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Functional genomics platform for pooled screening and mammalian genetic interaction maps

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Abstract

Systematic genetic interaction maps in microorganisms are powerful tools for identifying functional relationships between genes and defining the function of uncharacterized genes. We have recently implemented this strategy in mammalian cells as a two-stage approach. First, genes of interest are robustly identified in a pooled genome-wide screen using complex shRNA libraries. Second, phenotypes for all pairwise combinations of hit genes are measured in a double-shRNA screen and used to construct a genetic interaction map. Our protocol allows for rapid pooled screening under various conditions without a requirement for robotics, in contrast to arrayed approaches. Each stage of the protocol can be implemented in ~2 weeks, with additional time for analysis and generation of reagents. We discuss considerations for screen design, and present complete experimental procedures as well as a full computational analysis suite for identification of hits in pooled screens and generation of genetic interaction maps. While the protocols outlined here were developed for our original shRNA-based approach, they can be applied more generally, including to CRISPR-based approaches.

Keywords

RNA interference; genetic interactions; epistasis; pooled screen; shRNA; mammalian cells

INTRODUCTION

The recent revolution in sequencing technology has facilitated the collection of massive amounts of human genomic sequence and expression data. To understand the function of the human genome and its role in disease, a complementary set of functional genomics tools is required. In particular, there is an acute need for methods that systematically discover pathway relationships between genes. One such method is the use of high-density genetic interaction maps, which report on pairwise measurements of the extent to which perturbing

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AUTHOR CONTRIBUTIONS

M.K., M.C.B. and J.S.W. designed the study. M.K. and M.C.B. developed the experimental methods. M.K. developed the quantitative framework and scripts for computational analysis. M.K., M.C.B. and J.S.W. wrote the manuscript.

one gene's function impacts the function of a second gene. These maps have proven to be powerful tools in haploid microorganisms such as yeast for understanding coordinated gene function and to identifying novel roles for poorly characterized genes^{1–8}. Recently, we^{9,10} and others^{11,12} have described efforts to construct and analyze genetic interaction maps in mammalian cells. Here, we describe a step-by-step protocol to conduct genetic screens and create genetic interaction maps based on our approach^{9,10,13}.

Our current technology platform uses RNA interference (RNAi) to knock down genes. In the first stage, we define genes of interest in a pooled genome-wide screen, and in the second stage, we systematically determine all genetic interactions between these genes (Fig. 1). While RNAi proved a transformative technology for the study of mammalian cells, genome-wide RNAi screens have been plagued by false-positive and false-negative results¹⁴. To address these issues, we designed an ultra-complex genome-wide shRNA library in which each human gene is targeted by ~25 independent shRNAs. The library also contains thousands of matched negative controls. We have developed a quantitative framework for deriving shRNA phenotypes from pooled screens using such libraries, and for robustly detecting statistically significant hit genes, whose knockdown results in a phenotype of interest⁹. To determine genetic interactions between hits from the primary screen, shRNAs targeting the hit genes are individually cloned, batch-validated, and then used to construct a pooled double-shRNA library in a single ligation reaction. Double-shRNA phenotypes are determined in a pooled screen and used to construct a genetic interaction map.

Two other approaches for large-scale genetic interaction maps in mammalian cells were recently published by other groups^{11,12}. These alternative approaches are based on arrayed screening of combinations of siRNA or esiRNA reagents in separate wells. An advantage of arrayed approaches is the possibility to monitor complex phenotypes, such as cell morphology. However, specialized high-throughput equipment is required to implement them. Pooled screening strategies^{15–18}, including ours^{9,10}, have several important benefits, such as uniformity of growth conditions, and scalability, which is crucial to rapidly generate large genetic interaction maps and to compare genetic interaction maps across many different treatment conditions and genetic backgrounds. In addition, our library creation platform, quantitative framework and the computational pipeline for the analysis of pooled screens and the construction of genetic interaction maps^{9,10} applies more generally to other methodologies for interfering with gene expression, such as CRISPR-based modulation of gene transcription^{19–22}.

Applications of mammalian genetic interaction maps

We have demonstrated that a mammalian genetic interaction map can be used to identify new protein complexes and identify novel roles for known complexes involved in the biology of the toxin ricin¹⁰. Other groups have shown that genetic interaction maps in mammalian cells can be used to gain insight into chromatin regulation¹². We expect the potential uses of these maps to be very broad. The ability to query 100,000's of gene pairs in parallel for synthetic lethality in the context of cancer models promises to accelerate the identification of combination drug targets. The comparison of genetic interaction maps

obtained under different conditions or in cells with different genetic backgrounds can reveal the rewiring of pathways in normal and disease states.

