAS-targeting covalent inhibitors may synergize with upstream activators such as EGFR and IGF1R or downstream effectors such as MTOR while minimizing toxicity, suggesting that these combinations may result in more durable responses while mitigating the deleterious side effects of MAPK blockade [4][23].

In summary, studies targeting KRAS downstream signaling suggest that the inhibition of a single effector arm will be of limited efficacy due to compensatory feedback mechanisms. Thus, although the inhibition of KRAS effectors is a potential strategy to target *KRAS*-driven cancers, it remains a significant challenge, and successful targeting of *KRAS*-mutated tumors will likely require simultaneous targeting of multiple effector pathways [53][54].

3. Screening Approaches to Identify Synthetic Lethal Interactions with *KRAS*

3.1. RNA Interference Screens

In RNAi screens, exogenous short interfering RNAs (siRNA) or short hairpin RNAs (shRNA) are introduced into human cells. These small RNA sequences are then loaded into the endogenous RNA-induced silencing complex (RISC), allowing the knockdown of complementary target mRNAs [55]. This tool provided the first opportunity to carry out scalable genetic screens in human cells, and many studies have reported numerous genes as synthetic lethal interactors with oncogenic *KRAS* including *PLK1, TBK1, WT1, STK33, FGFR1, YAP1* and *XPO1*, among others [44][53][56][57][58][59][60]. In fact, some ongoing clinical trials are testing the efficacy of PLK1 inhibitors, CYC140 (phase I: NCT03884829) and BI-2536 (phase II: NCT00710710), in advanced leukemias and pancreatic cancers, respectively [61]. However, despite the vast amount of knowledge these RNAi-based screens have enabled, there are several limitations, including a substantial number of off-target activities of RNAi libraries, resulting in a lack of overlap in findings between independent screens [62][63]. The inconsistencies in the experimental results between studies is thus reflected in the relatively small number of robust synthetic lethal targets that have been identified by this type of screening. Such limitations likely contribute to false-negative and false-positive rates and are attributed to the use of different RNAi libraries, the use of cell lines with different genetic backgrounds as well as the different screening modalities and quantification methodologies [64][65]. The most informative RNAi screens in the context of *RAS*-mutant cancers have been previously reviewed by Ebi et al. [66], Downward et al. [63] and Aguirre et al. [67].

3.2. CRISPR/Cas9 Screens

Over the last decade, CRISPR/Cas9 technology has emerged as an alternative for uncovering new synthetic lethal partners in the biology and treatment of cancer, revolutionizing the field of loss-of-function screens [68][69][70]. CRISPR/Cas9 genome editing technology uses a 20-nucleotide guide RNA (gRNA) that guides the Cas9 nuclease to a specific target site generating precise DNA double-strand breaks [71]. A number of studies have confirmed that CRISPR-based screens have improved reproducibility compared to RNAi screening approaches, likely due to the lower off-target frequency of gRNAs and the higher efficiency of CRISPR reagents from creating knockout mutants rather than RNA-targeted knockdowns [72][73][74]. Thus, large-scale CRISPR/Cas9 screens have proven to be a powerful method identifying genetic defects in tumors harboring oncogenic mutations such as *KRAS* [75][76].

Pioneering work in the use of genome-wide CRISPR/Cas9 screens to identify synthetic lethal genes in the context of oncogenic *KRAS* was published by a team led by Sabatini and colleagues [75]. Six acute myeloid leukemia (AML) cell lines with mutations in either *KRAS* or *NRAS* against six *KRAS* wild type cell lines were compared. It was highlighted that the importance of targeting specific components of the RAS pathway itself in order to impact the viability of *RAS*-dependent tumor cells. Isogenic murine Ba/F3 (*NRAS*-mutant) cell lines were used to perform a parallel and independent CRISPR screen that showed a very high degree of overlap with the screen carried out in AML cell lines. Genes involved in the maturation of RAS (such as *RCE1* and *ICMT*) and genes related to MAPK pathway signaling (*RAF1* and *SHOC2*), supported the central role of MAPK signaling in *RAS*-mutant cancers. It was validated that PREX1, a GEF for the Rac GTPases, and described it as a novel *RAS* synthetic lethality [75].

Yau et al. [77] performed an in vivo pooled human genome-wide CRISPR/Cas9 knockout screen of tumor xenografts using a well-characterized isogenic pair of human colorectal cancer cell lines harboring either mutant or wild type *KRAS*. The primary aim of this screen was to extend the knowledge of the genetic vulnerabilities of mutant *KRAS* tumors to the in vivo setting. They identified approximately 250 gene candidates that were used to design a second smaller focused in vivo screen, with higher depth and coverage per construct, to validate the genome-wide screen. Comparing *KRAS*-mutant to *KRAS* wild type cells, they found gene knockouts that conferred selectively beneficial or detrimental viability effects in the context of KRAS activation. Pathway analysis identified multiple metabolic vulnerabilities (NAD kinase and ketohexokinase), highlighting the therapeutic potential of targeting cancer metabolism, associated with the rewiring of metabolic programs that promote tumor survival, growth and immune evasion in different *KRAS*-mutant cancer types [78][79]. Here, it was further identified *INO80 Complex Subunit C* (*INO30C*) as a novel *KRAS*-dependent tumor suppressor gene in both colorectal cancer and pancreatic adenocarcinoma isogenic xenografts.

