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# CRISPR approaches to small molecule target identification

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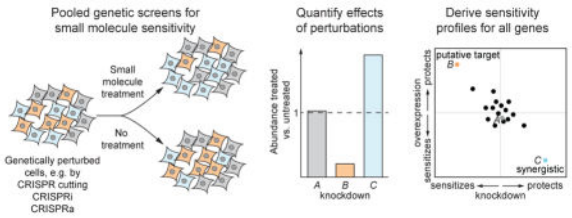
## Abstract

A long-standing challenge in drug development is the identification of the mechanisms of action of small molecules with therapeutic potential. A number of methods have been developed to address this challenge, each with inherent strengths and limitations. We here provide a brief review of these methods with a focus on chemical-genetic methods that are based on systematically profiling the effects of genetic perturbations on drug sensitivity. In particular, application of these methods to mammalian systems has been facilitated by the recent advent of CRISPR-based approaches, which enable one to readily repress, induce, or delete a given gene and determine the resulting effects on drug sensitivity.

## Graphical Abstract




## Main text

### The importance of identifying molecular targets

Small molecules provide critical tools both for basic biological discovery and therapeutic benefit. Traditionally, such small molecules were frequently derived from natural or man-made products with interesting physiological effects, but with advances in combinatorial chemical synthesis and screening methodologies, active compounds are now largely identified from cell-based phenotypic screens or *in vitro* screens for inhibition of a defined target. Regardless of origin, central to the utility of such a compound is an understanding of the molecular target(s) through which the compound exerts its physiological effect as well as a comprehensive understanding of off-target effects. For drug candidates, this knowledge is critical for subsequent development toward increased efficacy and for selection of patient

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populations that might respond most effectively to treatment. Understanding how drugs act is becoming particularly important with the interest in precision medicine efforts[1], in which therapies are precisely targeted at the genetic and environmental background of a patient and which therefore require drugs with high specificity for well-defined targets. For chemical probes, a lack of definitive functional characterization can severely confound results, limiting their utility[2].

Given the importance of target identification (also termed target deconvolution), it is perhaps not surprising that a number of experimental and computational methods have been developed to address it, each with its advantages and blind spots. Many of these methods have been reviewed in detail previously[3, 4, 5, 6, 7, 8, 9]. Experimental strategies largely fall into three major categories: chemical-genetic methods, affinity-based biochemical methods, and comparative profiling methods. We here provide a brief review of these methods while focusing in more depth on hypothesis-free chemical-genetic screening methods, which rely on systematically profiling the effect of genetic perturbations on sensitivity to small molecules (Figure 1). After pioneering work in yeast[10, 11], these chemical-genetic methods have now come to fruition in human cells with the advent of CRISPR-based genome-wide screening approaches[5, 12].

## Development of chemical-genetic strategies for target identification in yeast

The central tenet of chemical-genetic strategies is that sensitivity to a small molecule or drug is influenced by the expression level(s) of its molecular target(s)[10, 11]. This link between chemical sensitivity and gene dosage was clearly established in yeast: cells carrying a loss-of-function mutation in a specific pathway were typically found to be sensitive to drugs targeting that pathway[13], and inversely, increasing the dosage of a drug's molecular target by expression from a multicopy plasmid conferred resistance[14, 15]. These observations implied that for drugs with unknown mechanisms of action, target hypotheses should emerge from identification of genes whose expression levels modulate sensitivity. The next challenge was to rapidly and comprehensively identify such genes.

Methods to address this challenge first came about in yeast, specifically *Saccharomyces cerevisiae*, fueled by the completion of its genome sequence over 20 years ago[16] and the ensuing rapid advent of functional genomics methods. As part of efforts to understand the functions of poorly characterized genes, libraries of diploid strains carrying heterozygous deletions of genes were created[17, 18]. Such deletions result in reduced gene dosage; thus, strains with deletions in a gene or pathway targeted by a drug should exhibit hypersensitivity to the drug. Indeed, this relationship held for several known drug:target pairs such as tunicamycin:*ALG7* and benomyl:*TUB1*.[19]

At the same time, the development of methods to generate barcoded strain libraries[20] coupled with the advent of microarray technology[21, 22], which enabled quantification of the abundance of each individual strain in these libraries, allowed for parallel pooled screens in many conditions. Thus, drug sensitivity as a function of gene dosage could be determined for many genes and drugs simultaneously by exposing a pooled library of barcoded heterozygous deletion strains to a drug and quantifying the change in abundance of each strain after the treatment (Figure 1). In a proof-of-principle implementation of this

methodology, termed haploinsufficiency profiling (HIP), tunicamycin sensitivity was profiled for a panel of strains carrying heterozygous deletions of selected essential genes, which revealed depletion of *ALG7* heterozygotes, among others[19]. Genome-wide HIP has since been applied to reveal the molecular targets and mechanisms of action as well as modifiers of sensitivity of a wide diversity of drugs including the cancer therapeutics 5-fluorouracil and methotrexate and the antifungal agent fenpropimorph[23, 24].

