Chapter 2

Applying the Logic of Genetic Interaction to Discover Small Molecules That Functionally Interact with Human Disease Alleles

Ari D. Brettman, Pauline H. Tan, Khoa Tran, and Stanley Y. Shaw

Abstract

Despite rapid advances in the genetics of complex human diseases, understanding the significance of human disease alleles remains a critical roadblock to clinical translation. Here, we present a chemical biology approach that uses perturbation with small molecules of known mechanism to reveal mechanistic and therapeutic consequences of human disease alleles. To maximize human applicability, we perform chemical screening on multiple cell lines isolated from individual patients, allowing the effects of disease alleles to be studied in their native genetic context. Chemical screen analysis combines the logic of traditional genetic interaction screens with analytic methods from high-dimensionality gene expression analyses. We rank compounds according to their ability to discriminate between cell lines that are mutant versus wild type at a disease gene (i.e., the compounds induce phenotypes that differ the most across the two classes). A technique called compound set enrichment analysis (CSEA), modeled after a widely used method to identify pathways from gene expression data, identifies sets of functionally or structurally related compounds that are statistically enriched among the most discriminating compounds. This chemical:genetic interaction approach was applied to patient-derived cells in a monogenic form of diabetes and identified several classes of compounds (including FDA-approved drugs) that show functional interactions with the causative disease gene, and also modulate insulin secretion, a critical disease phenotype. In summary, perturbation of patient-derived cells with small molecules of known mechanism, together with compound-set-based pathway analysis, can identify small molecules and pathways that functionally interact with disease alleles, and that can modulate disease networks for therapeutic effect.

Key words Chemical screen, Functional genomics, Chemical genetics, Chemical genomics, Drug repurposing, Genetic interaction

1 Introduction

The growing catalogue of genetic variants that influence human disease risk has highlighted two related challenges: how to understand the biological function of a risk allele, and how to translate genetic and functional insights into new therapies. Targeted mutagenesis, RNA interference, and genome editing represent genetic approaches for dissecting genotype-phenotype correlations.

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Here, we present a complementary chemical biology strategy that applies the logic of synthetic genetic interaction.

Our approach is based upon chemical perturbation using small molecules with defined mechanisms to elucidate the functional and therapeutic implications of human disease alleles. If a small molecule causes a distinct phenotype in the presence versus absence of a disease allele, then the small molecule, or its protein target(s), may be inferred to have a functional connection with the disease gene and the phenotype. This rationale is borrowed from the notion of genetic interaction in classical genetics, in which mutations in two different genes interact to produce a phenotype that is unexpected based on the phenotype of each mutation in isolation. A common implementation of genetic interaction is synthetic lethal screening in model organisms, in which two mutations (each nonlethal in isolation) lead to lethality when coincident in the same organism (Fig. 1). Genetic interaction in model organisms has been a powerful tool to discover functional relationships among genes or their gene products [1, 2].

We have adapted this logic to identify small molecules, genes, or pathways that functionally interact with disease alleles. By analogy to genetic interaction screens, the first "hit" is a mutation in a gene of interest, such as a gene that influences disease susceptibility. The second "hit" is a small molecule with characterized mechanism(s), such as an FDA-approved drug or a tool compound (e.g., a kinase inhibitor). A synthetic phenotype can then be observed when a small molecule causes a qualitatively or quantitatively distinct phenotype in the presence of a wild-type