The protocol described here was used to produce our published genetic interaction map for factors controlling susceptibility of the human K562 leukemia cell line to ricin¹⁰. We are currently optimizing several aspects of our methodology for use in future studies. These aspects include the design of next-generation shRNA libraries, the use of bioreactors for large scale pooled screens, and the streamlined preparation of samples for deep sequencing, in which we are aiming to replace the gel purification step with size selection using solid-phase reversible immobilization beads.

Limitations—Our strategy for primary screens has performed very well for screens comparing growth of untreated cells to growth of cells in the presence of a drug or toxin that causes an intermediate selective pressure. By contrast, treatments that kill more than 50% of the cell population at a given time should be avoided, and instead, several rounds of moderate treatment should be used. In such screens, our method has successfully identified factors whose knock-down either sensitizes or protects cells from the treatment, compared to WT cells.

The single most important factor in obtaining reliable data from pooled screens is the representation of library elements in the cell population. While we aim for 1,000-fold to 4,000-fold representation, our pilot experiments have shown that higher representation results in even better signal-to-noise ratios¹⁰. A resulting limitation is that whole-genome screens using our methodology cannot easily be conducted where the scale of the pooled population is very limited – including screens such as mouse xenograft models.

Our strategy using ultra-complex libraries can robustly detect genes with even weak effects on the phenotype of interest¹⁰. However, it is possible that some relevant genes are not detected because the shRNA library does not contain enough shRNAs that knock down the gene sufficiently to observe the phenotype of interest. Furthermore, shRNAs that potently knock down genes essential for cell viability can get lost from the cell population before the beginning of the screen. This concern could be addressed by using inducible vectors (e.g. ref²³).

Finally, it is important to keep in mind that the typical selection protocol used in the primary screen (which often takes around 12–14 days) enables the detection of small differences in fitness or resistance to a selective pressure conferred by a given shRNA. This allows the identification of a wide range of genes of interest, which can then be characterized further in a systematic genetic interaction map. However, when validating hit genes individually, the observed effect in a short-term experiment (e.g. 2 days) may be comparatively small.

Experimental Design

Pooled shRNA library

It is assumed that the experimenter begins with a pooled high-coverage shRNA library in the lentiviral plasmid pMK1047 (ref. 10). Design and construction of such libraries has been

described elsewhere^{9,10,13}. For the purposes of screen design and analysis described here, essential features of these libraries are (1) ~25 shRNAs per gene, and (2) large numbers (~500–1000) of matched negative control shRNAs per sublibrary designed using the same rules as for the targeted shRNAs. The plasmids we are currently using for primary screens, batch retests of shRNAs and double-shRNA screens are annotated in Fig. 2. The vectors encode mCherry and puromycin, which allow monitoring of viral infection and selection for the presence of stably integrated virus.

Considerations for screen design

The protocol presented here applies to a screen based on growing cells under standard conditions or in the presence of selective pressure, such as addition of a drug or toxin¹⁰. Quantification of the frequencies of cells expressing a specific shRNA in starting populations, untreated and treated populations can be used to determine different shRNA phenotypes, which we have previously defined quantitatively⁹. Briefly, gamma phenotypes quantify the effect of an shRNA on cell growth in the absence of treatment, tau phenotypes quantify the effect of an shRNA on cell growth in the presence of treatment, and rho phenotypes quantify the effect of an shRNA on resistance to the treatment. While growth and selection conditions must clearly be optimized for each screen and cell type, several general principles should guide screen design:

- Selection should be set up so that a sufficient dynamic range between treated and untreated conditions can be expected. In our screens, a difference of around 6 net doublings between treated and untreated conditions worked well to see significant differences. With such a protocol, an shRNA conferring complete resistance to the selective pressure should be enriched $2^6 = 64$ -fold relative to a negative control shRNA.
- Given the complexity of the shRNA library, it is extremely important to avoid tight population bottlenecks at each stage of the experiment: lentiviral infection, screen selection, and preparation of the sequencing sample. We aim to maintain 1,000-fold representation of each library shRNA on average at each stage. For example, for a screen of an shRNA library with 50,000 different shRNAs, the cell population should be maintained at 50 million cells or more. In particular, when designing a screen, care should be taken to avoid selective pressures that are so high that a large fraction of the cell population is killed. We aim for selective pressures that do not kill more than 50% of the population at any time point during the screen.
- Considerations for library representation also affect the numbers of double-shRNAs that can be queried for the construction of a genetic interaction map. For example, if the screen is carried out in a volume of 2 l at a cell concentration of 500,000 cells/ml, i.e. a population of 1 billion cells, a representation of 1,000-fold would correspond to a complexity 1 million, or all pairwise combinations of 1,000 different shRNAs. To get better quantitative data, we typically choose a representation of 4,000-fold for double-shRNA screens, which would reduce the number of shRNAs in the above example to 500×500 . We typically include 12 independent negative control shRNAs, which then form combinations with other negative control shRNAs (used to derive the WT phenotype in the screen) and

combinations with gene-targeting shRNAs (used to calculate single-gene phenotypes).