In separate implementations of this concept, HIP screens were performed in arrayed format, in which strains carrying individual heterozygous deletions are robotically arrayed onto agar plates supplemented with compounds of interest and colony size is monitored to quantify growth rate.[25] Alternative approaches to modulating gene function or expression were also developed, such as isolating temperature-sensitive alleles with compromised function or tagging the 3′-UTRs of genes with antibiotic markers, which destabilizes the corresponding mRNAs and thereby reduces expression levels (decreased abundance by mRNA perturbation, DAmP)[26]. Similar to HIP, profiling the chemical sensitivities of temperature-sensitive or DAmP strains allows for identification of genes targeted by small molecules[27, 28, 29].

Methods to reduce but not entirely eliminate expression are required for essential genes, as by definition homozygous deletion of such genes is lethal. For non-essential genes, however, both alleles can be deleted. Profiling drug sensitivities of libraries of such homozygous deletion mutants (homozygous profiling, HOP), in analogy to the HIP approach, can provide additional information on drug mechanisms of action. In particular, although HOP rarely reveals the direct target of a drug because most cytotoxic drugs target essential genes, HOP can identify modifiers of sensitivity and mechanisms of resistance such as efflux pumps[30, 31]. More importantly, the measured sensitivity phenotypes can be used to generate profiles of genetic dependencies for each drug, which are similar for drugs with similar mechanisms of action. Thus, after generating reference profiles for a set of drugs with known mechanisms of action, a clustering or pattern matching approach can be employed to classify poorly characterized drugs by similarity of their genetic dependencies[30, 32]. This pattern matching approach was recently extended to phenotypes derived from both HIP and HOP for a large panel of >3,000 chemicals, generating an array of fitness signatures that allowed for large-scale assignment of molecular mechanisms of action[33].

Loss-of-function profiling approaches such as HIP and HOP have enjoyed substantial success in chemical genetics efforts. A potential drawback to such approaches, however, is that sensitivity or resistance to a drug upon reduced expression of a gene can stem from pleiotropic or indirect effects rather than direct interaction of the gene product with the drug. A complementary approach therefore is to profile the effect of targeted overexpression of genes on drug sensitivity (multicopy suppression profiling, MSP), as increased levels of the molecular target of a drug often lead to resistance[14] (with exceptions for subunits of multi-protein complexes or for cases in which the drug-target complex mediates toxicity such as for some topoisomerase-I inhibitors). Such MSP screens were used, for example, to demonstrate that phenylaminopyrimidine targets protein kinase C (Pkc1)[34] and to identify genes in the TOR pathway by profiling rapamycin sensitivity[35].

Combining deletion and overexpression profiling approaches further increases the sensitivity in identifying molecular targets. In particular, genes directly on the pathway targeted by a small molecule are expected to have anti-correlated phenotypes in deletion and overexpression profiling, whereas individual loss-of-function or gain-of-function screens may be prone to false negatives or false positives (see above). Indeed, in a proof-of-principle study, integration of HIP, HOP, and MSP data readily identified the molecular targets of several small molecules with unknown mechanisms of action with improved sensitivity over any of the individual approaches[36].

Yeast was a natural test bed for these chemical-genetic approaches, and further efforts are ongoing, including the mapping of structure-function relationships and drug-drug interactions as well as the profiling of human alleles without yeast counterparts *via* heterologous expression. Despite the advantages of yeast as a model system, it has intrinsic limitations in identifying the molecular targets of drug candidates or probes for use in human cells. In particular, yeast has a cell wall that influences drug activity, many essential genes or their isoforms or drug binding pockets are not conserved between yeast and humans, and yeast cannot recapitulate the complex organization of a multicellular organism. Thus, with the intellectual groundwork of chemical-genetic approaches laid by these pioneering efforts, attention turned increasingly to implementation in human cells.

