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Determining the mode of action of bioactive compounds

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ABSTRACT

Matching bioactive molecules with molecular targets is key to understanding their modes of action (MOA). Moving beyond the mere discovery of drugs, investigators are now just beginning to integrate both biochemical and chemical-genetic approaches for MOA studies. Beginning with simple screens for changes in cell phenotype upon drug treatment, drug bioactivity has been traditionally explored with affinity chromatography, radiolabeling, and cell-based affinity tagging procedures. However, such approaches can present an oversimplified view of MOA, especially in light of the recent realization of the extent of polypharmacology and the unexpected complexity of drug–target interactions. With the advent of more sophisticated tools for genetic manipulation, a flood of powerful techniques has been used to create characteristic drug MOA 'fingerprints'. In particular, whole genome expression profiling and deletion and overexpression libraries have greatly enhanced our understanding of bioactive compounds in vivo. Here we highlight challenges and advances in studying bioactive compound–target interactions.

1. Introduction

Small bioactive molecules are outstanding probes of biological systems; moreover, under the right circumstances, they can also act as drugs, further enhancing their attractiveness. Matching bioactive small molecules with their biological targets is therefore key to understanding their modes of action (MOA) and their resulting impact on physiology. Despite this importance, determining the MOA of bioactive molecules is often highly challenging. In fact, the MOA of even well-established bioactive molecules remains unknown. For example, despite its use as an antimalarial drug for more than 400 years, the primary molecular target of quinine is currently unknown. The reasons for the difficulty in establishing drug MOA include the biochemical and genetic intractability of many target species, chemical instability of some bioactive molecules (including their metabolism), and the fact that many compounds are bioactive by pleiotropic means. These challenges conspire to make the determination of MOA often laborious and difficult.

Historically, bioactive compounds have been identified by screening compound-treated cells or organisms for specific phenotypes. For example, antibiotics and anticancer drugs are often identified by screens for cell death. For the exploration of traditional medicines, for example, plant-derived natural products, compound-treated organisms are often screened for specific indications such as disease alleviation or pain relief. These efforts were then followed by strategies that probed drug MOA. The advantage of such whole cell/organism strategies was that all available targets (proteins, transporters, membranes, etc.) were simultaneously sampled in phenotype screens. Deconvolution of MOA, however, still remained difficult.

The advent of genome sequencing and facile heterologous protein expression methods ushered in an era of target-based approaches for the identification of compounds that interacted with specific targets deemed important. This method has been highly successful in identifying in vitro inhibitors, activators, and general probes of target structure and function. Unfortunately, very often these compounds failed to recapitulate their activity in vivo. This challenge has been especially vexing in the field of antibiotic discovery. Further, unanticipated in vivo 'off-target' interactions can dominate the desired compound–target interaction resulting in complex or unwanted phenotypes. The result has been a growing recalibration towards cell-based screens for phenotype, and an increased investment in the development of creative ways to determine MOA in follow-up experiments. Here, we review established and newer methods to identify the MOA of bioactive compounds.

2. Biochemical approaches to determine MOA

2.1. Measuring macro impacts of bioactive molecules

Using phenotype-based screens, clues to MOA may be discerned using a number of high-level techniques. At the whole organism

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level, changes in behavior or normal activity (e.g., tolerance to pain, eradication of infection, etc.) may be observed and quantified to establish tissues or systems likely affected by bioactive compounds. Similarly, at the cellular level, drug-induced changes in cell morphology, cell division, or other measures of cell growth may suggest likely subcellular targets of bioactive compounds. Here, the use of various forms of imaging—in particular, microscopy using fluorescent markers for the visualization of organelle structure and function—may be very informative. Further, the availability of genome-scale libraries of tagged proteins (e.g., green fluorescent protein (GFP)) and their associated imaging databases can provide a visual toolkit to monitor deviation from the wild-type phenotype.[1–3]

Traditionally, the impact of bioactive molecules on general metabolism, translation, transcription, and replication has been monitored using radioactive precursors such as ^{14}C-labeled glucose, leucine, thymidine, or uracil for in vivo feeding assays (or testing of cell-free extracts), followed by analysis of label incorporation in downstream products (CO_2, protein, DNA, RNA). Labeling of specialized precursors such as *meso*-diaminopimelic acid in peptidoglycan has been used to target experimental impact on specific organelles and structures.[4] By integrating the results of such studies, the molecular processes affected by bioactive molecules can be identified. However, primary molecular targets often remain elusive despite these efforts.

2.2. Affinity methods

In order to match molecular targets—particularly protein targets—with bioactive compounds, a direct unequivocal measure of compound interaction and impact on function is desirable. One well-established method to provide such a measure is affinity chromatography (Fig. 1A).[5,6] In this approach, the bioactive compound is immobilized either directly or indirectly through a linker molecule onto a solid matrix suitable for protein fractionation (e.g., agarose), and the cell-free extract is incubated with the labeled matrix. Washes with buffer of increasing stringency (e.g., increased salt concentration) serve to remove non-specific or weakly bound molecules. A final wash with buffer that includes the bioactive compound competes with the immobilized compound to release tightly-bound target. Separation of eluted proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis or an analogous analytical technique serves to confirm the release of target proteins that can then be identified by mass spectrometry or N-terminal peptide sequencing. Advantages of affinity chromatography include its relative technical simplicity, and its ability to identify proteins that have both medium- and high-affinity for a compound of interest, as established by wash stringency. In many cases, this method directly identifies a high-affinity target with little ambiguity.[6] As effective as affinity chromatography is, however, it has several drawbacks. First, many compounds cannot be readily grafted onto a solid matrix, or maintain their affinity for target molecules once immobilized. Second, compounds that irreversibly or covalently modify their targets (e.g., penicillin, wortmannin) will not release target proteins during column washes. Third, protein complexes such as transcriptional regulators, the ribosome, or proteins embedded in membranes are often very difficult to purify and solubilize in a fashion that is amenable to affinity chromatography.