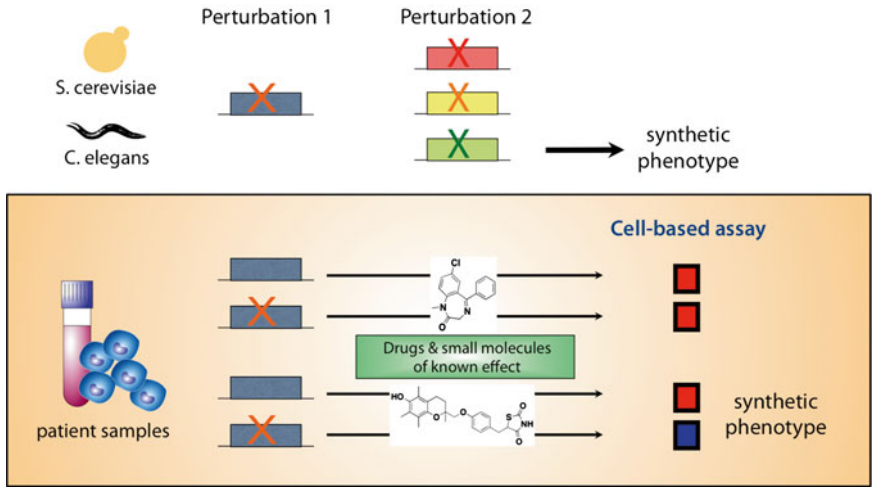

Fig. 1 Analogy between genetic interaction screens in model organisms and chemical:genetic interaction screen in patient-derived cells. In the latter, the second "hit" is provided by a small molecule with characterized mechanism; the synthetic phenotype is manifest as an assay phenotype that is distinct in mutant versus wild-type cells

versus mutant allele at the disease gene (Fig. 1). While operationally these experiments constitute a chemical screen, our annotated small molecules function as quasi-genetic perturbations. By screening FDA-approved drugs as part of a known bioactives collection, new therapeutic hypotheses can be developed that "repurpose" existing drugs and can be rapidly tested in proof-of-concept studies in humans.

To enable a synthetic interaction approach in patient-focused studies, we first developed a framework to analyze the results of chemical perturbation of multiple patient-derived cell isolates and rank compounds according to the degree that their induced phenotypes differ between cells mutant versus wild type at a disease gene. The resulting data set structurally resembles a gene expression dataset, in which multiple cell lines that belong to distinct classes (e.g., mutant versus wild type at a disease gene) are represented in columns, and each row corresponds to a different small molecule (rather than genes, in the case of gene expression) (Fig. 2). The values depicted within each cell of this data matrix (and thus the features that characterize each cell line) are assay phenotypes induced by a small molecule (rather than expression values of individual genes).

Taking advantage of this analogy between chemical screening and gene expression datasets, we developed methods to analyze chemical screening data using statistical methods used to examine gene expression data. For instance, identifying compounds that cause different assay phenotypes between mutant versus wild-type cell lines is analogous to identifying genes whose expression most differs between two classes of cell lines.

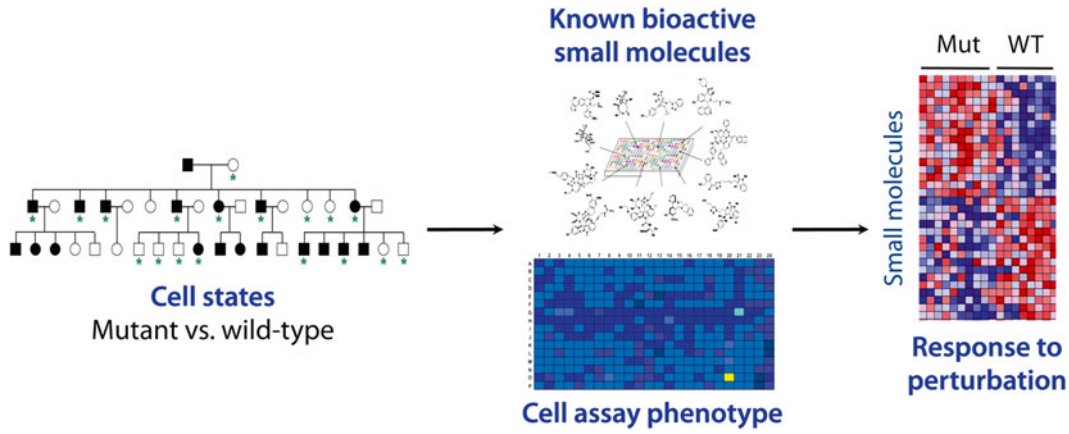

Fig. 2 Overview of chemical genetic interaction screen in patient-derived cells. The resulting dataset resembles that of gene expression. In the heatmap, individual cell lines (belonging to mutant and wild-type classes) are depicted in *columns*, and *rows* represent small molecules. The content of each cell is the quantitative assay phenotype for each small molecule in each cell line