- Suspension cell lines facilitate culturing of large cell numbers, since they can easily be grown in large spinner flasks or bioreactors, and do not require trypsinization for passaging, but can simply be diluted. We had good experiences with the human K562 leukemia cell line, which does not grow in clusters and grows stably over many generations. Other suspension lines, including B-cell, T-cell and myeloma lines have also worked well in our hands. Adherent cell lines can be seeded on microcarriers and then grown in spinner flasks or bioreactors.
- We have successfully detected both positive and negative selection. It can however be more challenging to confidently detect shRNAs that cause cells to drop out of the screen, especially if they also affect growth under standard conditions, since phenotypes for such shRNAs are then calculated based on low numbers of reads in the sequencing data, which inherently will increase statistical noise.

Considerations for sample preparation

Following the screen, genomic DNA is isolated from the different cell populations and the shRNA-encoding cassettes are PCR-amplified to generate a sample for deep sequencing. Our protocol was designed with the following goals:

- Adequate representation of the complex cell population should be maintained during library preparation. Genomic DNA is isolated from a number of cells that is 1,000-fold the number of different shRNAs in the screened library. To reduce the amount of genomic DNA used as a PCR template, we size-fractionate the DNA by a restriction enzyme digest (with PvuII) followed by gel purification of the size range around the expected fragment size of the shRNA-expression cassette. We are currently testing an alternative approach based on DNA size selection with solid-phase reversible immobilization beads, which may be implemented in future versions of this protocol.
- During sample preparation from the primary screen, the DNA region encoding the shRNA is PCR-amplified. We found that amplification of this region creates bias against GC-rich shRNAs – presumably because the DNA is more likely to form secondary structures that interfere with amplification. Therefore, it is important that the exact same PCR protocol is used to amplify samples that will be compared during data analysis. Future versions of our library will implement a restriction site in the shRNA loop-encoding region to enable linker ligation followed by half-mer amplification, thus circumventing amplification of the full shRNA-encoding region. Other groups have previously developed analogous strategies^{16,24}.
- In the PCR-amplified material, the shRNA-encoding region is highly enriched compared to its abundance in pre-PCR genomic DNA. Therefore it is critical to avoid cross-contamination of pre-PCR material with any post-PCR material. Ideally, the pre-PCR steps should be performed using separate equipment (e.g., pipettes, vacuum manifolds) from that used for post-PCR steps.

Controls

- For pilot experiments, it is helpful to use positive and negative control shRNAs to assess the dynamic range of screening conditions. Cells infected with control lentivirus expressing mCherry can be mixed with WT cells, and used to track changes in populations under mock selection conditions¹⁰.
- To distinguish the effect shRNA have on cell growth under standard (untreated) conditions from treatment-specific effects, experiments should be designed to compare the population at the time point before selection (t0), the treated population at the end point of the experiment, and a control untreated population that grew for the same time in parallel.
- After infection of cells with the lentiviral library, vials of cells representing the library diversity at ~1000-fold should be frozen to be used as t0 controls, backup vials, and replicate experiments.

MATERIALS

REAGENTS

- Genome-wide shRNA library (e.g. in plasmid pMK1047, as described in refs. 9,10. Plasmid pMK1047 will be provided on request.)
- Target cell line in which the screen is conducted
- 293T cells (ATCC cat # CRL-3216)
- Heat-inactivated FBS (Hyclone Characterized, Thermo Scientific, Cat# SH30071.03)
- DMEM-High glucose (Sigma, cat# D5796)
- RPMI w/HEPES modification (Sigma, cat# R5886)
- 100x Penicillin/Streptomycin (Sigma, cat# P4333)
- 100x L-glutamine (Sigma, cat# G7513)
- PBS (Sigma, cat# D8537)
- Trypsin (Sigma, cat#T3924)
- Puromycin (Sigma, cat#P8833-10MG)
- Mirus LT1 Transfection agent (Mirus, cat # MIR 2300)
- QIAamp DNA Blood Maxi Kit (Qiagen, cat # 51192)
- Qiagen miniprep kit (Qiagen, cat # 27106)
- Qiagen Buffer PB (Qiagen, cat # 19066)
- Qiagen Gel extraction kit (Cat No. 28704 or 28706)
- Qiagen Minelute kit (Cat # 28204)
- Qiagen QIAquick PCR purification kit (Cat No. 28104 or 28106)

