PrePAIRing Cas9s for screening success

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Orthogonal CRISPR–Cas9 nucleases enable more efficient high-throughput screens for deciphering genetic interactions.

Single-gene functions can be evaluated by either deletion or perturbation, but single-gene approaches cannot always assign genes to pathways or networks. Complete gene networks can be built by determining the phenotypes of all possible combinations of double mutants in yeast and bacteria. However, in mammalian cells the number of potential pairwise combinations needed for a genome-wide double-knockout screen is daunting, and current methods to target two-gene pairs suffer from low accuracy and efficiency. In this issue, two groups report dual-gene screens that use two orthologous CRISPR–Cas9 proteins to substantially increase the efficient targeting of gene pairs[1,2]. These approaches can identify synthetic lethal gene pairs, buffering gene-pairs and the directionality of genetic interactions. Going forward, they promise coverage of a much larger fraction of the combinatorial space of gene interactions in mammalian cells than is currently possible.

Synthetic lethal interactions, in which the simultaneous perturbation of two or more genes results in cell death, can identify genetic vulnerabilities in pathways and networks. These so-called synthetic lethal screens reveal vulnerabilities that are good targets for therapeutics, especially cancer therapeutics. For example, inhibition of poly (ADP-ribose) polymerase (PARP) is an effective treatment for breast cancer, but only in those patients that carry mutations in *BRCA* genes[3,4].

Identification of all the interacting gene pairs in higher eukaryotes is difficult. First, there is an immense number of potential two-way gene combinations (roughly 400,000,000 for the 20,000 or so protein-coding human genes); and second, synthetic lethal interactions are rare. RNA interference (RNAi)-based large-scale screens have been applied to identify synthetic lethal interactions[5]. However, RNAi screens are plagued with drawbacks, such as variable knockdown efficiency and extensive off-target effects, resulting in compromised efficiency and reproducibility.

CRISPR–Cas9 has previously been applied for paired-gene knockout screens in human cancer cells. For example, by making double knockouts with CRISPR–Cas9 in the same cell, Han *et al.* screened all possible two-gene combinations of 207 genes[6], and Shen *et al.* screened combinatorial knockouts of 73 genes[7]. Both screens identified previously unknown genetic interactions with therapeutic relevance. One problem that can arise when using two gRNAs in one cell is that homologous sequences present in vectors harboring gRNAs can recombine and reduce editing efficiencies. Indeed, identical sequences in promoters may lead to recombination among lentiviral constructs, resulting in compromised screen efficiency[8].

One way of trying to overcome the problem of recombination is to use two orthologous U6 promoters (hU6 from human and mU6

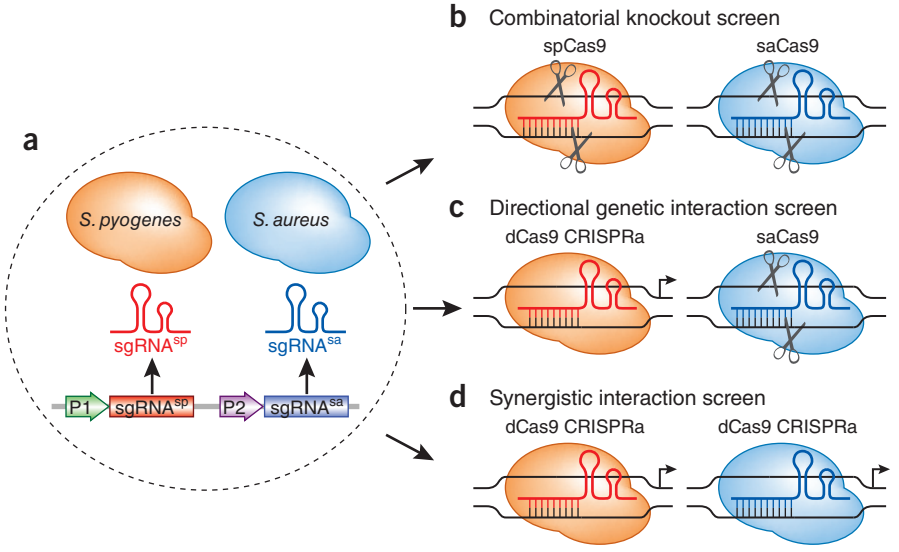

Figure 1 Using orthogonal CRISPR–Cas9 proteins for high-throughput genetic screens. (**a**) Before being loaded onto their cognate Cas9 proteins, the spCas9 sgRNA (sgRNAsp) and saCas9 sgRNA (sgRNAsa) are expressed from two distinct promoters (P1 and P2, respectively). This strategy overcomes two problems to achieve more efficient combinatorial screening: first, recombination between similar or repetitive promoters; and second, unequal targeting due to competition between two sgRNAs for a common Cas9. (**b**) Najm *et al.*[1] used a dual-gene knockout screen to identify synthetic lethal genepairs. (**c**) Both groups coupled gene knockout and gene transcriptional activation (dCas9 CRISPRa) to probe the directionality of genetic interactions. (**d**) Orthogonal CRISPR systems could, for example, map synergistic interactions of genes by CRISPRa-mediated dual gene activation.