## Functional genomics tools for chemical-genetic screens in human cells

The past decade has witnessed the rapid development of functional genomics tools for human cells such as RNA interference (RNAi), barcoded open reading frame (ORF) libraries, and most recently CRISPR-based methods[37, 38, 39, 40, 41]. Briefly, RNAi relies on short RNAs complementary to target mRNAs that are introduced into cells to mediate degradation of these mRNAs and thereby reduce target gene expression[37, 38]. ORF libraries overexpress human ORFs from standardized expression vectors with a strong constitutive promoter[39]. Finally, in CRISPR-based approaches, an effector protein such as Cas9 is programmed with a short-guide RNA (sgRNA) that directs the effector to a DNA locus of interest *via* sequence complementarity. Cas9 then introduces a double-strand break, which triggers DNA repair mechanisms that in protein coding regions frequently result in frameshift mutations[40, 41]. Other CRISPR-based approaches rely on a catalytically inactivated mutant of Cas9 (dCas9)[42] that is essentially a highly programmable DNA-binding protein and can be used to deliver transcription factors to target loci to mediate knockdown or overexpression without DNA cutting[41]. With the availability of these tools to systematically perturb gene expression, the concepts developed from chemical-genetic efforts in yeast can now be applied directly in human cells.

RNAi and CRISPR-based methods are readily amenable to pooled screening experiments akin to those developed for yeast. In particular, when the expression constructs for short-hairpin RNAs (shRNAs) in RNAi or sgRNAs in CRISPR-based methods are stably inserted into the genome, for example by lentiviral transduction, the DNA copies can directly serve as molecular barcodes because their sequences encode the identities of the targeted genes. In a pooled screen, a library of shRNA or sgRNA expression constructs, with elements targeting genes of interest such as all protein-coding genes in the genome, is introduced into

cells such that each cell stably integrates one construct (Figure 2A). The abundance of all shRNAs or sgRNAs in the cell population is then quantified, most commonly by next-generation sequencing, both at the outset of the experiment and after growth under a condition of interest such as drug-induced selective pressure. This quantification reveals the effect of expression of a given shRNA or sgRNA, and by proxy the effect of perturbation of the targeted gene, on growth in this condition[43]. In such pooled screens, a large number of genetic perturbations can be queried rapidly in a single experiment, allowing for coverage of the entire genome, and all cells are grown in identical conditions, avoiding batch effects. Thereby, pooled screens enable both the identification of genes required for growth in the absence of other selective pressures and chemical-genetic profiling.

Indeed, shortly after the development of large-scale shRNA libraries, shRNA screens were used to map out modifiers of sensitivity to small molecules[44, 45, 46]. For example, a screen for sensitivity to Nutlin-3, an MDM2 inhibitor, revealed that cytotoxicity depends on *TP53BP1*[44] and genome-scale shRNA screens were used to map out the genetic dependencies of sensitivity to established drugs including etoposide[45] and imatinib[45]. Subsequent studies highlighted how shRNA screens can be used to identify the molecular targets of drugs *de novo*: after a small-molecule screen had identified a promising anti-leukemia agent (STF-118804) with unknown mechanism of action, a genome-scale shRNA screen revealed that knockdown of nicotinamide phosphoribosyl transferase (NAMPT) strongly sensitized cells to STF-118804[47]. Subsequent validation confirmed that STF-118804 is a specific NAMPT inhibitor. Similarly, a reporter-based shRNA screen revealed that ISRIB, a compound found to enhance cognitive memory in mice[48], inhibits the integrated stress response by triggering dimerization and activation of eIF2B[49], a finding that was independently made by screening for ISRIB-resistant mutants[50]. Despite these successful examples and additional advances in library construction and pooled screening, shRNA methodologies suffer from technical limitations including off-target effects and limited knockdown efficiency[51], which confounds results and limits sensitivity and thereby has hampered their widespread application in characterizing the mechanisms of action of drug candidates.

CRISPR-based screening techniques have proven to be more specific and efficacious in most instances[41, 52, 53] and are similarly applicable to chemical-genetic screening. For example, early results from CRISPR cutting-mediated loss-of-function screens for resistance against 6-thioguanine[54], etoposide[54], and vemurafenib[55] recapitulated the known mechanisms of action, validating the ability of such screens to identify targets and genetic dependencies of known drugs. CRISPR cutting generally results in complete loss-of-function of target genes, whereas RNAi reduces target levels but does not ablate them. This mechanistic difference was noted in a recent study, in which combined CRISPR cutting screens and shRNA screens were used to determine that the antiviral compound GSK983 inhibits dihydroorotate dehydrogenase[56]. Although some genes appeared as hits in both screens, the majority only scored in one or the other, highlighting strengths and limitations of both approaches: CRISPR cutting screens for drug sensitivity do not perform well for essential genes, as loss of these genes is lethal, but the high efficacy of CRISPR cutting allows for accurate determination of sensitivity phenotypes of non-essential genes, even if the phenotypes are

weak in magnitude. By contrast, shRNA screening can be used to probe phenotypes of essential genes but may miss weaker phenotypes.

