Uncovering Tumorigenesis Circuitry with Combinatorial CRISPR

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Oncogenesis relies on the alteration of multiple driver genes, but precisely which groups of alterations lead to cancer is not well understood. To chart these combinations, Zhao and colleagues use the CRISPR-Cas9 system to knockout all pairwise combinations among 52 tumor suppressor genes, with the goal of identifying groups of alterations that collaborate to promote cell growth. Interaction screens are performed across multiple models of tumorigenesis in cell cultures and mice, revealing clear cooperation among *NF2*, *PTEN*, and *TP53* in multiple models. These and other strongly synergistic interactions are characterized further by single-cell transcriptomic profiling. This methodology presents a scalable approach to move beyond single-gene drivers to map the complex gene networks that give rise to tumorigenesis.

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Genes rarely act independently; instead, they usually function jointly as components of larger genetic circuits (1). A common technique for exploring this circuitry is genetic interaction mapping, in which interaction between two genes is detected by comparing the phenotypic effects when both genes are simultaneously perturbed versus the phenotypic effect following individual genetic perturbations (2, 3). While most genetic interaction studies aim to detect pairwise perturbations that result in surprising cell death, a phenomenon termed synthetic lethality, Zhao and colleagues now demonstrate that genetic interaction maps can also be used to study cancer progression (4).

The authors applied genetic interaction mapping to tumor suppressor genes to find pairwise perturbations that cause tumorigenesis, a process known to require multiple mutations. For instance, in the three-strike model of tumorigenesis, a mutation is responsible for each developmental stage: initiation, clonal expansion, and metastasis (5). Therefore, to obtain a malignant tumor, a cell must have accrued a series of mutations that drive tumorigenesis.

To systematically profile pairs of mutations that cooperatively drive tumorigenesis, the authors prepared several derivatives of a commonly used nontumorigenic breast epithelial model, MCF10A. These derivatives harbored *MYC* or *PIK3CA* amplification, which drives cancer cell proliferation, or alternatively *PTEN* knockout, which releases suppression of the PI3K-AKT-mTOR pathway, driving proliferation (6). In these backgrounds, the authors interrogated all pairwise interactions among 52 tumor suppressor genes using a 35,826-element combinatorial-sgRNA library. Derivatives of MCF10A were infected with this library and cultured *in vivo* by injection into murine mammary fat pads. These cells with diverse genotypes then competed with one another for 6 to 8 weeks, allowing for the identification of epistatic interactions, which are the pairwise perturbations that result in faster than expected cell growth.

Focusing on genotypes that promote runaway growth alleviates a common pitfall of most competitive growth assays, which seek to determine genotypes that cause cell death. In synthetic lethality assays, the screen risks complete drop out of the genotypes that produce interesting growth defects if the perturbed cells are not sampled early enough. As a result, to maintain dynamic range, CRISPR screens focused on profiling lethality need to be cognizant of the temporal dynamics of the underlying interactions (7). Focusing on genotypes that result in accelerated growth solves the problem of drop out by enriching for the most interesting phenotypes.

After transplanting perturbed cells in mice, cells with a select few pairwise perturbations grew rapidly and dominated the population. Some epistatic interactions, such as *NF2-SMAD4*, *NF2-TP53*, and *BAP1-TP53*, were recovered in all three MCF10A backgrounds, but many were found in only one. Such diversity highlights the gap in understanding why certain interactions cause tumorigenesis in one context but not another. Furthermore, conserved epistatic interactions clustered around hubs of frequently mutated tumor suppressor genes such as *NF2*, *PTEN*, and *TP53*. Interestingly, many hubs appeared to form cliques, where all members of the clique interact with one another, unlike most synthetic lethality networks in which hubs rarely interact with one another (8).

In addition to screening for epistatic interactions *in vivo*, Zhao and colleagues performed the screen *in vitro* in three different culture conditions (full medium, minimal medium, and medium containing TGFβ1) to determine whether they closely resemble the interactions discovered *in vivo*. Interestingly, the *in vivo* proliferation phenotype was better captured in cells cultured in minimal media or TGFβ1, likely because runaway growth is harder to detect when cells are already growing optimally in rich media. Another explanation for this finding is that cancer cells *in vivo* are generally in hypoxic, low-nutrient conditions, which are better reflected in minimal than rich media. Nevertheless, a significantly higher abundance of epistatic interactions was identified *in vivo* than *in vitro*. This suggests that while minimal media conditions may provide a closer approximation to *in vivo* growth conditions, studies that require modeling the complex interactions between the cell and its environment, such as tumorigenesis, are still better conducted *in vivo*.

Beyond identifying epistatic interactions among tumor suppressor genes, Zhao and colleagues applied single-cell transcriptomics to

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elucidate the mechanisms underlying the most significant interactions. Much like an interaction in fitness, transcriptomic interaction can be defined as gene expression levels in double knockout cells that cannot be explained simply by an additive combination of expression levels across the single knockouts (9). Among the pairs of tumor suppressor genes examined, transcriptional synergy was observed in approximately half of all differentially expressed transcripts. As cell proliferation is well known to be closely associated with sweeping changes in transcription (10), it is perhaps not surprising that epistasis at the fitness level corresponds to epistasis in gene expression. Indeed, Zhao and colleagues reported that the transcriptional interaction score correlated strongly with the genetic interaction score.

A challenge with interpreting transcriptomic profiles is that the sequence of events can be difficult to order. For instance, transcriptional epistasis among pathways, such as translational or cytoskeletal functions, may also be attributed to the fact that disruptions in these pathways exhibit a strong growth phenotype. Whether the transcriptional changes are driven by or are driving proliferation remains unclear. Consequently, it is important to investigate the mechanisms underlying the effect a genotype has on the transcriptome. The authors proposed a model where the disruptions of tumor suppressor genes like *NF2* may lead to proliferation through E2F1 activation, but further mechanistic studies are needed to elucidate this pathway.

In summary, the study by Zhao and colleagues provides one of the first examples of combinatorial CRISPR screening to study tumorigenesis *in vivo*, reaffirming that tumorigenesis is a complex process that requires the interaction of multiple genetic perturbations. This study highlights the importance of considering the interaction network that underlies any complex phenotype, not just growth or tumorigenesis. With single-cell RNA sequencing technology, many phenotypes that would not have been tractable to measure in a CRISPR screen can be approximated by transcriptional profiles, further expanding the phenotypes that can be studied. Given this work, one can expect that future studies will leverage similar strategies to study the genetic circuitry of cells.

Authors' Disclosures

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