To overcome some of these shortcomings, cell-based affinity tagging procedures have been developed.[7] This strategy was presaged by successful methods used to track bioactive compounds that covalently interacted with targets. For example, the β-lactam

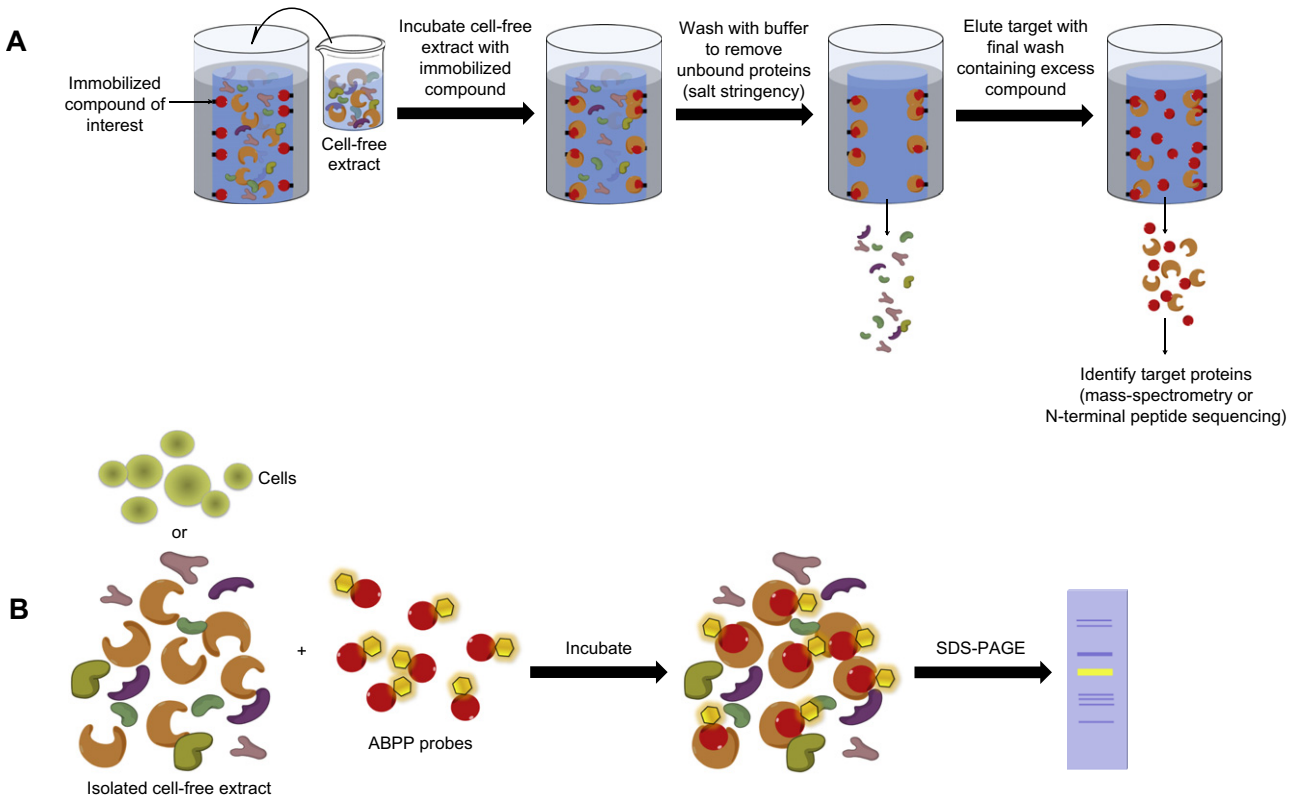

Figure 1. Affinity chromatography and ABPP. (A) In affinity chromatography, the bioactive compound is immobilized onto a gel matrix. Cell-free extract is incubated with the immobilized compound to allow for target molecules to bind. Non-specific binding molecules are washed away with increasing stringency (e.g., salt). A final wash with excess compound elutes target proteins that can then be identified by mass-spectrometry or N-terminal peptide sequencing. (B) Whole cells or cell-free extract are incubated with ABPP probes. In this case, the probes contain a fluorophore. Proteins are separated and visualized using in-gel fluorescence scanning.

antibiotics such as penicillin do not have a single protein target in the cell, but rather covalently inactivate a collection of so-called penicillin binding proteins (PBPs) that are important in bacterial cell wall biosynthesis and metabolism.[8] Here, the use of radiolabeled penicillins identified PBPs whose roles in cell wall metabolism were later deduced with follow-up biochemical and genetic experiments. The exploitation of the covalent interactions between penicillins and PBPs enabled the elucidation of their MOA.

By exploiting similar irreversible interactions in whole cells, targets for bioactive small molecules have been identified using a technique termed Activity Based Protein Profiling (ABPP), originated by the Cravatt group (Fig. 1B).[9] For this technique, chemical probes bind and label enzymes to monitor enzyme function. Bioactive chemical probes suitable for ABPP have the following properties: a moiety capable of covalently interacting with the target (e.g., electrophilic or photoactivatable groups), a target recognition element, and a tag that can either directly or indirectly be used to visualize and capture labeled targets (e.g., biotin or fluorescent tags such as rhodamine).[10] Such probes have been successfully directed towards the in vivo labeling and identification of proteases, lipases, cytochrome P-450s, glycosidases, kinases, phosphatases, and many other proteins (recently reviewed in Refs. 7,11–13).

The ABPP approach has also been adapted to establish ligand affinity and the MOA of biochemically challenging targets such as membrane receptors. For example, in order to probe a potential interaction between the antibiotic vancomycin and the integral membrane sensor His kinase VanS in drug-resistant bacteria, we prepared an affinity probe linking vancomycin to a biotin tag and a benzophenone photoactivatable group (Fig. 2).[14] Using this probe, we established that VanS binds vancomycin, the first step in the induction of vancomycin resistance.

These biochemical approaches offer excellent and robust opportunities to probe the MOA of bioactive compounds. Nevertheless, they can be challenging to implement and often require significant technical and chemical expertise. The advent of genome-scale tools to explore biology has presented an orthogonal series of methods to explore drug MOA.

3. Chemical-genetic approaches to determine MOA

3.1. Global microarray analysis

Whole genome sequencing has revolutionized the strategies used to identify targets of bioactive compounds. Since the genome of *Haemophilus influenzae* was initially sequenced in 1995,[15] genomics has uncovered many 'druggable' targets with proven or potential ability to interact with bioactive compounds. In particular, it was the sequencing of the yeast *Saccharomyces cerevisiae* that served as a springboard for current genetic methods into drug-target deconvolution. In a 1998 landmark study, Marton et al. introduced the concept of a drug-induced expression 'fingerprint'.[16] Genome-wide *S. cerevisiae* microarrays were used to compare expression fingerprints, or profiles, with the addition of the immunosuppressant FK506. As expected, the FK506 expression profile in wild-type strains was absent in treated mutants lacking the FK506-specific target calcineurin. However, at higher FK506 concentrations, changes in the expression of genes unrelated to the calcineurin pathway were observed. These results sparked an