An alternative to RNAi and CRISPR cutting that combines some of the strengths of both is provided by CRISPR-mediated transcription interference (CRISPRi), in which a fusion protein of catalytically inactivated Cas9 (dCas9)[42, 57] and the KRAB transcriptional repressor is targeted to promoters of genes[58]. CRISPRi consistently mediates strong transcriptional repression (85–>99%) of target genes and allows for determination of both sensitizing and protective phenotypes of essential genes[59, 60, 61, 62], making it an ideal technique for chemical-genetics efforts.

Similarly, the programmable DNA binding activity of dCas9 allowed for the development of CRISPR-based methods for overexpression of target genes (CRISPRa), in which transcriptional activators such as VP64 (a tetra-repeat of the viral transcription activation domain VP16) are delivered via dCas9. In a recent comparison of CRISPRa methods[63], three methods in particular were found to consistently mediate strong overexpression of target genes (10–10,000x): the SunTag method[59, 64], the SAM method[65], and the VPR method[66]. All of these methods rely on dCas9-mediated recruitment of multiple transcription activator domains. Briefly, in the SunTag method, dCas9 fused to ten copies of a GCN4 peptide epitope is co-expressed with the cognate single-chain variable fragment (scFv) fused to VP64, effectively recruiting ten copies of VP64[59, 64]. The key advance of the SAM method is an sgRNA constant region engineered to contain MS2 aptamers, allowing for recruitment of transcription factors via the MS2 coat protein (MCP). In the current implementation of SAM, a dCas9-VP64 fusion protein is co-expressed with MCP fused to both the NF-κB trans-activating subunit p65 and the activation domain of human heat shock factor 1 (MCP-p65-HSF1)[65]. In the VPR method, dCas9 is fused to a tripartite transcription activator consisting of VP64, p65, and the replication and transcription activator (Rta) of Eppstein-Barr virus[66]. Finally, a VP64-dCas9-VP64 fusion protein also appears to mediate strong CRISPRa activity[63, 67]. As is typical for CRISPR-based methods, sgRNAs can be rapidly generated against all genes in the genome, enabling genome-wide screens, and each sgRNA sequence can serve as a molecular barcode in pooled screening experiments (see above). Although ORF libraries provide an alternative method to target gene overexpression, such libraries contain elements of broadly varying size, posing issues for pooled lentiviral screening methods.

In general, CRISPR cutting, CRISPRi, and CRISPRa are specific for the target locus of interest and mediate loss-of-function or gain-of-function with high efficiency, with few exceptions: whereas CRISPR cutting may cause non-specific toxicity at amplified loci due to DNA damage responses, CRISPRi and CRISPRa are less specific at bidirectional promoters as they may affect both genes. Indeed, a recent analysis found that CRISPR cutting and CRISPRi yield complementary insights in screens[61]. For each of the CRISPR screening methods, algorithms have been developed to predict highly active sgRNAs with minimal off-target effects, enabling the generation of compact sgRNA libraries for pooled screening applications[60, 68, 69].

## Combined CRISPRi and CRISPRa screens for target identification

With CRISPR-based knockdown and overexpression methods available, it had become feasible to perform both loss-of-function and gain-of-function screens in a condition of interest, mirroring those performed in yeast[36] and with immediate implications for chemical-genetic screening. Indeed, a comparison of genome-wide sensitivity profiles obtained from proof-of-principle screens with a cholera-diptheria toxin fusion clearly revealed anti-correlated phenotypes for genes targeted by the toxin or on the pathway for toxin entry[59]. Similarly, measurements of sensitivity to CB-5083, an inhibitor of p97 (*VCP*), as a function of expression levels of p97 modulated by CRISPRi and CRISPRa revealed a strong correlation[70], validating p97 as the target of CB-5083.