- Ethidium bromide (Biorad, cat # 161-0433)
- BstXI (NEB, cat # R0113S)
- PvuII (NEB, cat # R0151S)
- High-sensitivity DNA kit (Agilent Technologies, cat. no. 5067-4626)
- TruSeq SBS v3 kit, 50 cycles (Illumina, cat. no. FC-401-3002)
- TruSeq SR Cluster Kit v3, cBot, HS (Illumina, cat. no. GD-401-3001)
- Phusion® High-Fidelity PCR Kit with 5X HF buffer (NEB Cat No. E0553L)
- dNTPs (e.g. NEB N0447L)
- Polybrene (Sigma cat # H9268-5G)
- DMSO (Sigma Hybrimax, cat# D2650-5X5ML)
- Mr. Frosty Freezing container (Fisher, cat# 15-350-50)
- Tris base (Fisher, cat # BP152-5)
- Boric acid (Sigma, cat # B6768)
- Acetic acid (Fisher, cat # A38-212)
- EDTA (Sigma, cat # 03660)
- Bromophenol blue (Bio-rad, cat # 161-0611)
- Xylene cyanol (Sigma, cat # X4126)
- Sucrose (Sigma, cat # 84097)
- HEPES (Sigma, cat # H3375)
- Magnesium acetate (Sigma, cat # M0631)
- Potassium acetate (Sigma, cat # P5708)
- Agarose (Research Products International, Cat # A20090-500)
- 20% Polyacrylamide/TBE gels, 1.0mm, 12-well (Invitrogen, cat # EC63152BOX)
- 1kb+ DNA ladder (Invitrogen, cat # 10787-018)
- D-tube midi electroelution columns (EMD cat # 71507-3)
- Milli-Q purified H₂O
- Oligonucleotides from IDT Technologies (see sequences in Table 1)
- MegaX DH10 T1R Electrocompetent Cells (Invitrogen, cat # C6400-03)
- Lentiviral packaging plasmids pRSV, pMDL, pVSVG (obtained from the Jacks lab, described in refs^{25,26})
- Plasmid pMK1200 (ref. 9), which we will provide on request.
- T4 DNA Ligase + buffer (NEB, cat # M0202T)

- DH5a cells, subcloning efficiency (Life Technologies, cat#18265-017)
- Carbenicillin (Research Products International, cat # C46000)
- agar (Fisher cat # DF0145070)
- bacto tryptone (Fisher cat # DF0123-07-5)
- sodium chloride (Fisher cat # S271-10)
- yeast extract (Fisher cat # DF0127071)
- SOC (Life Technologies, cat#15544-034)
- Pasteur pipets (glass) (VWR cat # 14672-380)
- Qiagen Gigaprep Kit (Qiagen, cat#12191)
- Qtray plates (Molecular devices, cat # X6023)
- 1-mm gap cuvette for electroporation (USA Scientific, cat # 9104-1050)

EQUIPMENT

- 15-cm Tissue culture plates (Fisher cat # 12-565-100)
- T150 flasks (Fisher, cat# 1012634)
- 6-well plates (Corning, cat# 3506)
- 30 mL syringe (BD, Fisher cat # 14-829-48)
- 0.45um syringe filter with luer lock (Millipore, SLHV033RB)
- 96-well PCR plates (Bio-Rad, cat # MLP-9601)
- 15 and 50 mL falcon conical tubes (Fisher, cat # 14-959-53A and 14-432-22)
- Owl A1 Large Gel System (Thermo Scientific, cat # A1)
- Gel combs creating large volume wells (e.g. 2 ml). These can be created by taping together standard gel combs, or, if a 3D printer is available, by constructing a customized comb. We will provide a file for this on request.
- Invitrogen Xcell *SureLock*® Mini-Cell (Invitrogen, cat # EI0001)
- Tissue culture benchtop centrifuge with plate buckets
- Qiagen vacuum manifold (Qiagen cat # 19413)
- Illumina HiSeq next generation sequencer (Illumina)
- Thermal cycler (Bio-Rad, cat. no. 170-9713)
- 2100 BioAnalyzer (Agilent Technologies, cat. no. G2940CA)
- Computer. The scripts described here have been developed for use on a computer with a MacOS operating system. Use on computers with other operating systems may require adaptation of the scripts.
- Razors (VWR, cat. no. 55411-050)

- Low-power UV gel box
- Electroporator (e.g. Bio-rad cat # 165-2662)
- Bunsen burner (e.g. Fisher cat # S49107)

REAGENT SETUP

293T growth medium—To 450mL DMEM-High glucose, add 50mL heat-inactivated FBS (10% (vol/vol) final), 5 mL Penicillin/Streptomycin (100x), and 5 mL Glutamine (100x). Store at 4C for up to 1 month.

4 mg/mL polybrene—Add 40 mg polybrene to 10mL Millipore water, filter through a 0.2 um filter, and freeze in aliquots at –80C, and store indefinitely.

Freezing medium—Prepare freezing medium by adding 5 mL DMSO to heat-inactivated FBS (10% DMSO final), and filter through a 0.2 um filter. Store at –20C for up to 1 year.