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from mouse) to express paired guide RNAs (pgRNAs), as reported by Han *et al.*[6] and Shen *et al.*[7], but problems may still occur. For instance, identical scaffold sequences of *Streptococcus pyogenes* gRNAs are likely to mediate recombination. In addition to problems caused by recombination, distinct gRNAs may compete for loading onto a common Cas9 protein, both of which will cause unequal targeting and decreased targeting efficiency. Najm *et al.*[1] and Boettcher *et al.*[2] devise solutions to these problems using orthogonal Cas9 nucleases from *S. pyogenes* (spCas9) and *Staphylococcus aureus* (saCas9) (**Fig. 1**) for perturbation of gene pairs.

Najm *et al.*[1] use spCas9 and saCas9 for dual-gene targeting. First, to increase SaCas9 utility, they optimized design of saCas9 sgRNAs by combining experimental data with machine learning, using anapproach similar to those previously applied to improve spCas9 sgRNA design[9]. Then they built a pgRNA library comprising random pairs of 96 $sgRNA^{sa}$ and $sgRNA^{sp}$ guides, to yield 9,216 pgRNAs in total. Synthetic lethal interactions were identified in several groups of functionally related genes, such as those in MAPK and AKT pathways, which served to validate their approach. In comparison with previously published CRISPR–Cas9 screens, the use of two sgRNAs for orthologous Cas9 enzymes by the authors improved screen efficiency, as demonstrated by the increased statistical confidence of their data[1]. Najm *et al.*[1] also report an orthogonal screen using simultaneous dead spCas9-mediated gene activation and saCas9-mediated gene knockout, which led to the discovery of new genetic interactions associated with the *TP53* gene.

Boettcher *et al.*[2] came up with a similar strategy to carry out a dual screen in which one gene is activated while a second gene is deleted in the same cell. They applied saCas9 and dead spCas9 together to identify directional dependencies in genetic networks. First, they carried out a genome-scale CRISPR activation (CRISPRa) screen in the chronic myeloid leukemia K562 cells to identify coding or non-coding genes that confer cell susceptibility to imatinib (Gleevec), a tyrosine kinase inhibitor. Then they built a CRISPR library that combined activating $sgRNAs^{sp}$ that targeted 87 candidate genes identified in their CRISPRa screen with knockout $sgRNAs^{sa}$ that targeted 1,327 known cancer-relevant genes. Using a screen for cell fitness in the presence of imatinib and simultaneously overexpressing and deleting genes in the same cell, they identified more than 2,000 gene–gene interactions. They coupled this with a new analytical approach for scoring directional genetic dependencies of the genes, and interpreted their data to produce a Ras-centric directional genetic interaction network.

Both studies exploit the use of orthologous Cas9 proteins to screen for genetic interactions more effectively than was possible using a single Cas9 editor. Najm *et al.*[1] tested whether using different Cas9 enzymes minimizes the competition between two sgRNAs, and found that this tactic increased overall targeting efficiency. Boettcher *et al.*[2] used two different Cas9 enzymes and two different genome engineering approaches (knockout and transcriptional activation), which enabled them to determine the hierarchy of genes in the same pathway.

Although valuable methods to identify genetic interactions, orthogonal CRISPR screens still have their limitations. For example, combinatorial knockout screens may introduce multiple double-stranded breaks (DSBs) in a chromosome, likely resulting in chromosome instability and cell death[10]. In a synthetic lethality screen for rare, underrepresented gene pairs, this non-specific cytotoxicity could substantially increase the false-discovery rate. Application of DSB-independent genome editing methods, such as CRISPR-mediated base editing, may help to address these problems.

In future, we envisage that the use of orthogonal genome editors could be exploited in myriad ways to probe genetic interactions. For example, activation of two genes in one cell could be applied to find synergistic gene pairs (**Fig. 1**). Or, gene inhibition and epigenome modification could be combined using orthogonal genome editors. It is feasible that more than two orthogonal genome editors could be applied to allow more complex genetic combinations to be tested.

Notwithstanding the advances published in this issue, it remains a daunting task to perform a genome-wide screen for genetic interactions because the number of combinations simply exceeds current methodological capabilities. Perhaps for now our choice of gene combinations should be focused on a subset of genes, especially for those screens that have potential therapeutic relevance. Combining orthogonal CRISPR screens with cancer genomics data sets and applying computational approaches to analyze these data[11,12] might help us to start to understand global genetic interaction networks.

Application of orthologous genome editor combination technologies may finally provide the efficiency that is needed to accelerate the discovery of gene networks in human cells. Robust gene networks will, in turn, shed light on gene function in disease and underpin the identification of better therapeutic targets.

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The authors declare competing financial interests: details are available in the online version of the paper.

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