These results motivated us to further develop a generalizable chemical-genetic screening strategy for the identification of molecular targets and mechanisms of action of small molecules [62]. The strategy uses genome-wide pooled CRISPRi and CRISPRa screens for sensitivity to a compound of interest, with selective pressure induced by several pulses of treatment around $LC_{50}$, to quantify for each gene how knockdown and overexpression affect sensitivity to the compound. (Figure 2A) Then, genes whose levels directly modulate sensitivity, such as the molecular target, are identified as those with anti-correlated phenotypes in knockdown and overexpression (Figure 2B). Application to rigosertib, a drug that had entered phase III clinical trials for high-risk myelodysplastic syndrome but whose mechanism of action had remained controversial with multiple proposed molecular targets[71, 72, 73], highlighted the potential of this approach. The combined CRISPRi/a sensitivity profiles identified microtubule destabilization as rigosertib's mechanism of action, as opposed to previously proposed mechanisms of action[62]. Subsequent follow-up experiments, including chemical-genetic comparison to known microtubule destabilizing agents and the isolation of a rigosertib-resistant tubulin mutant, confirmed that rigosertib kills cells by destabilizing microtubules.

A few observations from the rigosertib case are worth highlighting. The microtubule signature emerged in the CRISPRi/a profiles despite functional redundancy among tubulin genes and despite strong pleiotropic effects: knockdown of many essential genes conferred apparent protection against rigosertib, likely because these knockdowns prevent cells from reaching mitosis, when cells are highly dependent on microtubules and thus most sensitive to rigosertib. Overexpression of these genes, however, did not impact rigosertib sensitivity, and thus the protective phenotypes observed using CRISPRi could be attributed to pleiotropy. Such indirect effects likely contributed to the controversy over rigosertib's mechanism of action, during which evidence for several other targets had been obtained from targeted cellular assays, and they similarly often dominate sensitivity profiles especially if the small molecule targets a central biological process such as cell division or the microtubule network. This work illustrates the potential of the combined CRISPRi/a approach to separate direct and indirect effects on drug sensitivity, which provides a critical advantage over any individual screening approach.

## Other approaches to target identification

By and large, additional methods to identify the targets of bioactive compounds are either based on identifying or inferring binding partners, phenotypic comparison to compounds with known mechanisms of action, or screening for resistance-conferring mutations. We here provide a brief summary of these methods; the reader is referenced to other reviews for detailed discussions.

**Affinity-based methods—**Affinity-based methods to directly identify binding partners of small molecules have been mainstays in target identification for decades. Initially developed to purify enzymes[74], these methods rely on derivatization of the small molecule and immobilization on a solid-phase matrix. Incubation of this matrix with cell lysate and separation of non-binders allows for affinity purification and subsequent determination of the identities of binding partners. In one of the earliest examples of this approach, the cellular binding partner for the immunosuppressant FK506 was identified to be the *cis-trans* peptidyl-prolyl isomerase FKBP[75]. In a related tour-de-force, cyclophilin was identified as the binding partner of cyclosporin A by fractionation of lysates after incubation with radioactively labeled cyclosporin A[76]. Widespread implementation of such approaches was long limited by challenges in determining the sequences of the isolated proteins, but the development of high-throughput mass spectrometry techniques coupled with the availability of genomic sequence information now allows for straightforward identification of binding partners by proteomic methods. The resulting chemical proteomics workflow has been applied widely[7] and continues to evolve. For example, furnishing small molecules with both a photoactivatable diazirine crosslinker and an alkyne group allows for photo-crosslinking to non-covalent binding partners followed by affinity purification and mass spectrometry analysis. A panel of such molecules was recently employed in an elegant fragment-based screening approach that enabled mapping of thousands of small molecule-protein interactions[77]. Developments in multiplexed MS have furthermore given rise to the related proteome-wide thermal profiling approach, in which the melting curves of all proteins are determined in the presence and absence of a small molecule and proteins that are stabilized by the small molecule are inferred to be binders[78, 79].

**Comparative profiling—**Comparative profiling methods rely on matching phenotypes obtained with a compound in question to those obtained with a set of reference compounds with known mechanisms of action, with the expectation that compounds with similar mechanisms of action will elicit similar phenotypes. In a large-scale effort in the 1980s, the National Cancer Institute assembled a reference set of 60 cancer cell lines (NCI-60) and profiled sensitivity of each cell line to a large panel of compounds[80, 81]. Indeed, compounds with similar mechanisms of action showed similar patterns of sensitivity, and comparison to these patterns revealed, for example, halichondrin B as an inhibitor of microtubule polymerization. Steady profiling of additional compounds has produced large-scale data sets that are rich resources for target identification.