Second, we developed an approach that identifies sets of related compounds that are statistically enriched among the most discriminating compounds (i.e., those compounds that induce phenotypes that differ most between mutant versus wild-type cell lines). We call this method compound set enrichment analysis (CSEA) [3], after the gene set enrichment method (GSEA) on which it is based (http://www.broadinstitute.org/gsea/index.jsp) [4, 5]. GSEA is widely applied to gene expression data to identify sets of functionally related genes that are coordinately up- or downregulated across a class distinction (even if changes for individual genes are statistically modest). Screened compounds are first ranked according to quantitative difference between mutant versus wild-type cells; CSEA then tests if a prespecified set of compounds *S* are randomly distributed throughout the ranked list, or are enriched at the top or bottom (as would be expected if members of set *S* can discriminate between mutant and wild-type classes) (Fig. 3). CSEA calculates a Kolmogorov-Smirnov-like statistic by walking down the ranked list, and increasing a running-sum statistic whenever a member of set *S* is encountered, and decreasing the running-sum statistic whenever a compound that is not in set *S* is encountered. The enrichment score (ES) is defined as the greatest deviation

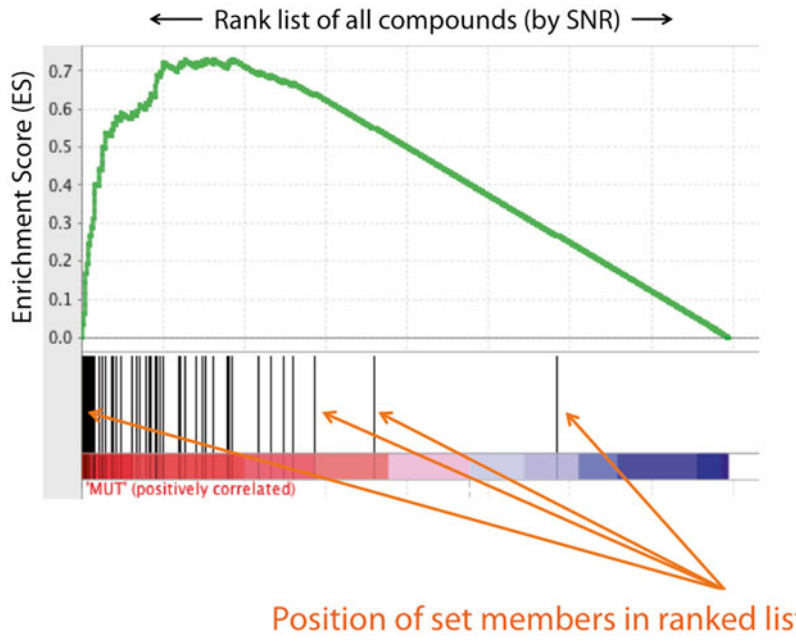

Fig. 3 Sample graphical output of CSEA. The algorithm steps through the ranked list of compounds (ranked according to SNR); at each position, the enrichment score increases if a member of the compound set is encountered, and decreases if a set member is not encountered. *Bottom panel*: The *red:blue horizontal bar* represents the ranked compound list (ranked by SNR); each *vertical line* represents the position of a member of the compound set within the ranked list. In this example, members of the compound set are highly enriched among compounds with the highest SNR; the enrichment score is 0.73

from zero (either positive or negative) achieved by the running-sum statistic (Fig. 3), and the normalized enrichment score (NES) adjusts the enrichment score for the number of compounds in a set. To help evaluate statistical significance, CSEA calculates a permutation *p*-value for the enrichment of each compound set by randomly permuting class assignments (i.e., which cell lines are mutant or wild type, preserving the number of cell lines in each class) 1,000 times, calculating the enrichment score for each permutation, and generating a null distribution from these permutations [5]. While we apply CSEA here to a screen across multiple cell lines belonging to two classes, CSEA may also be applied to traditional chemical screens in a single cell line. In this case, the screen results are inputted to CSEA as a ranked list based on assay *Z*-scores [6, 7]; to calculate statistical significance, CSEA randomly generates 1,000 compound sets with the same number of compounds as the query set, and generates a null distribution from the enrichment scores for these permutated compound sets. Compound sets can be defined by membership in the same metabolic pathway, or the same drug class, or any other shared property. Rather than choosing compound "hits" individually, CSEA identifies promising groups of functionally related compounds, increasing confidence in hit selection, and providing structural and/or functional insights into screen results. CSEA also allows statistical significance to be ascertained for compound sets, even if individual compound effects are statistically modest.