Although many studies have demonstrated the impact of targeting single KRAS downstream effectors, the appearance of resistance and compensatory signaling mechanisms highlights the need to use combination therapies. For this reason, multiple high-throughput CRISPR screening approaches have been applied to identify critical genes that contribute to drug resistance in *KRAS*-mutant human cancers [80]. Šuštić et al. [81] identified *IRE1*, a proteotoxic stress response gene, as a vulnerability in the context of *RAS* mutations in a *RAS* synthetic lethality screen in yeast. However, in human cells,

they found no difference in cell viability between the control and *ERN1* (*IRE1* mammalian ortholog) KO human cells, indicating the synthetic lethal interaction with *KRAS* is not conserved between human cells and yeast, which is surprising considering *RAS* is a highly conserved pathway. It was argued that this inconsistency between yeast and human cells could be due to the fact that yeast are missing the RAF/MEK/ERK MAPK cascade [82]. To corroborate their hypothesis, they investigated the effect of knocking *ERN1* out in cell proliferation in combination with a MEKi (selumetinib) and found increased MEKi sensitivity in ERN1 KO cells. This result encouraged them to perform a genome-wide CRISPR/Cas9 MEK inhibitor resistance screen to identify a mechanistic link between *ERN1* and the MAPK pathway using ERN1 KO LoVo colorectal cancer cells. This screen established a relationship between *ERN1* and *JUN* and highlighted the relevance of the ERN1-JNK-JUN pathway as a novel regulator of MEKi response in human *KRAS*-mutant colorectal cancer, providing a therapeutically exploitable vulnerability. Similarly, Szlachta et al. [83] described large-scale in vivo and in vitro CRISPR/Cas9 KO screens that also identified genes whose genetic deletion synergistically increased the cytotoxic effect of a MEKi (trametinib). They carried out the CRISPR screening using an sgRNA library enriched for epigenetic regulators, transcription factors and nuclear proteins, in a *KRAS*-mutant patient-derived xenograft (PDX) model of pancreatic ductal adenocarcinoma. Here, it was identified multiple genes, such as *CENPE*, whose depletion creates a synthetic lethality in combination with MEK inhibition. They complemented by demonstrating that overall drug responses could be modeled using the DREBIC approach, which captures the relative essentiality of the drug target (gene specific CRISPR viability scores) and their basal expression levels (mRNA) for specific cell types.

In another report on MEKi synthetic lethalities, Sulahian et al. [84] performed a genome-scale CRISPR/Cas9 screen in the presence of trametinib that identified *SHOC2* as a synthetic lethality when combined with MEK inhibition in *KRAS*-mutant lung and pancreas cancers. *SHOC2* is a positive regulator of *RAF1*-mediated MAPK signaling. It was demonstrated that *SHOC2* loss conferred a consistent attenuation of MAPK pathway re-activation in response to trametinib. These data further validated results described by Wang et al. [75], where *SHOC2* was essential for proliferation specifically in *RAS*-mutant leukemia cells. Another example of combinatorial CRISPR/Cas9 and MEKi screening is the work recently published by Yun et al. [80]. Here the authors focused on *KRAS*-mutant colorectal cancer and found the RTK pathway was a resistance driver to MEK inhibitors. They showed that a combinatorial inhibition of the RTKs-GRB7-PLK1 axis and MEK could be a promising strategy in the context of *KRAS* tumors. Taken together, these studies provide support for novel treatment combinations for refractory *KRAS*-driven tumors.

CRISPR/Cas9 loss-of-function screens have become a very useful and valuable tool for identifying synthetic lethal genes that do not cooperate just with MEK inhibitors and other therapies. For example, recent work described a genome-wide CRISPR/Cas9 screen performed in both 2D and 3D conditions [85]. Here, it was to identify synthetic lethal targets for *KRAS*-driven lung adenocarcinoma tumors, as well as synthetic vulnerabilities in combination with a KRAS inhibitor to combat the resistance mechanisms associated with these drugs [22][23][86][87]. While 2D in vitro models have been broadly used to investigate cancer biology and drug sensitivity, 2D cultured cells are unable to truly reproduce the natural proliferation, migration, drug response and/or rewired metabolism taking place in the complex 3D environment [88][89][90] of a tumor. To overcome some of these limitations, 3D cancer cell culture systems are a valuable resource that may provide a more accurate and relevant preclinical testing model. Nevertheless, 3D models have not been widely used to perform CRISPR screening because they are much less scalable [85]. Han et al. developed a scalable method to propagate *KRAS*-mutant lung cancer spheroids that allowed them to carry out a genome-wide CRISPR screen in 3D conditions. They found a module composed of genes correlated with *carboxypeptidase D* (*CPD*) was significantly depleted in the 3D versus 2D phenotype and showed a strong synthetic lethality with the KRAS inhibitor in 3D, suggesting that CDP and its interactors could be potential therapeutic targets.

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