Phenotypes for comparative profiling can also be derived from chemical-genetic profiling (see above), which has been used to identify or validate drug targets both in yeast[32] and in human cells[62, 82], or from various other methods such as transcriptional profiling or high-

content microscopy. Methods to compare transcriptional profiles were again pioneered in yeast, both by comparing expression profiles of cells treated with panels of drugs[83] or by comparing expression profiles from drug-treated cells with those from deletion strains, with the assumption that inhibiting a protein with a drug would be equivalent to deletion of the gene[84]. Improvements in transcriptome profiling methodologies led to large-scale efforts to comprehensively profile the transcriptional responses of human or other mammalian cell lines to large panels of small molecules[8]. These efforts have resulted in public databases such as the Connectivity Map[85] that can be queried with transcriptional data derived using a compound of interest. The ability to read out both genetic perturbations and transcriptomes from pools of cells using recently developed single-cell sequencing methodologies such as perturb-seq now additionally allows for simultaneous comparative profiling and chemical-genetic screening for hundreds to thousands of candidate genes[86, 87, 88, 89]. A recent study further demonstrated that even basal gene expression, i.e. expression in the absence of treatment, can be used to predict mechanisms of action. In particular, a gene whose basal expression correlates strongly with sensitivity to a small molecule across cell lines frequently encodes the molecular target, a transporter, or an enzyme that activates the small molecule[90]. Other comparative profiling approaches have used phenotypes measured by high-content microscopy such as various measurements of cell morphology and cellular contents[9, 91]. Many other implementations of comparative profiling have been developed that are increasingly relying on computational inference and machine learning trained on multi-dimensional datasets.

**Resistant mutants—**All of the discussed methods readily generate hypotheses for the molecular target of a compound of interest, but require subsequent verification that the compound indeed exerts its physiological effect through this target. One of the most compelling demonstrations of physiological relevance is the identification of a mutation in the target that confers resistance to the compound. This concept has been used to directly identify molecular targets of compounds by screening for resistant mutants and then identifying the resistance-conferring mutation. In particular, this approach has been widely employed in bacteria, which are haploid by nature and carry small genomes that can be mutated to near-saturation, as well as in yeast. In one of the most influential examples, the TOR kinases *TOR1* and *TOR2* were discovered in yeast by characterizing rapamycin-resistant yeast mutants[92]. Saturation screens, however, have been more difficult to implement in higher eukaryotes due to the difficulty in generating dominant resistant mutations as well as the subsequent identification in genomes containing billions of base pairs. Approaches to overcome these challenges exist: the use of near-haploid cell lines can mitigate the former challenge; the latter challenge was recently addressed by identifying resistance-conferring mutations by exome sequencing or RNA-seq rather than genome sequencing, which drastically reduces the required sequencing space[93, 94, 95].

## Strengths and limitations of target identification strategies

Each of the approaches described here comes with a set of inherent strengths and caveats, consideration of which is critical to successful implementation. At the same time, no single approach will provide the perfect solution, and instead a set of complementary approaches are needed to ultimately identify the molecular target of a small molecule in question.

Improvements in mass spectrometry technology such as increases in throughput and sensitivity, new statistical toolboxes, and workflows to reduce background have made affinity-based methods increasingly robust. Such approaches can, for example, distinguish different isoforms of binding partners, which other methods for target identification are largely insensitive to. To successfully identify binding partners, however, chemical proteomics workflows require derivatization of the compound, which is frequently a bottleneck or may introduce artifacts by changing the binding profile. In addition, detecting binding to membrane proteins or other biochemically poorly behaved proteins and complexes can be challenging, as they can be difficult to maintain in a native state. These concerns are somewhat alleviated in the thermal profiling approach, but this approach may fail if small molecule binding does not stabilize its binding partner or if there is a route to unfolding even when the small molecule is bound. Vice versa, low-affinity but high-abundance binding partners in the cell can complicate deconvolution of the functional target and off-target binders or even prevent detection of the true target. More generally, affinity-based methods test binding and not necessarily activity; the highest-affinity binding partner need not be the target through which a small molecule exerts its physiological effect.

Comparative profiling methods generally do not require compound derivatization or specialized cell models, as endogenous phenotypes can be used as a readout. Such methods are particularly powerful once a large array of compounds has been profiled to generate reference data sets for accurate pattern matching and are amenable to deep learning approaches to identify more complex relationships between small molecules or biological pathways. Such data sets, however, may not be available in the model of interest. More broadly, inhibition of diverse targets in the cell may result in similar phenotypes, for example due to pleiotropic effects; in such cases it can be challenging to identify the proximal mechanism of action. This approach may also fail for compounds with novel mechanisms of action as by concept this approach relies on comparing phenotypes to those of compounds with known mechanism of action.

