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COMMENTARY

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Defeat mutant *KRAS* with synthetic lethality

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ABSTRACT
Ras proteins are considered as the founding members of a large superfamily of small GTPases that control fundamental cellular functions. Mutationally activated *RAS* genes were discovered in human cancer cells more than 3 decades ago, but intensive efforts on Ras structure, biochemistry, function and signaling continue even now. Because mutant Ras proteins are inherently difficult to inhibit and have yet been therapeutically conquered, it was designated as "the Everest of oncogenes" in the cancer genome landscape, further promoting a "renaissance" in *RAS* research. Different paths to directly or indirectly targeting mutant Ras signaling are currently under investigation in the hope of finding an efficacious regimen. Inhibitors directly binding to $KRAS^{G12C}$ to block its downstream signaling have been revealed, supporting the notion of Ras' druggability. An alternative indirect approach by targeting synthetic lethal interactors of mutant *RAS* is underway. We recently employed a synthetic lethal drug screen plus a combinatorial strategy using a panel of clinical agents and discovered that *KRAS*-mutant cancers were fragile to the combined inhibition of polo-like kinase 1 (Plk1) and RhoA/Rho kinase (ROCK). The combined regimen of BI-2536 (a Plk1 inhibitor) and fasudil (a ROCK inhibitor) promoted a significant inhibition of patient-derived lung cancer xenografts and prolonged the survival of LSL-$KRAS^{G12D}$ mice. In this commentary, we will summarize the state-of-the art for the direction of synthetic lethality, and also speculate on the future development of this approach.

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Anti-RAS: The battle continues

According to the Ras history, *RAS* genes were first identified as viral genes, and Ras proteins are founding members of a large superfamily of small GTPases, including Ras, Rho, Rab, Arf and Ran families.[1] With some variation and exceptions, Ras superfamily proteins function as GDP/GTP-regulated binary on-off switches. Ras proteins (KRAS4A, KRAS4B, NRAS and HRAS) control cytoplasmic signaling networks and regulate diverse normal cellular functions. Mutationally activated *RAS* genes were discovered in human cancer cells in 1982, but drugging the Ras proteins was considered as "the Everest" to climb.[2] Gain-of-function missense mutations in *RAS* genes occur in approximately a third of all human solid tumors. As the principal of the 3 isoforms of *RAS*, *KRAS* (KRAS4A and KRAS4B) mutations are particularly prevalent in malignancies with the highest mortality rates, such as pancreatic (90%), colorectal (30–40%) and lung (15–20%) tumors.[3] Due to the high frequency of *RAS* mutations in a wide spectrum of human cancers, intensive efforts on Ras structure, biochemistry and biology have been made during the last 3 decades for "anti-RAS" therapy.

However, a clinically effective Ras inhibitor has eluded drug-discovery efforts for many years. Ras proteins lack pockets to which small molecules bind with high affinity, and also, a large family of related protein members share similar GDP/GTP-binding domain, making Ras therapeutic attack extremely challenging. Six strategies for targeting Ras signaling have been proposed[4]: targeting Ras proteins directly, upsetting Ras membrane association, exploiting synthetic lethal partners of mutant *RAS*, targeting Ras downstream pathways, disrupting the greedy metabolic habit of *RAS*-mutant cells, and harnessing the immune response. These anti-RAS strategies could be generally divided into "direct" and "indirect" approaches.