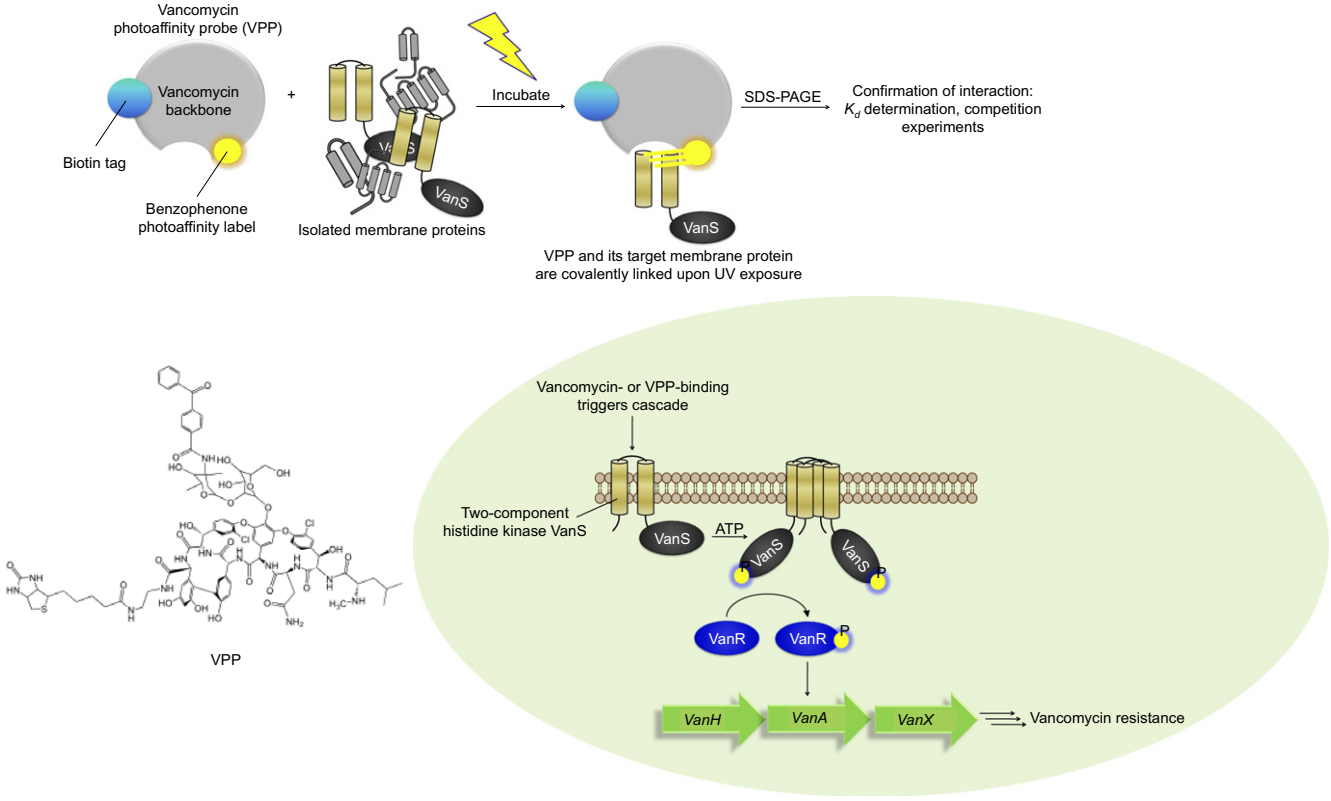

Figure 2. Outline of the ABPP approach we used to determine that the two-component histidine kinase VanS is a target of vancomycin.[14] The synthesized ABPP probe contained the vancomycin backbone, a biotin linker, and a benzophenone photoaffinity label. Vancomycin or the vancomycin photoaffinity probe (VPP) both trigger the phosphorylation of VanS. This results in the phosphorylation of the VanR transcriptional activator, which in turn induces the transcription of the *vanH*, *vanA*, *vanX* genes for vancomycin resistance. The induction of these genes eventually leads to the cell wall terminating in vancomycin-resistant D-Ala-D-Lac units. Isolated membrane proteins including histidine-tagged VanS were incubated with VPP, irradiated with UV light, and separated by SDS–PAGE. VanS–VPP binding was confirmed using competition experiments with vancomycin and teicoplanin.

interest in the detection of off-target drug targets, and the development of more sophisticated uses of whole genome expression profiling in drug discovery.

The power of large-scale expression profiling was originally demonstrated by Hughes et al.[17,18] Because drugs with diverse MOA often correspond to unique expression profiles, these can be used to find targets of novel bioactive compounds. The following assumption underlies expression profiling studies: deletion of a drug target gene produces an expression profile corresponding to the inhibitory effect of that drug. With this assumption in mind, the authors analyzed a compendium of microarray profiles of yeast mutants and wild-type strains subjected to drug treatments. By 'pattern matching' profiles of mutants and drug-treated wild-type strains, the functions of uncharacterized genes were revealed. The target of the topical anesthetic dyclonine was also identified. Numerous studies have since used a similar pattern matching approach to uncover drug targets.[19–21]

Currently, there exist a number of databases that compare expression data and correlate transcript profiles to diseases and pathogens, namely the Gene Expression Omnibus (GEO) and Connectivity Map (C-MAP) databases.[22] The C-MAP database has proven particularly useful in predicting drug MOA based on patterns of up- and down-regulated genes.[23–28] In a recent study, expression profiles of lung adenocarcinoma were used to query the C-MAP database to find compounds that reversed expression direction in cancer cells.[29] One of the most promising compounds generated by C-MAP, the HSP90 inhibitor 17-AAG, was shown to inhibit lung adenocarcinoma cell growth by inducing apoptosis and cell cycle arrest. Costanzo et al. recently constructed a large-scale network of gene–gene interactions in *S. cerevisiae*.[30] Genetic interactions were quantified via a synthetic genetic array (SGA) method: when the combination of two mutations results in cell lethality, functional relationships are gleaned between genes.[31] Because densely clustered genetic interactions were often linked to the targets of bioactive compounds, this global gene network could be used to identify novel drug targets and chemical-genetic interactions.