Chemical-genetic approaches do not require reference data sets or small molecule derivatization and probe function rather than binding and therefore are less prone to identifying non-functional binding partners. In human cells, CRISPR approaches are relatively easy to implement and mediate robust knockout, knockdown, or overexpression. The cell lines generated thus far as well as the libraries are freely available. Nonetheless, loss-of-function and gain-of-function approaches individually are prone to false negatives and false positives. Loss-of-function approaches, for example, may fail in cases of redundancy or pleiotropy, whereas overexpression of a drug target may not provide resistance if the target functions as part of a multiprotein complex. Combining loss-of-function and gain-of-function screens can mitigate these caveats[36, 62]. In particular, the combined data provide a filter to separate direct and indirect effects and can reveal targets in the presence of redundancy or pleiotropy (Figure 2B). Even if the direct molecular target does not score strongly in such screens, as was the case in the rigosertib screen, the availability of sensitivity phenotypes for all genes in the genome typically identifies the process targeted by the compound. Two key requirements need to be fulfilled for the screening approach: a model system amenable to genetic manipulation needs to be available to implement the CRISPR effectors, and the compound of interest must generate a selectable

phenotype. In the simplest manifestation, this phenotype may be growth, but more complex phenotypes such as expression levels of a reporter gene, cell migration, or differentiation can also be probed. As long as these requirements are fulfilled, the combined CRISPRi/a approach provides a high-throughput and sensitive method to probe small molecule mechanisms of action.

Regardless of approach, independent validation of the putative mechanism of action is usually required, most compellingly by identification of a resistant mutant. Direct identification of such mutants is technically challenging especially in diploid organisms in which they need to be dominant to be identified, but target hypotheses generated from other target identification approaches can greatly reduce the search space. For non-essential putative molecular targets, the resistant mutant may be a homozygous deletion mutant; in other cases rational structure-guided approaches or random mutagenesis approaches such as those enabled by newly developed CRISPR-mediated base editors[96, 97] may prove successful.

### Perspective

The rapid development of high-throughput functional genomics tools has enabled large-scale chemical-genetic screening efforts to identify the molecular targets and mechanisms of action of therapeutic candidates and chemical probes. Newer CRISPR-based approaches in particular can be applied in a wide variety of cellular models, including cancer cell lines or primary cells as well as animal models. The underlying principles furthermore can be readily combined with emerging methods for targeted mutagenesis such as CRISPR cutting[98] or base editing[96, 97] to enable target identification with amino acid resolution. With the ability to probe drug mechanism of action directly and *in vivo*, chemical-genetic approaches provide natural complements to other approaches in target identification such as affinity-based methods.

Application of the approaches described here is not limited to eukaryotic models, which have classically been the main targets of drug development efforts. Implementations in bacteria are simultaneously revealing drug mechanisms of action and new biology, for example by comparative profiling of drug sensitivity in the *Escherichia coli* Keio knockout collection[99], by chemical-genetic screening of *Bacillus subtilis* strains carrying CRISPRi-mediated knockdowns of essential genes[100], or by microscopic profiling of drug-treated cells[101]. With increasing appreciation for the central role of bacteria in human health and disease, such efforts may prove critical toward developing treatments for microbiome-associated deficiencies or infections with multidrug-resistance bacteria. Regardless of ultimate target, there now is a large suite of tools at our disposal to usher in a new era in the development of precisely targeted therapeutics.

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## Glossary

**Chemical-genetic screen**
Systematic profiling of small molecule sensitivity for a library of strains or cell lines carrying genetic perturbations.

**Haploinsufficiency profiling (HIP)**
Chemical-genetic screen in a library of yeast heterozygous deletion strains.

**Homozygous deletion profiling (HOP)**
Chemical-genetic screen in a library of yeast homozygous deletion strains.

**Multicopy suppression profiling (MSP)**
Chemical-genetic screen in a library of yeast strains with multicopy expression plasmids.

**RNA interference (RNAi)**
Inhibition of gene expression by a complementary short RNA.

**CRISPR**
Clustered regularly interspaced short palindromic repeats, a bacterial and archaeal adaptive immune system that mediates degradation of DNA or RNA molecules based on sequence complementarity using effector proteins and short RNAs.

