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Spotlight

Mapping Effector–Phenotype Landscapes in KRAS-Driven Cancers

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Oncogenic KRAS can activate numerous effector pathways to drive malignant progression. However, the relationships between specific effectors and oncogenic phenotypes, and the extent to which these relationships vary across heterogeneous tumors, are incompletely understood. Recently in *Cell Reports*, a team of scientists described an innovative, combinatorial siRNA-based approach to functionally link KRAS effectors and phenotypes in a large panel of cancer cell lines. Central to this work was the identification of two major subtypes of *KRAS*-mutant cancers with distinct effector landscapes and tractable therapeutic vulnerabilities.

It is well established that oncogenic KRAS can activate numerous effector pathways that contribute to tumor progression, including most notably the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and RalGDS pathways. Through its effector pathways, KRAS drives oncogenic phenotypes like proliferation, survival, metastasis, and metabolic reprogramming. However, we lack a comprehensive understanding of which KRAS effector pathways regulate these phenotypes, a difficult knowledge gap to traverse owing to two central challenges. First, various lines of evidence suggest that the key KRAS effector pathways driving malignancy are likely to vary across, and even within, tumors arising from distinct tissue lineages. Second, genetic approaches to dissect KRAS-driven effector–phenotype relationships are hampered by the fact that genes encoding nodes in the RAS pathway are highly redundant and thus single-gene knockdown or knockout is likely to underestimate the impact of targeting multiple effector node paralogs simultaneously.

In a study recently published in *Cell Reports*, a team led by Frank McCormick and Cyril Benes made major strides toward the goal of mapping the effector–phenotype landscapes in *KRAS*-mutant cancers [1]. They designed and validated potent siRNA molecules against 84 RAS pathway genes then used these to create siRNA combinations targeting each of the paralogs in 41 major KRAS effector nodes. To control for variable transfection efficiency, they stably expressed enhanced GFP (EGFP) in each cell then cotransfected all siRNA combinations alongside siRNA targeting EGFP so that the abundance of EGFP could be used to infer knockdown efficiency on a single-cell basis with flow cytometry. For each node knockdown, they then measured multiple phenotypic outputs, including cell viability, abundance of reactive oxygen species (ROS), proliferation, growth, and death, each using multicolor flow cytometry. Together, this siRNA effector node (siREN) screen allowed the construction of highly resolved, single-cell-derived dose–response curves for each phenotypic output in a panel of 92 lung, pancreas, and colon cancer cell lines, 64 of which were *KRAS* mutant (Figure 1A).

This *tour de force* effort revealed that every cell line harbors a unique combination of effector dependencies, with only four nodes affecting viability in >80% of lines and five nodes failing to affect viability in any line. However, despite this heterogeneity most lines fell into one of two major, largely nonoverlapping functional groups. Approximately two-thirds of *KRAS*-mutant lines were dependent on KRAS and canonical downstream RAF/MAPK activity for survival. Consistent with prior evidence, these 'KRAS-type' lines were more epithelial than their KRAS-independent counterparts and also dependent on wild-type HRAS and NRAS, RAF kinases, and, to an extent, RAC, RGL, and autophagy [2–4]. Interestingly, the remaining one-third of *KRAS*-mutant cell lines were KRAS independent and instead dependent on the RSK p90 S6 kinases (RSKs), with dependency landscapes that more closely resembled *KRAS*-wild-type lines than *KRAS*-mutant, KRAS-dependent lines. These 'RSK-type' lines were more mesenchymal in nature and were dependent on glutaminase, mTOR, and KSR. Interestingly, they retained a dependence on wild-type HRAS and NRAS, suggesting potential noncanonical RAS effector activation mechanisms. By expanding the study of RSK-type lines using gene-expression profiling and small-molecule screens, it was revealed that these lines have increased sensitivity to PDK1, RSK, mTOR, and S6K inhibitors, a finding corroborated by analysis of data from an independent small-molecule profiling effort [5]. Further, they have higher expression of genes used in oxidative phosphorylation and greater sensitivity to inhibitors of DNA repair enzymes, perhaps a result of increased ROS levels and consequent DNA damage in these cells. Critically, gene-expression signatures of the KRAS- and RSK-type cell lines can be projected onto human *KRAS*-mutant tumors analyzed by The Cancer Genome Atlas (TCGA) project, suggesting that these lines model authentic states found in the human disease. Paired with information about differential drug sensitivity from small-molecule screens, these signatures could, in theory, help guide treatment decisions (Figure 1B).