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$KRAS^{G12C}$ selective inhibitors that target the thiol to inhibit GTP binding were recently identified.[5-7] Binding of these inhibitors to $KRAS^{G12C}$ subverts the native nucleotide preference to favor GDP over GTP, therefore preventing subsequent downstream activation. Most recently, a novel cell-active and mutant-specific inhibitor targeting the allosteric switch II pocket of $KRAS^{G12C}$ showed improved potency and selectivity, further revealing that $KRAS^{G12C}$ rapidly cycles its nucleotide substrates.[8,9] These breakthroughs provide novel insights into the function of $KRAS^{G12C}$. Although further work is still needed to determine whether these inhibitors suppress the *in vivo* growth of $KRAS^{G12C}$-driven tumors, the development of $KRAS^{G12C}$ selective inhibitors opens a landmark discovery that changes the perception of Ras proteins from "undruggability" to "druggability." Ras mimetics has also been recently revealed that the clinical small-molecule inhibitor rigosertib disrupts the association of Ras with Raf and other effector proteins.[10] The discovery of new mechanism of rigosertib shed light on the translation of a clinical drug to directly treat *KRAS*-mutant cancers. Other drug development of targeting mutant *RAS* by inhibiting proteins that facilitate Ras trafficking to the plasma membrane has progressed as well. Two classes of inhibitors that direct bound to the prenyl-binding pocket of PDEδ were synthesized by the same group.[11,12] Additionally, based on the rationality that the Ras protein degradation occurs together with the β-catenin degradation, novel chemical molecules that bound directly to the regulators of G-protein signaling domain of axin and promoted Ras degradation were discovered.[13] Collectively, the "best" path to inhibit Ras has yet to be determined, as these molecules through directly targeting Ras proteins or Ras signaling have a long way before going into the clinic. Attempting to find "synthetic lethal" interactions between activated mutant *RAS* and other genes to which the cancer cells heavily addict shows enormous potential in recent years.

Synthetic lethal strategy: Challenging but promising

Synthetic lethal studies, the indirect strategy to defeat *RAS*-mutant tumors, have made another big splash, changing the landscape by uncovering vulnerabilities in tumors with *RAS* mutations. The existence of oncogene-specific synthetic lethal interaction is supported by the notion that oncogenic transformation substantially alters the cell phenotype.[14] Synthetic lethal screens uncover multiple "oncogene addiction" and "non-oncogene addiction" pathways that were required for the survival of *RAS*-mutant cells.[15] This direction has been raised on the concept of synthetic lethality that was described in invertebrate genetics one century years ago. At its simplest, the 2 or more separate genes (or pathways) are synthetic lethal if the mutations in any one of them will not change the viability of a cell but simultaneous mutations in both of them will result in a lethal phenotype. Therefore, targeting a gene that is synthetic lethal to a cancer-relevant mutation should kill only cancer cells and spare normal ones. Based on this principle, mutations in candidate genes might be either loss-of-function or gain-of-function defects. This approach has been inspired most strongly by the successful use of poly (adenosine diphosphate [ADP]-ribose) polymerase inhibitors to treat BRCA defective cancers in the clinic.[16,17] During the last decade, synthetic lethality has been intensively exploited for identification of novel anticancer targets, development of new genotype-selective anticancer agents, and characterization of genes associated with drug responses.[18,19]

In the Ras research community, several studies from different groups have identified synthetic lethal interactors with mutant *KRAS* by using large-scale RNA interference (RNAi) screens.[14,20,21] Since normal cells lack mutant *KRAS*, genes or inhibitors identified in this manner should in principle be selectively lethal for tumors but not for untransformed cells. Although the first generation of screens led to more novel acknowledgment of the dependency of mutant *KRAS* but not many tractable drug targets, potential interactors of mutant *KRAS* identified by synthetic lethal RNAi screens, such as the anti-apoptotic protein BCL-XL, cyclin-dependent kinase CDK4 and serine/threonine-protein kinase TBK1, have already been translated to clinical settings to treat *KRAS*-mutant cancers.[3]

RNAi-based synthetic lethal screens

Synthetic lethal screens have now progressed from drosophila model system to genome-wide short hairpin RNA- and small interfering RNA-mediated drug-sensitization screens and novel small-molecule inhibitor screens. This approach is particularly attractive for those oncogenic drivers or tumor suppressors, such as *KRAS, MYC* and *TP53*, which were usually thought "undruggable."