**Cas9/dCas9**
A CRISPR effector protein that binds to target DNA via sequence complementarity of an associated short RNA and cleaves the target DNA. dCas9 is a nuclease-dead variant that binds but does not cleave.

**Short-guide RNA (sgRNA)**
a short RNA, generated by fusion of a targeting sequence and a constant region, that binds Cas9 and directs it to target DNA.

**CRISPR interference (CRISPRi)**
transcription repression mediated by dCas9 or fusion proteins of dCas9 or other CRISPR effectors.

**CRISPR activation (CRISPRa)**
transcription activation mediated by fusion proteins of dCas9 or other CRISPR effectors.

**Pooled screen**
A screen in which cells with different genetic perturbations are grown in a single pool and phenotypes are determined by quantifying cells with each perturbation at the end of the screen, for example by deep sequencing.

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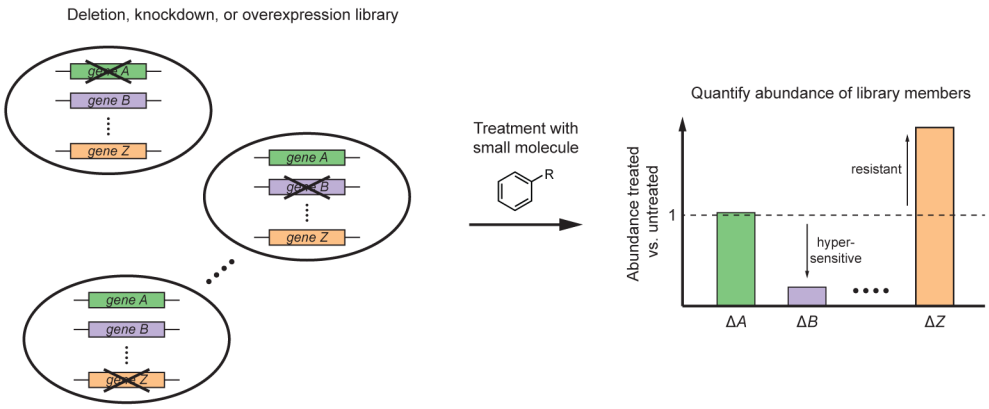


**Figure 1.**

Schematic of chemical-genetic screen. A library of cells each with a specific genetic perturbation, such as deletion, knockdown, or overexpression of a gene, is exposed to a compound of interest, either in pooled or arrayed format. Quantification of the abundance of library members before and after treatment reveals the effect of each genetic perturbation on sensitivity. In this example, deletion of *gene B* confers hypersensitivity; thus, *gene B* likely operates in the pathway targeted by the compound. By contrast, deletion of *gene Z* confers resistance; thus, *gene Z* might be required for uptake of the compound.

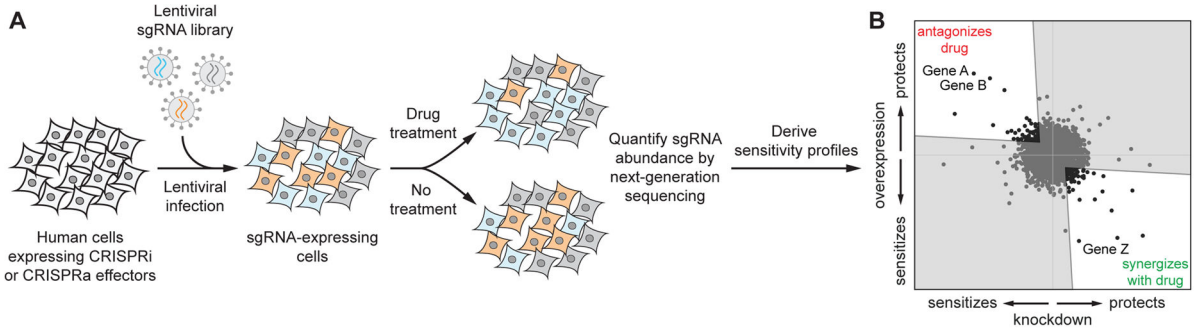


**Figure 2.**

Schematic of combined CRISPRi/a screens.

A: Schematic of pooled CRISPRi and CRISPRa screens for drug sensitivity. Other pooled screens can be conducted analogously using the corresponding cell lines and libraries.

B: Comparing knockdown and overexpression data such as those from combined CRISPRi and CRISPRa screens reveals genes with anti-correlated sensitivity phenotypes. These genes are likely to be directly involved in the pathway targeted by the drug.