Although global expression analysis has played an important role in drug-target deconvolution, this method has its drawbacks. Importantly, it can be difficult to mine the associated large volumes of complex data and extract relevant expression patterns. Depending on how the data is experimentally obtained, organized, or clustered, different conclusions may be reached regarding drug MOA.[32,33] Further, compounds that target different proteins of the same cellular pathway, or proteins involved with similar cellular processes, might produce overlapping expression profiles.[19] Conversely, different drugs affecting the same protein target do not always produce the same expression profiles.[34,35] For example, although two inhibitors of cyclin-dependent kinase 2 in yeast showed similar in vitro activities, their microarray profiles were different meaning they had unique MOA.[36] One issue that must be resolved is the standardization of expression data within existing databases. Subinhibitory and therapeutic concentrations of bioactive compounds have been shown to produce different expression profiles, with additional variations in expression data stemming from differences in the experimental organisms, specific growth conditions, and genetic techniques used.[21,32,37–40]

Using subinhibitory drug concentrations may paint a simpler picture of drug–target interactions by eliminating off-targets and downstream transcriptional effects.[19,40] For example, to probe the poorly understood quinine MOA against *Plasmodium falciparum*, a relatively low concentration of the antimalarial was used for yeast microarray analysis to limit the expression of genes involved with a general nutrient starvation response.[41] The use of a low concentration of quinine resulted in the overexpression of genes involved with carbohydrate transport and metabolism, and a resultant decrease in the uptake of glucose. Yeast deletion strains for hexose transporters showed a resistance phenotype and a decrease in the uptake of quinine. These data pointed to quinine being a potential inhibitor of hexose transporters in *S. cerevisiae*, and therefore a potential inhibitor of the highly homologous hexose transporter (PfHT1) in *P. falciparum*. To obtain a more holistic fingerprint of drug MOA, a range of drug concentrations and specific growth conditions should be used.

3.2. Gene dosage

The efficiency and accuracy of genome-wide expression profiling was greatly enhanced with the use of barcoded yeast deletion collections. Given the ease with which yeast may be genetically manipulated and cultivated, yeast deletion collections consisting of haploid strains for both mating types, and/or heterozygous and homozygous diploid strains, have been widely used to decipher drug MOA.[21,42–45] For example, the *S. cerevisiae* deletion collection includes ~6000 heterozygous diploid and ~5000 haploid deletion mutants, leaving ~1000 genes that are essential for yeast survival under standard laboratory conditions.[44,46] Each gene is replaced with a *kanMX* selectable marker and tagged with an identifiable 'barcode': an upstream and downstream 20 base-pair sequence tag, both of which are flanked by universal primers (Fig. 3).[47,48]

In a typical fitness or competitive growth experiment, barcoded mutants are grown in specific conditions (e.g., compound treatment) (Fig. 3).[49,50] Genomic DNA is extracted from the deletion pool at specific time intervals. Using universal primers, the upstream and downstream tags of every mutant within the deletion pool are amplified simultaneously by PCR. The relative abundance of each barcode or deletion strain is then quantified by sequencing or DNA microarray analysis. If a particular gene confers a selective advantage or disadvantage in the presence of compound, then this mutant will grow faster or slower, respectively, relative to other deletion strains within the pool. In this way, barcoding genes allows for the fitness of each deletion strain to be analyzed in parallel in response to compound treatment or specific growth conditions.[42,51–53] Numerous variations of this method have been developed. By manipulating the copy number of drug target genes (e.g., deleting one copy of a drug target gene in a heterozygous organism), the amounts or dosages of drug targets produced by an organism can be controlled. Gene dosage assays paved the way for a more detailed understanding of drug–target interactions.

3.2.1. Haploinsufficiency profiling (HIP): Reducing gene dosage from 100% to 50%

Using this competitive growth strategy, Giaever et al. reported that reducing the copy number of a drug target gene in diploid *S. cerevisiae* by 50% resulted in drug hypersensitivity.[43] This approach assumes that upon drug treatment, heterozygous deletion strains that encode a drug target will have a reduced fitness versus strains that do not encode a drug target. This drug-target discovery tool, coined drug-induced haploinsufficiency profiling (HIP), has since been extensively used to unveil compound–target interactions.[43,44,54,55] Importantly, HIP identifies drug targets that are essential for growth in a given environment. This means that downstream off-targets such as those associated with non-essential responses may not be identified. This limitation is balanced by the advantages of producing a smaller and potentially less complex pool of expression profile data, identifying 'direct' drug targets with a higher frequency, and using smaller amounts of inhibitors and other reagents. A variation of HIP or other gene dosage assays involves patching mutants onto agar containing the compound of interest, and monitoring colony size over time as an indicator of strain fitness.[56,57] This strategy was used by Carroll et al. to find