During the last 10 years, intensive studies have taken advantage of synthetic lethal approach to identify genes that maintain the tumor phenotype with "addiction" to mutant *KRAS*. Genome-wide RNAi screens and other technologies have identified a list of candidate genes, including TANK-binding kinase 1,[20] serine/threonine-protein kinase 33,[22] heat-shock protein 90,[23] polo-like kinase 1 and cell mitotic regulators,[14] the cyclin-dependent kinase 1 and 4,[24,25] transcription factor GATA2,[21] evolutionarily conserved gene enhancer of rudimentary

homolog,[26] transforming growth factor β-activated kinase 1,[27] anti-apoptotic BH3 family member BCL-XL,[28] proteasome and topoisomerase components,[14,29] genes that are involved in glucose metabolism[30] and SUMO E2 ligase Ubc9.[31] These hit genes span diverse different cellular regulations, including cell mitosis, cell apoptosis, cell growth, cell metabolism, and gene replication, transcription and modification (Fig. 1). These important works broaden the view of the biology and function of mutant *KRAS*. In our recent paper, a combinatorial clinical drug screen based on synthetic lethality revealed a preclinical feasibility for combining a polo-like kinase 1 inhibitor and a Rho signaling inhibitor to conquer *KRAS*-mutant lung cancer.[32] Such combined regimen activates the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, a critical regulator of cell cycle and cell mitosis. Because mutations in *KRAS* have been suggested to contribute to chromosome instability and mitotic stress,[33] further load of mitotic stress by activating $p21^{WAF1/CIP1}$ would cause susceptibility to apoptosis in *KRAS*-mutant cells.

Synthetic lethal studies based on *KRAS* mutations illustrate the great potential to improve our understanding of Ras signaling and open new possibilities for blocking the Ras' functions. Although only a modest overlap is found among these identified synthetic lethal targets, and some targets are intractable and still lack effective inhibitors, the strategy based on synthetic lethality dose open up a new avenue to understand the dependency features of *KRAS*-mutant cancers.

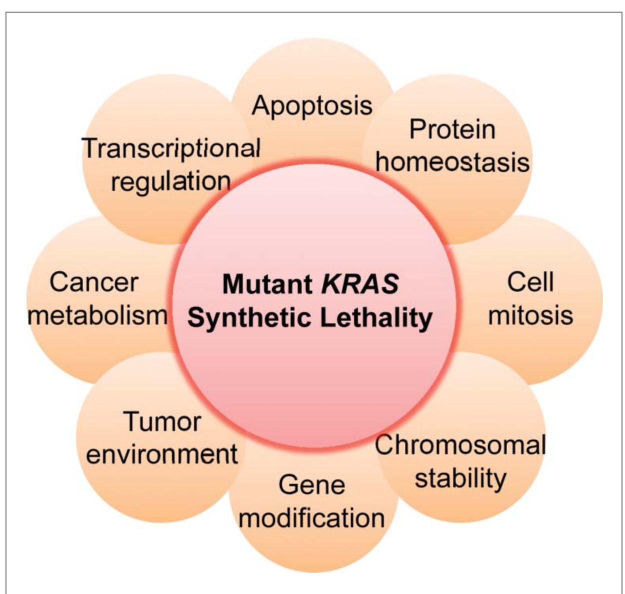

Figure 1. The dependency of mutant *KRAS* on diverse cellular regulations. Synthetic lethal interactors of mutant KRAS identified by several synthetic lethal screens span different cell phenotypes, such as cell apoptosis (e.g. *BCL-XL*), cell mitosis (e.g., *PLK1*, *CDK4* and *CDKN1A*), transcriptional regulation (e.g. *GATA2*) and protein homeostasis (e.g., *HSP90*). Clinical inhibitors targeting some of these synthetic lethal partners of mutant KRAS have been tested in clinical settings for *KRAS*-mutant cancers through a combinatorial strategy with a MEK inhibitor (e.g. NCT02079740 NCT02258607 and NCT02022982).