1X TAE buffer—Prepare 1L 1X TAE by combining 4.84g Tris-base, 1.14 mL acetic acid, and 2mL 0.5M EDTA pH 8.0; adjust to 1L with milli-Q water [40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0 final]. Store at room temp 6 months.

10x DNA loading buffer—Prepare 10 mL 10x DNA loading buffer by combining 6.5g sucrose, 100 uL 1M, Tris pH 7.5, 200 uL 0.5 M EDTA pH8.0, 2 mg bromophenol blue, and 2 mg xylene cyanol; adjust to 10mL with milli-Q water [0.65 g/ml sucrose, 10 mM Tris pH 7.5, 10 mM EDTA, 0.02% (w/v) bromophenol blue, 0.02% (w/v) xylene cyanol final].

1X TBE buffer—Prepare 1L 1X TBE by combining 10.8g Tris, 5.5g Boric acid, 4 mL 0.5M EDTA (pH 8.0), and adjust volume to 1L with milli-Q water. [89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0 final] Store at room temp for 6 months.

2X Annealing buffer—Prepare 100mL 2X Annealing buffer by combining 1.96 g potassium acetate, 12mL 0.5 M HEPES-KOH pH 7.4, and 0.86 g magnesium acetate, and adjust to 100mL with milli-Q water. [200 mM potassium acetate, 60 mM HEPES-KOH pH 7.4, 4 mM magnesium acetate final]. Store at room temp for 6 months.

Qtray Agar plates—Prepare LB agar: 15 g/l agar, 10 g/l bacto trypton, 5 g/l sodium chloride, 5 g/l yeast extract, autoclave, cool down while stirring. When the temperature is below 50 °C, add 75 ug/ml carbenicillin. Pour into Qtray plates, 200 ml per plate.

LB Agar plates—Prepare LB agar: 15 g/l agar, 10 g/l bacto trypton, 5 g/l sodium chloride, 5 g/l yeast extract, autoclave, cool down while stirring. When the temperature is below 50 °C, add 100 ug/ml carbenicillin. Pour into 10 cm round plates.

Glass spreading rod—In the flame of a Bunsen burner, heat the region of a glass Pasteur pipet about 5 cm below the tip. After a few seconds, the pipet will bend in the heated region, remove it from the flame. Now heat the very tip of the pipet to bend it towards the thick

stem of the pipet. The resulting glass spreading rod allows scraping of colonies from agar plates without scratching the agar surface.

EQUIPMENT SETUP

Software and file installation

- Install bowtie²⁷, which can be downloaded for free at <http://sourceforge.net/projects/bowtie-bio/files/bowtie/1.0.0/>.
- Install python (www.python.org), or, in order to use interactive graphics, install the Canopy Python package from Enthought, Inc., which is free for academic use (<https://www.enthought.com/products/canopy/academic/>).
- To generate tab-delimited text files, we recommend the use of a command-line text editor, like vim (<http://www.vim.org>).
- Download an archive containing the custom scripts used in this protocol, as well as example data, from gimap.ucsf.edu. Unzipping of the archive will create the subdirectory structure referred to in this protocol.
- Download Homo_sapiens.gene_info.gz from ftp://ftp.ncbi.nlm.nih.gov/gene/GENE_INFO/Mammalia/, unzip the file and move it to subdirectory LUT.
- Download and install the Pycluster module for python from <http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#pycluster>
- Download and install Cluster 3.0 (ref. ²⁸) from <http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>.
- Download Java Tree View²⁹ from <http://sourceforge.net/projects/jtreeview/files/>.
- Create a fasta file for the shRNA library you used for the primary screen as follows: Each entry describes one shRNA. Each shRNA name should begin with the Entrez Gene ID, followed by a double underscore, and a number or other information that uniquely identifies the shRNA, e.g. 9064__NM_004672.3__24. Names of negative control shRNAs should have a 0 instead of an Entrez Gene ID, e.g. 0__0__1. The nucleotide sequence included for each shRNA is the reverse-complement of the shRNA guide strand for the shRNA in your shRNA library. (The length of each sequence should be 22 nt, so in case your guide strand is shorter, include the reverse-complement sequence of the constant nucleotides following the guide strand in your shRNA construct.) To create a bowtie index from this file, use the bowtie-build command. For example, if the path to bowtie-build on your computer is /Applications/bowtie-0.12.3/bowtie-build, and the name of the fasta file for your shRNA library is LibraryName.fa, enter the following on the command line:

```
/Applications/bowtie-0.12.3/bowtie-build LibraryName.fa LibraryName
```

Six output files are created, move them to the subdirectory indices/. In the script align_primary.py (subdirectory scripts), specify the path to bowtie on your computer in line 4.

PROCEDURE

Lentivirus preparation and infection – TIMING 7–10 d

! CAUTION All lentivirus work should be performed in BSL2 containment; wear appropriate protective clothing, and dispose of lentivirus-infected materials using bleach.