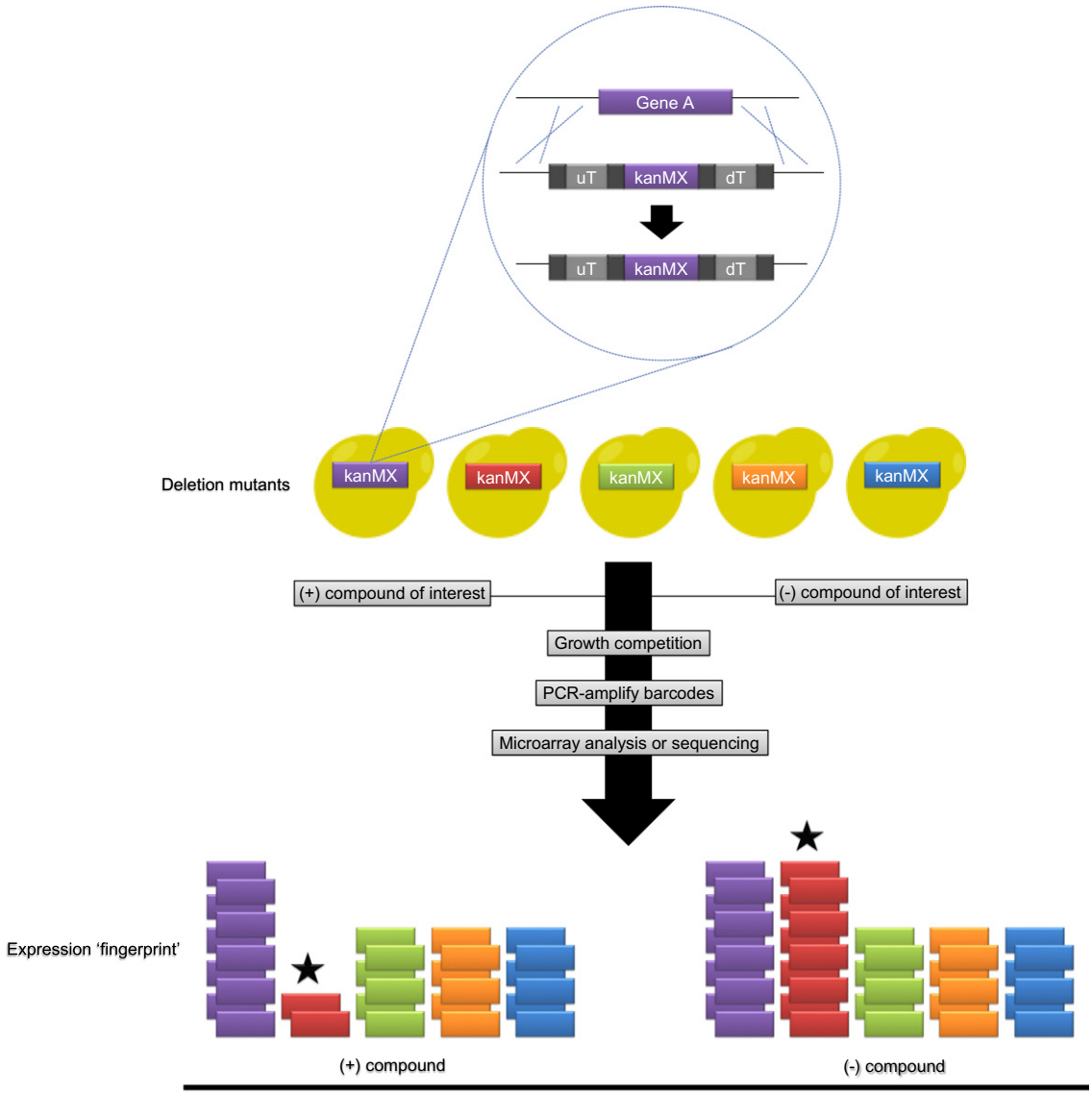

Figure 3. Overview of global microarray analysis. A barcoded yeast deletion library is constructed by replacing each ORF with a deletion cassette consisting of a kanamycin selectable marker, and an identifiable 'barcode': an upstream (uT) and downstream (dT) 20 base-pair sequence tag, both of which are flanked by universal primers. Mutants are grown competitively either in the presence or absence of a compound of interest. Barcodes from pooled mutants are isolated, PCR-amplified, and quantified using sequencing or microarray analysis. In this example, 'pattern-matching' the microarray expression profiles of compound-treated and -untreated mutants revealed that the 'red' gene (designated by a star) may be targeted by the compound.

genes that mediated hypersensitivity or resistance to the yeast A/B toxin, K28: a dense spot of K28-secreting cells was plated onto a lawn of a deletion strain, and zones of inhibition were measured.[57]

Given its unique pathology and complex yeast-to-hypha switches, *Candida albicans* genetics has only recently been coupled to HIP for drug-target identification.[58–61] For example, HIP in *C. albicans* identified inhibitors of an essential fungal fatty acid desaturase (OLE1), as well as novel genetic interactions linked to the unsaturated fatty acid biosynthetic pathway.[62] Interestingly, using HIP, one of the identified drug targets of the vesicular transport inhibitor brefeldin A[63] in *C. albicans* (Sec7) was not identified in *S. cerevisiae*, despite this inhibitor having been reported to inactivate *S. cerevisiae* Sec7 complexes in vitro.[64,65] This result highlights another potential drawback to this technique: although it is possible that brefeldin A is readily inactivated or effluxed by *S. cerevisiae* (or that *S. cerevisiae* has widespread genetic redundancy for the pathways inhibited by brefeldin A), HIP assumes that allele transcriptional feedback—an in vivo phenomenon where mRNA expression levels are maintained despite a reduction in gene dosage—does not occur. This effect can be limited through the use of conditional shut-off strains. Alternatively, 'decreased abundance by mRNA perturbation' (DAmP) may be used in cases where haploinsufficiency does not follow a 50% reduction in gene dosage.[48] The DAmP technique is based on the observation that an essential gene whose 3′ untranslated region is disrupted will encode ~5–50% less protein than the wild-type strain.[66] Another obstacle common to HIP is the paralogue problem, where a drug inhibits a highly redundant target with a similar function.

3.2.2. Homozygous deletion profiling (HOP): Reducing gene dosage from 100% to 0%

An alternative to HIP is homozygous profiling (HOP), where haploid or homozygous diploid deletion collections are screened simultaneously for drug resistance.[44,67–69] Strain fitness is similarly quantified by microarray analysis or sequencing, or on agar plates where fitness is measured by colony size. Unlike HIP, which is used

for the study of essential genes, HOP is restricted to non-essential genes due to cell viability. Because gene dosage is reduced to 0%, this technique by itself cannot directly identify drug target genes since they are completely removed from the mutant pool. This means that HOP often generates a larger pool of expression data or drug target candidates. One advantage of HOP is the complete elimination of allele transcriptional feedback. Further, the HOP assay has proven particularly useful for compounds that do not have a specific protein target. This technique has identified genes that 'buffer' the actual drug target pathway, such as those involved in transport, drug metabolism, and general stress responses.[44,51,54,67,68,70–72] For example, to better understand the DNA-repair response, *S. cerevisiae* homozygous deletion strains were treated with DNA-damaging agents.[72] Subsequent microarray analysis and individual strain growth analysis revealed novel DNA-repair genes, compound-specific expression profiles, and the relative importance of different DNA-repair mechanisms for resistance to each compound.

An interesting application of HOP was demonstrated by Noble et al.[73] To study the genetic correlations between *C. albicans* yeast-to-hypha morphological switching and pathogenicity, *C. albicans* homozygous deletion mutants corresponding to a variety of largely uncharacterized cellular processes were screened in vivo for infectivity of mice, and in vitro for colony morphology and growth rates. These screens identified candidate mutants that were infectivity-defective, but normal in terms of colony morphology and growth rates. Biochemical characterization of these mutants identified the glycolipid glucosylceramide: the first virulence effector discovered to function independently of yeast-to-hypha switches. Recently, HIP and HOP were integrated[74] to elucidate the secondary targets of commonly prescribed psychoactive drugs.[71] Although both methods are complementary, they cannot definitively identify drug targets without additional genetic and/or biochemical confirmation (e.g., binding assays).

3.2.3. Multicopy suppression profiling (MSP): Increasing gene dosage to >100%

Using the same logic as in HIP and HOP, increasing drug target gene dosage from 0% to >100% was therefore expected to confer drug resistance.[20,45,75,76] Multicopy suppression profiling (MSP) identifies drug targets based on the assumption that mutants harboring multiple copies of a drug target will be resistant to drug treatment. Drug resistance in multicopy (overexpression) mutants arises from either the overexpression of proteins involved with inactivating or effluxing the drug, or the overexpression of the drug target itself.[77] Similar to HIP and HOP, strain abundance or fitness is quantified by microarray analysis or sequencing, or by plate-testing for colony size.