Chemical-based synthetic lethal screens

To advance the discovery of drugs against *KRAS*-driven tumors, synthetic lethal small-molecule inhibitor screens have concomitantly emerged and represent a complementary approach to directly identify drugs that target the essential signaling for the growth of *KRAS*-mutant tumors. Compared with RNAi-based screens that only downregulate gene expression, chemical-based screens show great advantages in regulating genes' functions. For example, drug-induced inhibition of enzymatic activity functions distinctly from loss of expression of a protein. At this point, chemical drugs are likely to be better at regulating multiple related isoforms of a protein. Therefore, chemical screens are expected to give significantly different insights to functional genomic screens.[34] Additionally, the use of well-known, clinical approved agents in a synthetic lethal screen in *KRAS*-mutant and wildtype cells has the advantage of sorting out both gain-of-function as well as loss-of-function candidate factors for synthetic lethal interaction with mutant *KRAS*. Such approach provides a shortcut to utilize drugs that are already in clinical use. Here we will briefly list some novel-structure compounds and clinical drug combinations discovered based on synthetic lethality to treat *KRAS*-mutant cancers.

Chemical libraries were usually utilized by the synthetic lethality technology to identify novel-structure anticancer drugs for killing *RAS*-mutant cells.[35] Novel compounds triphenyl tetrazolium and a sulfinyl cytidine derivative were identified through a drug screen based on isogenic cells lines of *KRAS*-mutant colon cancer. This class of compounds displayed selective activity in *vitro* against tumor cells and inhibited tumor xenografts containing mutant Ras.[36] Compound erastin exhibited lethal selectivity in human tumor cells harboring mutations in the HRAS, KRAS or BRAF oncogenes by modulating mitochondrial voltage-dependent anion channels.[37] Other compounds, such as oncrasin-1,[38] lanperisone,[39] and oncrasin analogs[40] all induced cytotoxic effects in *KRAS*-mutant tumor cells by triggering oxidative stress and regulating oxidative stress-related pathways. However, the precise target and target specificity of these compounds are still unclear.

Tumor initiation, progression and high heterogeneity are primarily driven by multiple genetic mutations rather than by a single defect.[41] Resistance and partial responses to targeted monotherapy are major obstacles in cancer

treatment. To achieve more potent efficacy and less toxicity, a "cocktail" of drugs is now often given.[42] Based on this rationale, many significant works have been conducted to search for effective drug combinations to defeat *KRAS*-mutant cancers through synthetic lethality. Inhibition of *KRAS*-mutant tumors by using surrogate drugs already approved for clinical use is the fast way for translational research. By RNAi library screening, transcriptional factor GATA2 was identified as a synthetic lethal gene of mutant *KRAS*. Pharmacological inhibition of GATA2-mediated pathways with bortezomib (a proteasome inhibitor) and fasudil (a ROCK inhibitor) results in dramatic tumor inhibition.[21] By far, MEK inhibitors have showed potential effectiveness in patients with *KRAS*-mutant cancer and have been approved by the US Food and Drug Administration. A large-scale screen of short hairpin RNA with MEK inhibitor as a backbone identified that BCL-XL inhibitor ABT-263 in combination with a MEK inhibitor led to dramatic apoptosis in many *KRAS*-mutant cell lines from different tissue types.[28] This study promotes the initial clinical trial for *KRAS*-mutant cancer (NCT02079740) by using a combined regimen of navitoclax (an inhibitor of BCL-2 and BCL-XL) and trametinib (an MEK inhibitor).[3] In a similar screen system, the tyrosine kinase TBK1[20] and interphase cyclin-dependent kinase CDK4[24] were identified as synthetically lethal partners of mutant *KRAS*. These works lead to ongoing clinical trials combing momelotinib (a dual JAK2 and TBK1 inhibitor; NCT02258607) or palbociclib (a CDK4/6 inhibitor; NCT02022982) with a MEK inhibitor for treating *KRAS*-mutant cancers. Another study showed that agents enhancing proteotoxic stress, such as the Hsp90 inhibitor IPI-504, induce tumor regression in aggressive mouse models when combined with rapamycin (a mTOR inhibitor).[33] Our recent work show that dual inhibition of polo-like kinase 1 and RhoA/Rho kinase leads to the synergistic effects in *KRAS*-mutant lung cancer. Mechanism study revealed a new synthetic lethal interaction between *KRAS* and *CDKN1A* (encoding p21),[32] as genetic or pharmacological increase of $p21^{WAF1/CIP1}$ level preferentially impairs the growth of *KRAS*-mutant cells. Most recently, a combinatorial strategy by combining a FGFR inhibitor and a MEK inhibitor for treating *KRAS*-mutant lung cancer has been reported.[43] These synthetic lethal chemical screens based on available clinical drugs and drug combinations unveil new armamentaria to fight *KRAS*-mutant cancer.