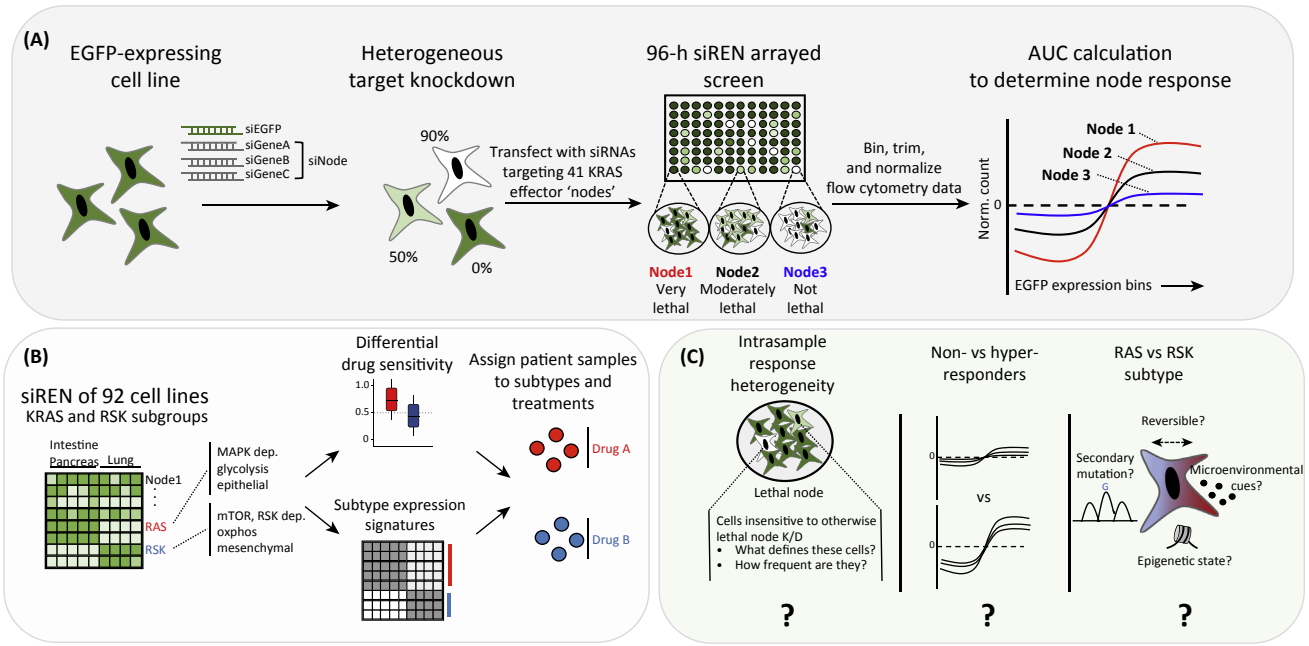

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Figure 1. An siRNA-Based Approach to Mapping *KRAS* Effector Phenotypes. (A) The siRNA effector node (siREN) assay utilizes enhanced GFP (EGFP)-expressing cells to link the effects of heterogeneous target knockdown to a phenotype of interest. Pools of siRNAs are cotransfected targeting the relevant paralogs for a given 'node' along with an siRNA targeting EGFP. The arrayed screen is read by flow cytometry and relative enrichment/depletion is calculated across 75 bins of increasing EGFP expression, allowing calculation of the area under the curve (AUC) to determine node response. (B) The siREN assay was conducted across 92 cell lines and revealed two distinct subtypes of *KRAS*-mutant cancer, those dependent on either *KRAS* or *RSK*. These subtypes mapped to differential drug responses and gene-expression signatures and revealed distinct metabolic and developmental states for each subset. (C) Some outstanding questions from this study concern intrasample and overall response heterogeneity as well as what determines a given tumor's subtype (*KRAS* vs *RSK*).

These findings support and extend the results of several influential studies from recent years. For example, the idea that *KRAS*-mutant lines are sometimes KRAS independent is consistent with earlier findings from Jeff Settleman and colleagues, as is the concept that these lines tend to be more mesenchymal in nature [2]. The concept of KRAS independence is also consistent with the observation, in both patients and cell lines, that tumors frequently exhibit unexplained intrinsic resistance to oncogene-targeted therapy. The fact that node dependencies varied in a tissue-dependent manner is consistent with the idea that KRAS activation in different tissues is driven by different mutational mechanisms and occurs in the context of different cooperating genetic events and signaling topologies, stages of tumor development, and microenvironmental selection pressures [6]. Further, these data are consistent with recent evidence from CRISPR–Cas9 screens demonstrating that patterns of cooperativity in KRAS effector pathways vary in a tissue-dependent manner [7]. Finally, as the authors point out, the heterogeneity in effector signaling across *KRAS*-mutant cell lines underscores the challenge of finding universal KRAS synthetic lethal partners. However, these data also validate previous findings of context-specific synthetic lethality involving, for example, RAC1 and autophagy in KRAS-type tumors and oxidative phosphorylation and NF-κB signaling in RSK-type tumors [3,4,8–10].