- 1 | Trypsinize healthy, logarithmically dividing 293T cells and plate 7.5×10^6 in a 15-cm plate in 30ml of 293T growth medium. Steps 1–5 describe the handling of one 15 cm plate, which is on appropriate scale for an shRNA library with a complexity of up to ~20,000. Scale up the number of plates for larger shRNA libraries as necessary.
- 2 | Transfection: 24 h after plating the cells, prepare transfection mix by add 48 μ l Mirus LT1 to 1300 μ l DMEM (no additives), mix and wait 5 min. Add a mixture of 8 μ g pooled shRNA library (e.g. in plasmid backbone pMK1047) and 8 μ g of an equal mix of packaging plasmids (pMDL, pRSV, pVSVG). Mix, wait 20 min, and then add dropwise to the 15 cm plate.
- 3 | 72 h after transfection, harvest virus by aspiration into a 30 ml luer-lock syringe (no needle), and then filtration through 0.45 μ m filter into 50 ml conical tube. We prepare virus fresh for each infection, although in principle virus can be concentrated, titered, and stored at -80°C .

PAUSE POINT

- 4 | Transduction: for suspension cells, infect cells with the virus library by resuspending 35×10^6 cells in 60ml lentiviral supernatant supplemented with 8ug/ml polybrene, and distributing this mixture into the wells of a 6-well plate. Centrifuge at 1000xg for 2h at 33°C . If using adherent cells, split cells the day before infection, and infect cells by simple addition of virus supernatant to the growth media; no centrifugation or polybrene is necessary (skip step 5). The exact parameters may need to be adjusted based on the target cell line.

CRITICAL STEP Optimize infection for each cell type by adjusting the amount of virus added to the cells. The initial multiplicity of infection (monitored as the fraction of mCherry-expressing cells by microscopy or flow cytometry) should be ~30–40% to minimize double-infection of cells.

- 5 | Carefully pipet the suspension cells in the 6-well plate up and down with a 5 ml serological pipet. Transfer all cells to a 50 ml conical tube and spin them at 400g for 5 min at room temp. Remove viral supernatant by aspiration and resuspend cells in fresh growth medium. Transduction during spin step is sufficient for infection.
- 6 | Allow the cells 2 days of recovery before selecting them for stable integration of virus with puromycin.

CRITICAL STEP Titer the dose of puromycin for each cell line to identify the minimum necessary dose to kill ~80–90% of cells to avoid selection for superinfected cells.

- 7 | The selection of cells with stably integrated virus can be monitored as an increase in the fraction of mCherry-expressing cells by flow cytometry or microscopy. Once ~80% of cells are mCherry-positive (typically after 1–3 days of selection), cells should be removed from puromycin and expanded in fresh growth medium. Vials of cells should be frozen to be used as t0 controls, backup vials, and for replicate experiments. The number of cells in each aliquot should be chosen such that the average representation of shRNAs in each library is 1,000-fold. (E.g., for a library with 50,000 shRNAs, 50 million infected cells should be frozen in each aliquot.)

PAUSE POINT After expansion and freezing vials, cells can be stored indefinitely in liquid nitrogen before screening.

? TROUBLESHOOTING

Conducting a screen – TIMING (varies from 1–21 d depending on selection strategy)

- 8 | Cells are subjected to selective pressure (duration, dose, strategy are dependent on individual experiment); see experimental design section for considerations.
- 9 | For a screen based on growth or survival under a selective pressure, keep track of the number of cell doublings for the treated and untreated (control) cell populations. The fraction of mCherry-positive cells may change during the screen, as monitored by flow cytometry. In that case, calculate the number of doublings for the uninfected (mCherry-negative) cells, since these represent the wild type.
- 10 | At the end of the screen, freeze down multiple vials of cells for selected and unselected conditions. As for t0 aliquots, the number of cells in each aliquot should be chosen such that the average representation of shRNAs in each library is 1,000fold. (E.g., for a library with 50,000 shRNAs, 50 million infected cells should be frozen in each aliquot.)

Preparing samples for deep sequencing – TIMING 3 d

- 11 | Genomic DNA is prepared using Qiagen DNA Blood Maxi kits. We chose this method for ease of use and reproducibility, although alternative methods for DNA purification could also be used. 1 column is used to extract DNA from 100×10^6 cells, and number of columns is scaled accordingly.
- 12 | Preheat a water bath to 70°C.
- 13 | Thaw cell aliquots by incubating them in a 37°C water bath.
- 14 | Resuspend cells in 25 ml PBS into a 50 ml-Falcon tube and pellet by centrifugation 5 min at 400g.