Synthetic lethality: System development

As mentioned above, synthetic lethal partners of mutant *KRAS* identified by the first generation of gene screens lacks of reproducibility. Changes in context of cell model and analytic approaches could easily affect the outcome of the results, making the efforts difficult to bear fruits. Considering the complexities of the Ras proteins and several problems in the current experimental system of synthetic lethal screens, several optimization strategies have been addressed.

KRAS specific mutations and cancer sub-classification

There is now emerging recognition that the human Ras proteins are not functionally identical, and there are mutation-specific consequences on Ras structure, biochemistry and biology.[44] Although *KRAS* mutation is prevalently present in pancreatic, colon and lung cancers, the hot mutations and mutation-specific signaling pathways of *KRAS* in these cancer types are dramatically different. Not all mutant K-Ras proteins affect patient survival or downstream signaling in a similar way. Most mutations of *KRAS* occur at codons 12 and 13, and the $KRAS^{G12C}$ mutation is the most common mutation in lung cancer, which is quite different from other cancer types. Difference in mutation frequency may reflect different biological characteristics of a mutant protein. For example, $KRAS^{G12C}$ and $KRAS^{G12V}$ mutations in lung adenocarcinoma preferentially activate the RalGDS pathway, whereas *KRAS*^{G12D} prefers the MAPK and PI3K pathways.[45,46] The heterogeneous behavior of mutant K-Ras proteins suggests that therapeutic interventions may need to take into account the specific mutant *KRAS* expressed by the tumor. Therefore, mutation-selective cell models are needed for setting up a synthetic lethal screen. Recent identification of $KRAS^{G12C}$ inhibitors also inspires us at this point.

Multiple cancers have altered metabolic processes, and oncogenic *KRAS* has been shown to be a key player in promoting metabolic rewiring. It has been demonstrated that mutant $KRAS^{G12D}$ is responsible for orchestrating metabolic phenotype of pancreatic cancer cells in part through its role in reprogramming anabolic glucose metabolism.[47] Mutation in *KRAS* has also been reported to facilitate pancreatic cancer cells to addict to glutamine for maintaining their redox homeostasis.[48] However, the specific actions of oncogenic *KRAS* on metabolic regulation may differ depending on tumor types and genetic context (including difference in mutant *KRAS* copy number).[49] For example, the *in vivo* evidences of metabolic rewiring during lung cancer malignant progression showed that mutant $KRAS^{G12D}$ homozygous cells exhibited an increase in glucose metabolism toward the tricarboxylic acid cycle and glutathione synthesis, leading to enhanced glutathione-mediated detoxification.[49]

Therefore, effective sub-classification of *KRAS*-mutant cancers will be required to improve anti-RAS therapy through personalized medicine in the near future.