This overall screening and analytic approach was applied to patient-derived cells from a family pedigree whose members were diagnosed with maturity-onset diabetes of the young type 1 (MODY1), a form of monogenic type 2 diabetes due to highly penetrant loss-of-function mutations in the orphan nuclear hormone receptor HNF4α [8–10]. Despite the monogenic cause of MODY1, how mutations in HNF4α lead to impaired insulin secretion and diabetes remains poorly understood. Selecting a surrogate cell line for screening involves balancing physiologic fidelity, and the accessibility and availability of cells. We opted to screen Epstein-Barr virus-transformed lymphoblasts, primarily because the ubiquity of these cell lines in association with clinical cohorts (generally created as a renewable source of DNA) makes them an attractive cell resource for high-throughput screens [11]. As a cellular phenotype, we selected a commercially available assay for cellular ATP content for two main reasons: (a) ATP is a key intracellular sensor in the pancreatic β-cells that can help initiate a series of ionic fluxes that ultimately lead to insulin release; (b) lymphoblasts grow in clumps and are only partially adherent, and the ATP assay used does not involve any wash steps that could result in cell loss. Note that for our synthetic genetic interaction screen, interesting interactions between the disease gene (HNF4α) and a small molecule or its target are revealed regardless of the direction of the small

molecule's effect on assay phenotype; that is, we do not require a specific direction of effect.

We screened lymphoblasts from 18 members of a MODY1 family (10 with the diabetes-causing HNF4α mutation and 8 without). CSEA identified several classes of small molecules that interact with HNF4α (including a series of fatty acids that likely physically bind HNF4α). Several small molecules showed a synthetic interaction with HNF4α genotype in both human lymphoblasts and a murine pancreatic β-cell model, indicating that a subset of interactions between HNF4α and the small molecule (or its protein target) are conserved across lymphoblasts and β-cells [3]. Analysis of the pathways modulated by discriminating compound sets supported a functional connection between the causative disease gene, HNF4α, and "metabolism-excitation coupling" (a pathway crucial for insulin secretion in pancreatic β-cells) [12]. As validation, some of the drugs identified in our screen also modulated insulin secretion from β-cells, a critical disease phenotype in MODY1 [3]. None of the drugs identified in our study have been studied in association with MODY1, and none were approved for diabetes-related indications. These data together demonstrate how perturbation with small molecules of known mechanism, together with compound-set-based pathway analysis, can identify pathways that functionally interact with disease alleles, and that can modulate disease networks for therapeutic effect. More broadly, this approach identifies small molecules that induce phenotypes that are dependent on the presence of disease alleles, and thus reveals the functional consequences of disease alleles in the native genetic context of cells from individual patients.

Recently, the Cancer Therapeutics Response Portal has catalogued both allele- and lineage-specific effects of 354 small molecules on cell viability in 242 genomically defined cancer lines [13]. As datasets incorporating genomic and chemical screening data become more widely available, chemical:gene synthetic interaction analysis will yield mechanistic and therapeutic insights for a variety of diseases and susceptibility genes.

In this chapter, we describe a protocol for the systematic perturbation of patient-derived cell lines using small-molecule probes, which is both scalable to high-throughput workflow and generalizable to a variety of assays. In the protocol presented here, multiple patient-derived lymphoblastoid cell lines either mutant or wild type at a defined genetic locus (e.g., HNF4α) are perturbed by an annotated chemical library. After sufficient incubation, cells are subjected to a phenotypic assay, in this case a luminescence-based readout of cellular ATP content, that aims to quantify the effect of compounds on oxidative phosphorylation, viability, or other relevant traits. The effect of each compound is expressed as a metric that reflects the difference in compound-induced phenotypes between mutant and wild-type cells. These ratios are then

ranked. The resulting rank list is further analyzed using CSEA (described above). The pattern of enriched compound sets, mechanisms of which are extensively annotated, may provide insights into the function of the gene in question.