- 15 |** Based on the number of total cells per sample, extract genomic DNA using the QIAamp DNA Blood Maxi kit according to the manufacturer's instructions, with the specifications described in the table below. If more than 300×10^6 cells are present in the sample, split the cells into > 1 Falcon tube before starting. To the manufacturer's instructions apply the following minor modifications: repeat step 6 (leave first eluate in tube) and pipet up and down to ensure genomic DNA is in solution; in step 9, spin 2 minutes at $3800 \times g$; in step 10, spin 20 minutes at $3800 \times g$; in the elution step add 0.8 ml EB per column, incubate for 5 min, and centrifuge for 5 min at $3800 \times g$.

Number of cells:	$< 100 \times 10^6$	$< 200 \times 10^6$	$< 300 \times 10^6$
Resuspend in PBS	5 ml	10 ml	15 ml
Add Qiagen protease	500 μ l	1,000 μ l	1,500 μ l
Add buffer AL	6 ml	12 ml	18 ml
Add ethanol	5 ml	10 ml	15 ml
Split to how many columns:	1	2	3

- 16 |** Measure genomic DNA concentration (typically $\sim 1 \text{ mg}/100 \times 10^6$ cells or more).

PAUSE POINT Eluted genomic DNA can be frozen at -20°C indefinitely.

- 17 |** Set up a restriction digest of the genomic DNA in a 15-ml Falcon tube. For each 1 mg genomic DNA, adjust volume with Qiagen buffer EB to 1,500 μ l, mix with 169 μ l NEB Buffer 2 and add 20 μ l of PvuII. Incubate overnight at 37°C .

- 18 |** Pour 400 ml 0.8% agarose gel in TAE containing 32 μ l 10mg/mL ethidium bromide in an Owl A1 large gel system using gel combs creating large wells.

!Caution Ethidium Bromide is mutagenic, and should be handled with care and disposed of properly

- 19 |** After gel has solidified, slowly and carefully remove gel comb. Add TAE to the gel chamber so that buffer surface is $\sim 1\text{--}2\text{mm}$ below the top surface of gel.

CRITICAL STEP 1.5 ml volume wells generated with custom gel combs are very fragile. Tearing of the wells can cause sample leak and cross-contamination of samples.

CRITICAL STEP Large sample volumes cannot be neatly loaded if gel is under buffer, so it is necessary to maintain the gel surface exposed.

Carefully add sample in loading buffer to well, and run alongside 2.5 μ g 1kb+ DNA ladder (in an adjacent small well) at 220V for ~ 80 min.

- 20 |** Visualize PCR products on a low-power UV gel box. Cut a wide gel slice spanning between the 1kb and 1.6kb markers (Fig. 3a) and transfer to a 50-mL tube. Gel slices are typically $\sim 12\text{-mL}$ in volume.

- 21 | Add 3 gel volumes of buffer QG to the gel slice and incubate in a water bath at 55°C with occasional agitation until the gel is fully dissolved.
- 22 | Add 1/100 of the total buffer QG + gel volume of 3M sodium acetate pH 5.2 and mix.
- 23 | Purify genomic DNA by collecting the DNA/QG mixture onto Qiagen gel extraction columns (4 columns per 100×10^6 cells starting material), using a Qiagen vacuum manifold.

CRITICAL STEP Separate vacuum manifolds should be used for pre-PCR and post-PCR DNA purification to avoid cross-contamination with trace amounts of DNA from other experiments. Great care should be taken to thoroughly wash the manifold with water after each use.

- 24 | Wash columns twice with 800 μ l Qiagen buffer PE, then remove from vacuum manifold and spin 1 min at 14,000 rpm in an eppendorf centrifuge to dry membranes.
- 25 | Elute each column with 50 μ l EB.
- 26 | Measure concentration of post-digest DNA (typically ~15–20 μ g per 50×10^6 cells).

PAUSE POINT Eluted DNA can be frozen at –20°C indefinitely.

- 27 | PCR amplify the shRNA-encoding cassette, introducing Illumina adapter sequences with different indexes. For each sample that will be sequenced in the same lane, choose a unique forward primer index from the following set described in Table 1: oMK312, oMK313, oMK314, oMK315, oMK316, oMK317, oMK318, or oMK319. Indices should be chosen so that unique barcode sequences (see Table 1) can be distinguished when samples are pooled for Illumina sequencing. For all samples, use the same reverse primer, oMCB800 (Table 1). A typical PCR reaction total volume for DNA from 50×10^6 cells is between 1 and 2 ml, i.e. between 10 and 20 times the 100 μ l PCR reaction described below. Assemble the following PCR reaction, scaling the total volume to amplify the full amount of genomic DNA from Step 26:

Compound	amount per sample	final concentration
Size-fractionated DNA	1.5 μ g	150 ng/ μ l
Forward primer (100 μ M stock)	0.8 μ l	0.8 μ M
Reverse primer oMCB800 (100 μ M stock)	0.8 μ l	0.8 μ M
DMSO	3 μ l	3%
dNTPs (10 mM stock)	2 μ l	200 μ M
%X HF buffer	20 μ l	1X
Phusion polymerase	1 μ l	
Water	up to 100 μ l	N/A

CRITICAL STEP Maximum of 1.5 µg genomic DNA can be amplified in 100 µl.