One of the first studies to showcase multicopy suppression was reported in 1983.[76] Yeast expressing high-copy plasmids with random genomic inserts were plated onto media containing the antibiotic tunicamycin. Plasmids isolated from resistant colonies were sequenced to identify the target of tunicamycin: *ALG7*. In a more current study, screening ~9000 compounds for growth inhibition of hyperpermeable *Escherichia coli* revealed 49 lead compounds that were subsequently used for MSP.[77] These lead compounds were used to screen an *E. coli* clone set containing random genomic sequences on a high-copy plasmid for suppression of the inhibitory phenotype. Two of these inhibitors selected for multicopy clones encoding dihydrofolate reductase (DHFR). Follow-up kinetics analysis showed that these compounds competitively inhibit DHFR. However, an overwhelming majority of the inhibitors selected for clones encoding the multidrug efflux pump AcrB.

This forward genetics approach was therefore modified to screen for high-copy suppressors using an ordered, high-expression clone library corresponding to all essential genes in *E. coli*.[78] Assuming that targets of most growth inhibitory compounds are products of essential genes since these are needed for pathogen viability, it was reasoned that MSP using essential genes would reveal a more diverse set compound–target interactions. Multicopy *E. coli* clones were grown on microplate-sized agar slabs, and treated with increasing concentrations of known antibiotics or novel growth inhibitory compounds. Interestingly, no two antibiotics produced identical chemical-genetic interactions. This means that MSP, similar to microarray profiling, may produce a mechanistic fingerprint of drug MOA. One of the identified growth inhibitory compounds, MAC13243, was suppressed at up to 16-fold its minimum inhibitory concentration (MIC) by the high-copy *lolA* clone. The LolA protein is a novel drug target that is involved with transferring lipoproteins to the outer membrane. Subsequent molecular assays and structure–activity analysis confirmed the MAC13243-LolA interaction, and showed that MAC13243 is not a substrate of the efflux pump AcrB.

Although MSP has provided invaluable insight into drug MOA, it is not without its faults. One problem with MSP is that efflux pumps and other indirect targets linked to global resistance mechanisms may dominate over other high-copy suppressors.[77] To solve this problem, MSP in mutants harboring or lacking efflux genes may be compared.[77,78] Arita et al. probed the MOA of the anticancer drug etoposide by comparing microarray profiles of etoposide-treated and -untreated hypersensitive multicopy yeast strains—drug-hypersensitive strains were constructed by disrupting *bfr1* and *pmd1*, two non-essential genes encoding ABC transporters.[79] This problem may also be minimized by profiling a more diverse set of efflux-resistant mutants. Another issue with MSP is that some genes are toxic when overexpressed. In *S. cerevisiae*, approximately 15% of overexpressed genes inhibit growth.[80] However, this limitation can also be an advantage in that cell toxicity may be used to identify novel drug targets.[81–84] In one study, yeast mutants overexpressing *Pseudomonas aeruginosa* putative virulence factors were grown and monitored for toxicity.[81] Strains overexpressing a gene of interest were then screened for compounds that rescued yeast growth. Further in vitro and in vivo assays identified the first inhibitors of the *P. aeruginosa* toxin ExoS.

Table 1 summarizes each of the three gene dosage assays.

A more specific and sensitive assay for drug bioactivity has been realized using combinations of HIP, HOP, and MSP.[45,85] Kemmer et al. demonstrated that increasing gene dosage from 0% to 50% to >100% via HIP, HOP, and MSP, respectively, produced distinct information regarding drug MOA.[85] Because most MOA studies have focused on nuclear-encoded genes rather than mitochondria-encoded genes, the authors instead screened using the known mitochondria-dependent inhibitor, dhMotC. This tumor cell invasion inhibitor targets components of sphingolipid biosynthesis. Using HIP, genes encoding proteins involved with sphingosine/ceramide biosynthesis were identified as targets of dhMotC. Using MSP, however, identified genes encoding mitochondrial proteins. In fact, HIP and MSP with dhMotC resulted in only one overlapping gene: *SUI2*, a subunit of a translation initiation factor associated with amino acid starvation. Using HOP, genes involved with the regulation of vacuolar pH were identified. These data provided a more complete understanding of dhMotC MOA, and elegantly demonstrated the importance of using all three gene dosage strategies for MOA determination.

Hoon et al. used one single miniaturized TAG microarray to integrate data generated from genome-wide, drug-induced HIP, HOP, and MSP in yeast.[74] This composite strategy revealed unknown targets of established antibiotics and uncharacterized growth inhibitors based on the following assumption: genes that confer both resistance from MSP, and sensitivity from HIP and HOP, are more likely to be directly related to the MOA of a given drug. A major advantage of this 'one-pot' approach is that gene

Table 1
Comparison of HIP, HOP, and MSP for the identification of drug targets

	Haploinsufficiency profiling (HIP)	Homozygous deletion profiling (HOP)	Multicopy suppression profiling (MSP)
General purpose	Directly identifies drug targets	Indirectly identifies drug targets; typically identifies targets that buffer primary drug MOA (e.g., general stress responses)	Identifies targets that inactivate/efflux the drug, as well as the drug target itself
Phenotype screened	Sensitivity	Resistance	Resistance/sensitivity (toxicity profiling)
Used for essential/non-essential genes	Essential	Non-essential	Essential/non-essential
Pool size of putative drug targets	Smaller (restricted to essential genes)	Larger	Larger
Pros	- Typically results in a smaller, less-convoluted pool of putative drug targets - Can directly identify drug targets	- Eliminates allele transcriptional feedback - Works well for understanding MOA of drug's that do not have a specific protein target (e.g., DNA-damaging agents)	- Both resistant and sensitive strains may be screened (toxicity profiling)
Cons	- Non-essential, off-targets may not be identified - Allele transcriptional feedback may occur (solved with DAmP or conditional shut-off strains)	- Cannot directly identify drug targets - Drug-target pools may overlap or be highly similar due to shared off-target pathways	- Drug-target pool may be overwhelmed with common resistance proteins (e.g., multidrug efflux pumps) (solved by using mutants devoid of efflux pump genes) - Some genes are toxic when overexpressed

dosage assays are performed simultaneously and therefore without bias. In addition, this study explored the use of drug synergy for target identification. Although the two phosphatase inhibitors cantharidin and calyculin A have similar in vitro effects, their gene dosage profiles were surprisingly different. In line with their synergistic activity, using both drugs in combination for HIP and HOP identified genes that were not targeted by either drug alone. An interesting note made by the authors was the observation that MSP could be used to identify drug–target binding sites: the most abundant DNA fragment generated from rapamycin MSP corresponded to the C-terminal fragment of this drug's target. The C-terminus of this protein contains the rapamycin binding domain.