Cell models

Cell model is another limitation in the first generation of mutant *KRAS* synthetic lethal screens. Most of previous screens relied on isogenic cell lines or a panel of laboratory cancer cell lines, which could hardly mimic tumor heterogeneity. Isogenic cell lines were usually generated by ablation or overexpression of mutant *KRAS*. It is quite possible that such gene editing causes adaptive alterations in other oncogene drivers or other signaling pathways. As our experiences, it is better to use early passages of isogenic cells generated by the CRISPR/Cas9 system-mediate-knockout or by adeno-associated virus-mediated overexpression of a *KRAS* mutation. Additionally, it is also strongly suggested to rigorously detect the activation of Ras proteins (Ras-GTP state) in isogenic cell lines during synthetic lethal screens or in cell functional assays. RNAi screening technology with low potency of knockdown usually causes a high level of false negatives and off-target effects. The recently developed gene editing technology, the CRISPR/Cas9 system, could be applied to *KRAS* synthetic lethal screens by using a genome-scale lentiviral single guide RNA library.[34] Additionally, all previous screens have utilized *in vitro* anchorage-dependent culture conditions. Future synthetic lethal screens will benefit from *ex vivo* organoid cultures or *in vivo* xenograft tumor assays, which more accurately model tumor heterogeneity and tumor microenvironment.[4] Nowadays, patient-derived xenograft models are largely acceptable for cancer research, and it offers a powerful tool for developing anticancer therapies and personalized medicine for cancer patients.[50] It is strongly recommended to utilize patient-derived xenograft models to validate the efficacy of anticancer drugs or drug combinations identified by synthetic lethal chemical screens for targeting mutant *KRAS*.

Data validation and mechanism elucidation

The hits identified from the first generation of synthetic lethal screens for mutant *KRAS* span many different cellular processes, including protein homeostasis, mitotic modulation, chromosomal stability, transcriptional regulation, gene modification, apoptosis and cancer metabolism (Fig. 1), revealing targeting "non-oncogene addition" for efficacious cancer therapies.[15] However, most of these hits are not been validated by rescue experiments and other rigorous experimental assays. It seems that analytic approaches could easily affect the outcome of the results, therefore making it hard to achieve reliable candidate hits. Additionally, the mechanism for synthetic lethal interaction of mutant *KRAS* and these identified hits are largely unknown. Although some synthetic lethal hits are roughly mapped to the Ras signaling pathway network,[19] the detailed interaction between these hits and mutant *KRAS* are still elusive. Therefore, the candidate genes from the next generation of screens should be rigorously validated, and how these genes to map to mutant *KRAS* signaling also should be clarified.

Conclusions

Despite more than 3 decades of intense efforts, an effective anti-RAS therapy has yet to reach the clinic. A better understanding of Ras structure, biochemistry, processing and signaling will open the novel possibility to defeat *RAS*-driven tumors. Several strategies to target mutant Ras proteins are progressing, but each of them has pitfalls and the best way has not been determined. It has been gradually recognized that all the human Ras proteins are not functionally identical, and therefore, mutation-selective therapeutic strategies are accordingly appreciated. Recent studies of small-molecule approaches to directly inhibit oncogenic KRAS^{G12C} have invigorated the Ras community, raising the possibility of drugging Ras that has been long considered "undruggable." However, these direct inhibitors are more considered as chemical probes to understand Ras' biology, rather agents reaching clinical application. First-generation synthetic lethal screens have identified several synthetic lethal interactors of mutant *KRAS*, and these efforts are extremely important to dissect the signaling addiction of *KRAS*-mutant cells. Some inhibitors of *KRAS* synthetic lethal partners, such as CDK4, TBK1 and BCL-XL, are currently tested in clinical trials in combination with a MEK inhibitor to treat *KRAS*-driven cancers. Given the complexity of Ras proteins in different tumor tissues and the less reproducibility of those identified *KRAS* synthetic lethal interactors, the experimental system of synthetic lethal screens should be properly improved by taking into account *KRAS* specific mutations, cancer sub-classification, cell models, data validation and mechanism elucidation. The major challenge with current cancer treatments is drug resistance, making combination therapies by simultaneously targeting multiple cancer-associated pathways a necessary for efficacious anti-RAS treatments.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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