2 Materials

1. Chemical library in 96- or 384-well format (*see* **Note 1**).
2. 384-Well assay plates: Solid white plates are recommended for luminescence-based assays whereas black plates are recommended for fluorescence assays.
3. Pin-transfer robot with 96-well and 384-well pin-tool, to add compounds to assay plates, such as the CyBI-Well Vario robot (CyBio US) (*see* **Note 2**).
4. CellTiter-Glo (Promega Corporation) assay for cellular ATP. Depending on the experimental question, different assays may be selected (*see* **Note 3**).
5. Plate reader with stacker: For example, an Analyst HT plate reader (LJL Biosystems, Molecular Devices) but many other comparable devices are available.
6. Liquid handler that can add cells and reagents rapidly to a 384-well assay plate (e.g., Multidrop Combi, Thermo Scientific).
7. Lymphoblastoid cell lines (LCLs) (Coriell Cell Repositories).
8. Cell culture medium: For the LCLs, culture medium is RPMI medium 1640 supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, and 1 % penicillin/streptomycin (10,000 U/mL) solution.
9. GenePattern software available at http://www.broadinstitute.org/cancer/software/genepattern.
10. KNIME software available at https://www.knime.org/knime.

3 Methods

3.1 Cellular Assay

While many of the specific steps are unique to the assay described in the protocol, certain general principles apply to many cell-based phenotypic assay screens.

1. Thaw the LCLs from frozen stocks in 5 mL of culture medium in 6-well cluster plates or T25 flasks. Expand the cells, which grow in suspension, until the desired number of cells is attained. Count the cells daily and dilute with medium or passage cells as needed to maintain a concentration of 100,000–300,000 cells/mL (*see* **Note 4**).

2. Plate the LCLs in 384-well assay plates in 40 μL of medium at a density of 300,000 cells/mL (using a liquid-handling device such as a Multidrop Combi).
3. Pin-transfer compound stocks (~50–100 nL) from the compound library using a CyBi-Well Vario robot. Test each compound dose in each cell line in at least two replicates (*see* **Note 5**).
4. Incubate the plates at 37 °C and 5 % CO_2 for 40 h.
5. Remove the assay plates from the incubator and allow the plates to equilibrate to room temperature for approximately 10 min (*see* **Note 6**).
6. Vortex each plate briefly (30 s), moving the plate across the vortexer in a "Z" pattern (*see* **Note 7**).
7. Prepare the CellTiter-Glo reagent according to the manufacturer's recommendations and allow the reagent to equilibrate to room temperature (*see* **Note 8**).
8. Add 40 μL of CellTiter-Glo to each well of the assay plates (an equal volume as culture media). Place the plate on a vibrating platform for 2 min.
9. Allow the plate to incubate at room temperature for 10 min to stabilize the luminescent signal.
10. Read luminescence values using a microplate reader (*see* **Note 9**).

3.2 Data Analysis

1. Raw screening data from the plate reader and files mapping library wells to compound identity are loaded into KNIME for data transformation and analysis. These steps can be done manually in a spreadsheet program such as Excel, but using KNIME (or other similar programs, such as Pipeline Pilot) is more rapid for larger screens with less chance for human error.
2. The baseline distribution of luminescence values for DMSO-treated control wells is calculated utilizing Statistics and Data View KNIME modules.
3. After data processing, small molecules' effects are assessed by calculating a *Z*-score, which expresses each small molecule's effect in units of standard deviation of the distribution of DMSO-treated wells. Several approaches to preprocessing of screening data have been published elsewhere [14, 15].
4. At this point, the screening dataset consists of multiple cell lines (mutant or wild type at a disease gene), and each cell line is described by thousands of features consisting of small-molecule *Z*-score values. In a gene expression experiment, each cell line is described by features consisting of gene expression values. Subsequent analysis steps will take advantage of the analogy to gene expression and use a publicly available suite of gene expression analysis tools to analyze our small-molecule data (GenePattern; publicly available at http://www.broadinstitute.