4. Recent advances in determination of MOA of bioactive compounds

4.1. Compound–compound interactions and network analysis

Subjecting cells to combinations of unknown bioactive compounds and known drugs (with well-characterized bioactivities) has provided an indispensable amount of knowledge into uncharacterized drug targets, gene functions, and drug MOA.[86–89] Combinations of different bioactive compounds may result in synergistic, additive, or antagonistic effects (e.g., growth inhibition) depending on whether the combined effect is larger than, equal to, or smaller than their individual effects, respectively.[90] For example, high-copy suppression was recently combined with chemical–chemical profiling to uncover the MOA of uncharacterized growth inhibitors in *E. coli*.[90] Compound bioactivity was probed by measuring *E. coli* growth inhibition following treatment with pair-wise combinations of ~200 unknown growth inhibitors with 14 known drugs. Most of the combinations were synergistic, where chemical–chemical profiles were highly unique for each combination. The uncharacterized inhibitor MAC-0003199 was highly synergistic with norfloxacin, an inhibitor of DNA gyrase. Given that MAC-0003199 is a quinilone carboxylic acid, additional experiments were performed with this compound to confirm its inhibitory effect against DNA gyrase. However, MAC-0003199 was also synergistic with lincomycin, a protein synthesis inhibitor. The chemical-genetic mechanisms underlying this particular synergy are currently unknown. Chemical–chemical profiling is useful for understanding drug bioactivity within organisms that are genetically intractable.[86] This strategy also has the advantage of circumventing the use of tedious genetic techniques such as those associated with gene dosage assays. Chemical–chemical profiling using promiscuously synergistic compounds (e.g., compounds that depolarize the membrane) should be used with caution.

Yeh et al. constructed a drug network based on additive, synergistic, and antagonistic drug interactions.[88] Drug interaction networks have become increasingly common for perturbing drug MOA, especially with the realization of the extent of polypharmacology.[17,88,91] For example, pair-wise combinations of ~750 marketed drugs were clustered based on shared side-effects and other characteristics (similar structures or targets, therapeutic category, etc.), and the resultant side-effect-driven network was used to predict novel drug targets.[92] Another study computationally screened ~4000 known drugs against 1400 protein targets to generate a predictive drug-target network, where each target was defined by its unique set of known ligands.[93] Databases such as DrugBank provide detailed chemical and pharmacological information of known drugs and their targets, and they are often mined for network analysis. However, it is important to note that these repositories are often incomplete and/or biased, meaning that conclusions drawn from network analysis should be validated experimentally.[17,94] To avoid the inherent redundancy in drug-target networks, we recently constructed and experimentally validated a network of synergistic interactions between the antifungal fluconazole and compounds that were not previously known to have antifungal activity.[95] Synergistic combinations of antibiotics and non-antibiotics is an important and relatively untapped chemical space for drug discovery.[96]

4.2. Combining chemical genetics and imaging

As a proof-of-concept study, the targets of four known bioactive compounds were validated by coupling HOP to a fluorescence image profiling system.[97] The CalMorph program allows for high-throughput fluorescence image processing and analysis of multiple cellular events in yeast mutants.[98,99] Mutants were triple-stained for the cell wall, actin, and nuclear DNA. Targets of bioactive compounds were identified using CalMorph in conjunction with an algorithm that correlated ~500 morphological characteristics in

compound-treated mutants with wild-type strains.[97] This technique is largely limited to non-essential genes, as no defined set of morphological parameters for heterozygous deletion mutants currently exists. However, this technique could be applied to multicopy mutants, where certain morphological phenotypes have been characterized.[100,101] Compounds that fail to induce an observable phenotypic change in cells cannot be detected with this method. Although this technology is still in its infancy, it could someday serve as an effective front-line identification of potential drug targets.[102–104] Comprehensive morphological databases should accompany the development of image-based screening for drug targets.

4.3. RNAi

Many current studies have demonstrated the use of RNA interference (RNAi) in drug-target deconvolution.[22,105–109] Recently, short hairpin RNA (shRNA) expression profiles in mammalian cells generated from exposure to known chemotherapeutics were used to predict the MOA of poorly characterized chemotherapeutics.[106] This approach assumes that cells with RNA-silenced cell death regulators (Bcl2 family, p53, and its activating kinases) will exhibit distinct expression profiles following drug treatment. Cells were therefore infected with retroviruses coexpressing a given shRNA for an individual cell death regulator gene and GFP, and then treated with different individual chemotherapeutics. Cell flow cytometry was used to determine the relative abundance or 'resistance index' of each gene knockdown following drug treatment. This sensitive and robust approach showed that drugs with unique targets but similar downstream pathways (e.g., topoisomerase I and II) resulted in distinct shRNA profiles. This approach was simplified to employ only 8 loss-of-function genes to produce a large but manageable template for target elucidation.

For drug targets that are poorly conserved from yeast to mammals, RNAi is advantageous in that it is a relatively simple approach that can be used in mammalian cells, or in organisms that are difficult to genetically modify. Currently, custom-made pooled and unpooled (well-format) RNAi libraries exist.[22] For pooled RNAi libraries, cells are split into drug-treated and -untreated groups. Genomic RNA is isolated, and shRNA is amplified via PCR and quantified by microarray analysis. An RNAi strategy was recently adopted to identify drug synergy.[110] Screening the anticancer compound gemcitabine against a kinase siRNA library selected for siRNA constructs targeting the CHK1 kinase. Adding known CHK1 kinase inhibitors potentiated gemcitabine inhibition in cancer cells. Important pitfalls of RNAi might include varying levels of gene knockdown, the lack of target specificity, and cell-line specific expression profiles. One emerging technique that