This work also points to important, unresolved questions in KRAS tumor biology and therapy. From a heterogeneity perspective, this study nicely demonstrates the variation between KRAS-driven cell lines, but it may also be powered to inform on the variation within cell lines. For example, rare cells that survive ablation of an otherwise 'lethal' node could provide information on the treatment-refractory subset of cells capable of driving resistance. At the level of effector–phenotype heterogeneity, understanding the drivers of non- versus hyper-responding lines could provide useful insights on how effector signaling topologies are differentially wired and regulate survival downstream of *KRAS*. Additionally, the manner in which tumor lineage,

cooperating mutations, expression or energetic states, and microenvironmental cues give rise to KRAS and RSK subtypes is still not understood. Similarly, it remains to be determined whether these subtypes can coexist in individual tumors or patients and whether a single cell can transition from one subtype to the other in response to cell-intrinsic or -extrinsic changes (Figure 1C). Finally, the dependencies of the subset of *KRAS*-mutant human tumors from the TCGA that could not be mapped to KRAS and RSK subtypes remain to be determined.

Yuan *et al*. provide an innovative strategy and dataset to help unravel *KRAS*-associated heterogeneity, assigning much-needed biological and clinically actionable structure to the effectors downstream of this classically intractable oncogene. Future studies will need to continue in this vein by understanding how these phenotypes vary within single tumors and how that influences the rational design of therapeutic strategies.

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Spotlight

Turning Cold Tumors Hot by Blocking TGF-β

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Novel immune therapeutic tools are rapidly expanding the anticancer arsenal. Despite this progress, patients with colorectal cancer (CRC) that spreads to vital parts of the body still have a dismal outcome. Transforming growth factor-β (TGF-β) plays a pivotal role in the development of CRC and metastasis. Important new work by Tauriello and colleagues has revealed that inhibition of TGF-β prevents tumor metastasis by enhancing a cytotoxic T cell response, suggesting that TGF-β inhibition is a promising pro-immunogenic therapy.

Despite major advances in early detection and novel therapies, CRC remains a leading cause of cancer-related death worldwide [1]. One major factor that precludes the efficacy of these new therapies is the heterogeneous nature of CRC. Recently, CRC has been classified in four consensus molecular subtypes (CMS1–4) based on gene expression profiling [2]. Each of these subtypes is associated with a specific biological program. Of particular clinical interest is CMS4 (±25% of CRCs), which is characterized by poor disease outcome owing to frequent occurrence of metastasis and resistance to therapy [3]. Cancers belonging to CMS4 or the 'mesenchymal CRC subtype' display a rich stromal infiltrate (mostly tumor-associated fibroblasts), and high expression of genes associated with epithelial–mesenchymal transition (EMT), matrix remodeling, and TGF-β. In a recent issue of the journal *Nature*, the laboratory of Eduard Batlle elegantly scrutinized the role of TGF-β in the process of colon cancer metastasis [4]. Tauriello *et al.* generated mice bearing mutations in intestinal stem cells affecting four key signaling pathways involved in the process of colon carcinogenesis: WNT, EGF, TP53, and TGF-β, defined by conditional alleles $Apc^{fl/fl}$, $Kras^{LSL\text{-}G12D}$, $Tp53^{fl/fl}$, and $Tgfbr2^{fl/fl}$ respectively. Evaluation of mice displaying different combinations of mutations showed that four mutations were required for the development of metastasis and to reproduce key features of the tumor microenvironment in advanced human CRCs, such as abundant activated fibroblasts, high levels of TGF-β, and importantly, T cell exclusion. Next, mouse tumor organoids (MTOs) derived from the quadruple mutants were established and transplanted into the cecum of syngeneic mice. Transcriptomic analysis indicated that quadruple mutants display a CMS4 phenotype *in situ* when transplanted, but not when cultured *in vitro*. These data strengthen previous work from the same laboratory indicating that the stroma plays a pivotal role in regulation of the mesenchymal features in CRC, as well as in identifying the poor-prognosis molecular subtype [5]. More generally, these findings emphasize how the tumor cell–microenvironment interaction is crucial in shaping the phenotype of cancers as well as their clinical behavior.

The highlight of the study by Tauriello *et al.* is the characterization of the effects of TGF-β inhibition in CRC. It was demonstrated that blockade of the TGF-β receptor I with galunisertib,