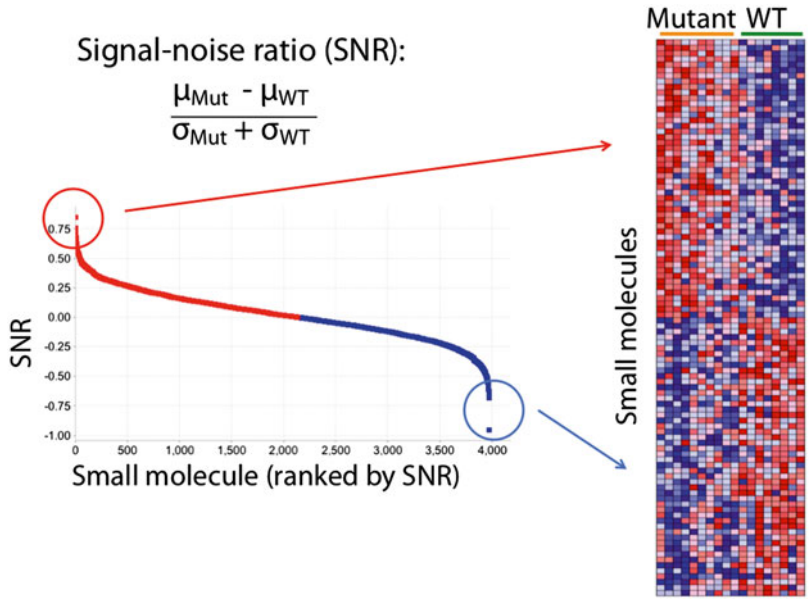

Fig. 4 Calculation of SNR and heatmap representation. The signal-to-noise ratio (SNR) represents the degree to which a compound induces a phenotype that is distinct in mutant versus wild-type cell lines. Compounds with the most positive and most negative SNR represent the compounds that best discriminate between the two classes of cells, and may be depicted in a heatmap (analogous to gene expression data)

org/cancer/software/genepattern) [16]. Other gene expression analysis tools that achieve the same purpose are widely available.

5. Run CSEA. CSEA calculates a "signal-to-noise ratio" (SNR) for each small molecule, which reflects the extent to which small molecule-induced assay values differ between mutant and wild type: $SNR = (\mu_{MUT} - \mu_{WT})/(\sigma_{MUT} + \sigma_{WT})$, where μ_{MUT} and σ_{MUT} are the mean and standard deviation, respectively, of the small molecule's composite *Z*-scores in all mutant cell lines (with analogous definitions for μ_{WT} and σ_{WT} in wild-type cell lines) (Fig. 4). CSEA requires three input files: (a) a tab-delimited data table listing composite *Z*-scores for each small molecule in each cell line (*.gct file); (b) a "class assignment" file that specifies which cell lines belong to which class (mutant versus wild type; *.cls file); and (c) a compound set file listing the name of each set and what compounds belong to each set (*.gmx or *.gmt file; compound identifiers must match in this file and the *.gct file) (*see* **Notes 10** and **11**). For each compound set, CSEA generates an enrichment score, normalized enrichment score, a permutation *p*-value, a false discovery rate (FDR), and a visual depiction of where members of the compound set fall within the ranked list (Fig. 3).

6. The compound sets that are used in CSEA analysis can be curated according to structural or functional similarity. For instance, the majority of the sets used in our synthetic interaction analysis were based on the World Health Organization Anatomical Therapeutic Chemical Classification System (http://www.whocc.no/atc_ddd_index) which classifies drugs according to therapeutic use and chemical properties; additional sets can be curated based on membership in pathways based on Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and Ingenuity Pathway Analysis (Ingenuity Systems). Compound set files were formatted as described in the GSEA documentation.
7. To identify the most discriminating small molecules by SNR without performing CSEA, we used the ComparativeMarker Selection module of GenePattern; the algorithm requires the same file types as CSEA but without the compound set file. Results of ComparativeMarkerSelection can be viewed graphically using the ComparativeMarkerSelectionViewer module (including heatmap views), and the ExtractComparative MarkerResults module creates a table listing the SNR and *p*-values for all compounds that can be exported to Excel. These analyses identify the most discriminating compounds between mutant and wild-type classes as the small molecules with the greatest magnitude of SNR, either positive or negative (*see* **Note 12**).