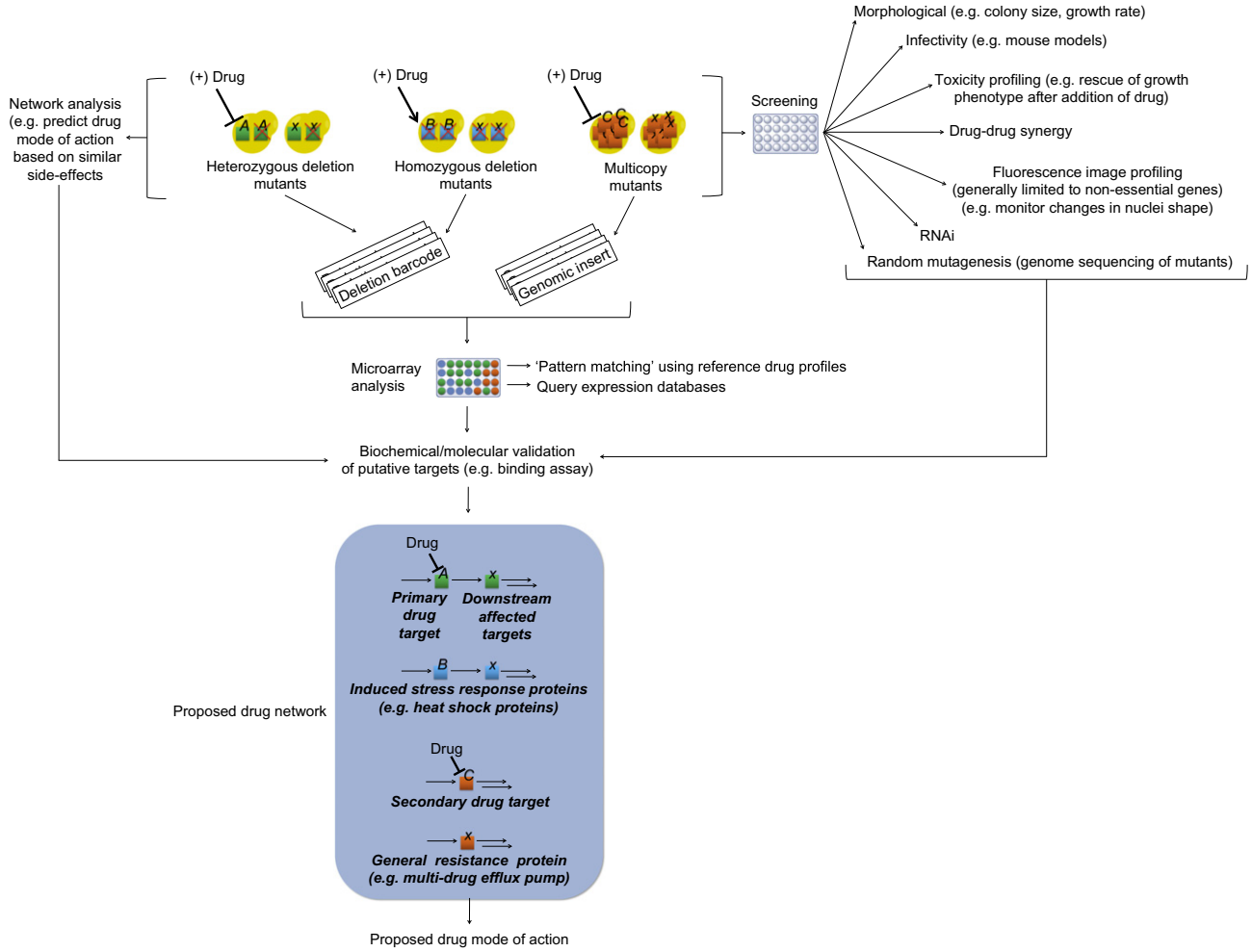

Figure 4. Summary of the chemical-genetic approaches used to determine drug MOA. Yeast deletion mutants are grown competitively in the presence of the compound of interest, and relative strain fitness is quantified by isolating and amplifying deletion barcodes and/or genomic inserts. Labeled products from all three gene dosage assays are hybridized to a single TAG microarray. 'Pattern-matching' microarray profiles to reference drug profiles may identify putative drug targets. Alternatively, yeast mutants may be screened either computationally to construct a predictive drug-target network, or screened using various in vivo techniques to investigate drug–target interactions. Regardless of the method used, further experimental validation is necessary to confirm drug–target relationships. To obtain a detailed, more holistic understanding of a drug's MOA, a compendium of chemical-genetic approaches should be used.

was posed as a solution to cross-species gene function comparison is the use of engineered zinc-finger nucleases (ZFNs) and truncated transcription activator-like effector nucleases (TALENs) with custom-made DNA binding domains.[111–113] Nuclease-mediated gene deletion is generally a highly accurate technique that could be used to gain tremendous insight into drug–target interactions, especially in organisms that are difficult to modify via other genetic tools.

4.4. Phage display biopanning

Phage display biopanning is a powerful tool for drug-target discovery.[114,115] A phage library is constructed, with each phage displaying a unique exogenous peptide fused to the N-terminus of a coat protein. In the biopanning step, the phage library is exposed to potential targets either in vitro or in vivo. Unbound phage is washed away, and bound phage is eluted with an excess of target ligand, or by lowering pH. Eluted phage is then amplified, and the biopanning process is repeated 3–6 times. To identify bound drug targets, the final pool of phage clones is analyzed via DNA sequencing. Phage display biopanning can also be used in combination with column chromatography. This relatively simple procedure has been adapted to study enzyme–substrate, antibody–antigen, and ligand–receptor interactions.[114] Recently, this technology was used to identify novel inhibitors of *Clostridium difficile* toxins.[116]

4.5. Genome sequencing of mutants

The relative ease of whole genome sequencing is ushering in a new approach for the determination of drug MOA, especially in microbes where the genome sequence is known and relatively small. Here, strains resistant to the effects of bioactive compounds are identified followed by sequencing of the genome to identify sequence differences between the wild-type, drug-sensitive organism. Using this approach, the target for R207910, a novel antimycobacterial compound, was identified to be the ATP synthase in *Mycobacterium tuberculosis* through the characterization of several single nucleotide polymorphisms (SNPs) in the target gene.[117] Given the rapid decrease in genome sequencing costs, this approach will undoubtedly increase in use. Similarly, where clues to the identity of the target may be already known, deep sequencing of known alleles of resistant organisms may identify SNPs that can support MOA studies.

Figure 4 summarizes the chemical-genetic approaches to drug MOA determination.

5. Summary

Identifying bioactive compounds is a relatively straight-forward process when compared to establishing their MOA. Traditional biochemical approaches such as impact on global biochemical pathways (metabolism, replication, transcription, or translation) and affinity methods remain important strategies for the determination of MOA. Orthogonal tools including ABPP, and chemical-genetic methods (HIP, HOP, MCS, transcript profiling) have greatly improved our ability to investigate the MOA of bioactive compounds. The addition of RNAi and whole genome sequencing adds significantly to this tool set. In the end, however, establishing MOA requires the use of many strategies to confirm compound–target interactions. A recent example in antibacterial drug discovery is informative.[118] Here, researchers performed a phenotypic screen of 250,000 molecules for antibacterial activity. The compound PNU-286607 was identified, monitored for antimicrobial spectrum, determined to affect DNA synthesis (this was determined by monitoring radioactive precursors (Section 2.1 above)), and confirmed to be a target of DNA synthesis by microarray analysis (Section 3.1 above). Resistant mutants were selected and partially sequenced to identify SNPs (Section 4.4 above). Finally, MOA was confirmed using purified enzymes and well-established assays. These efforts are not trivial, but they must be pursued to unequivocally establish MOA. As small molecule screening increases and becomes more widely available to researchers, efforts to establish MOA must keep pace; there are multiple approaches to determine MOA that should be used in concert to fully exploit the activity of bioactive compounds.

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