4 Notes

1. The compound collections used in the MODY1 study [3] included the following libraries: Prestwick Chemical library of marketed drugs (Prestwick Chemical, 1,120 compounds, 2 mg/mL stock concentration); Spectrum Collection of known bioactives, including drugs, tool compounds, and natural products (MicroSource Discovery Systems, 2,000 compounds, 10 mM stock concentration); Institute of Chemistry and Cell Biology Bioactives collection (Enzo Life Sciences, 480 compounds, variable concentrations); BioMol-NT (Neurotransmitter) collection of neurotransmitter drugs and bioactives (Enzo Life Sciences, subset of 287 compounds, 10 mM stock concentration); and 86 discretes (variable sources, 10 mM stock concentration). In addition, other bioactive sets are commercially available, such as the LOPAC collection (1,280 compounds, Sigma-Aldrich), or collections focused on specific protein families or pathways (e.g., ScreenWell compound libraries, Enzo Life Sciences).

2. While a pin-transfer robot was used in the cited study, liquid transfer from compound stock plates for low- to medium-throughput chemical screens can be conducted by hand using a precision manual pin-tool. Models are available in 48- to 1,536-well formats through manufacturers such as V&P Scientific, Inc.
3. The choice of phenotypic assay is one of the most flexible decisions of this protocol and depends on the biological question being asked. In other investigations, instead of using a biochemical readout like ATP content, we have employed high-throughput epifluorescence microscopy and high-content image analysis software to quantify image-based phenotypes, such as numbers of intracellular organelles and co-localization of intracellular proteins. *See* ref. 7 for more details.
4. We observed that reproducibility of small-molecule phenotypes was increased if the lymphoblast cells were maintained (through counting and dilution daily) at a concentration associated with exponential growth for at least 2 weeks from the initial thaw to harvest for screening [11]. Note that LCLs grow as non-adherent clumps.
5. Each compound plate is pinned into two identical plates containing cells, so that each compound is assayed in duplicate. Also, for every 6–8 compound plates, we include a plate containing only DMSO that is also pin-transferred in duplicate. The specific volume pinned is less important than the final concentration of compound (typically ~10 μM) and DMSO (ideally <0.4 % v/v), and depends on the pin set, volume in the 384-well pates, and other operational parameters.
6. To avoid systematic bias, cell lines that are mutant or wild type at the disease allele are processed in random order.
7. We found that this vortexing step significantly decreases the coefficient of variation of the CellTiter-Glo assay (to <10 %) [11]. We believe that the vortexing helps break up the clumps of LCLs, allowing more complete cell lysis by the CellTiter-Glo reagent.
8. We used the CellTiter-Glo reagent diluted 1:3 in phosphate-buffered saline to save on costs with comparable assay performance [3].
9. Because the luminescence values for the assay are time dependent, we coordinated the timing of addition of CellTiter-Glo addition with the time needed for each plate to be read by the plate reader. For instance, if the plate reader can read a plate every 30 s, then we added the CellTiter-Glo reagent to plates at 30-s intervals.

10. The table of small-molecule screening data (containing small-molecule composite *Z*-scores for each cell line screened) must be reformatted according to the requirements of whatever software is used for analysis. For GenePattern software, detailed descriptions of file formats are described within the online documentation (http://www.broadinstitute.org/cancer/software/gsea/wiki/index.php/Data_formats). While our analysis used the default SNR metric, a *T*-statistic yielded similar results.
11. To apply CSEA to a conventional small-molecule screen in a single cell line, CSEA can be run in its "pre-ranked" mode. In this case, CSEA inputs include a rank-ordered list of small molecules and their assay scores (*.rnk file) and the file containing compound sets (*.gmx or *.gmt file).
12. By analogy to gene expression, the most discriminating small molecules for the class distinction (i.e., most positive and most negative SNR) can be used to define "small-molecule signatures" that are characteristic of two classes of cell lines.

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