- 28 | Distribute the PCR mix into a 96-well PCR plate, 100 µl per well, and perform PCR with following conditions:

Cycle number	Denature	Anneal	Extend	Hold
1	98 °C, 2 min			
2–24	98 °C, 30 s	56 °C, 15 s	72 °C, 15 s	
25			72 °C, 10 min	
26				4 °C

- 29 | Collect the PCR reactions from the 96-well plate into a 15 ml conical tube. Once the material has been mixed, only a fraction (typically 800 µl) needs to be processed, the rest can be stored as a backup sample. Mix 1 part PCR reaction with 4 parts Qiagen buffer PB, and add 1/100 volume of 3M Sodium acetate pH 5.2.
- 30 | Purify the sample/PB mix over a Qiagen PCR purification column, following the manufacturer's instruction, and elute in 50 µl buffer EB.
- 31 | Load the PCR product in DNA loading buffer onto a 20% polyacrylamide/TBE gel, distributing 20 µl into adjacent wells of a 12-well gel, and run for 1 h at 180 V in 0.5X TBE. Once the sample has been mixed, only a fraction of the sample (typically 20%) needs to be loaded onto the gel.
- 32 | Remove gel from casing and stain in 50ml 0.5X TBE with 5 µl of 10mg/ml ethidium bromide for 5 min on a rocking platform.
- 33 | Visualize PCR products on a low-power UV gel box (Fig. 3b). The correct products should run at ~250 bp. Excise this band using a razor blade.

? TROUBLESHOOTING

- 34 | Combine all slices from a single sample into a D-tube midi electroelution column (pre-soaked in 800 µl 0.5xTBE for 5 min and emptied), add 800 µl fresh 0.5X TBE and close.

CRITICAL STEP Make sure the lid is closed finger-tight.

- 35 | Electroelute in TBE for 1.5 h at 160 V; reverse electrode polarity for the last 2 minutes of electroelution to ensure DNA does not stick to membrane.

CRITICAL STEP Make sure the tubes stay submerged in TBE throughout the electroelution time.

- 36 | Collect electroeluted DNA, mix 1 part PCR product with 4 parts Qiagen buffer PB, and add 1/100 volume of 3M sodium acetate pH 5.2.

- 37 |** Purify PCR product on 1 Qiagen gel extraction column using a vacuum manifold, wash column with 1 ml Qiagen buffer PE, and elute in 100 µl buffer EB.
- 38 |** Verify the size and concentration of the purified PCR product using procedures recommended by the sequencing facility (such as qPCR or bioanalyzer), and sequence on an Illumina HiSeq using primer oMK132. This primer should be added to the primer mix provided by Illumina as follows: for each lane, 240 µl of sequencing mix HP1 (provided with Illumina sequencing reagents) should be mixed with 1.2 µl of 100 µM oMK132. A 1000-fold representation of library shRNAs by sequencing reads is recommended, e.g. for a library with 50,000 different shRNAs, 50 million reads should be obtained.

Data analysis for the primary screen – TIMING 1 – 4 d

- 39 |** In the subdirectory growth, create a tab-delimited text file containing, on the first line, the number of doublings untreated WT cells completed during the experiment (Step 9) ('G'), and, separated by a tab, the difference between that number and the number of doublings treated WT cells completed during the experiment ('K'). As an example, we have provided the file Example_Primary_GK.txt.
- 40 |** Move the sequencing results files, in fasta or Illumina fastq format, to the subdirectory sequencing.
- 41 |** Open a Terminal window and on the command line, change to the directory scripts, and enter the IPython console with the following command:

```
ipython --pylab
```

The Enthought python distribution enables interactive graphical data display. If you do not want to use this feature, you can execute all scripts directly from the terminal command line by replacing “run” with “python” in the following command line examples (Steps 42–46). In that case, where graphical output is created as part of the program pipeline, the pop-up window with the graph needs to be closed for the program to proceed.

- 42 |** Run the script crop_reads.py to process the sequencing data with the following command-line arguments:
- Name of the sequencing data file, e.g. ../sequencing/Sample.fastq
 - Name of the output file, e.g. ../cropped/Sample.fa
 - Number of bases after which the sequencing read should be truncated. For the analysis of primary screen samples, 22 is sufficient.

Example command line:

```
In [1]: run crop_reads.py ../sequencing/Sample.fastq ../cropped/Sample.fa 22
```

- 43 |** Run the script `align_primary.py` to align the cropped reads to the shRNA library reference with the following command-line arguments:
- Cropped sequencing data file, e.g. `../cropped/Sample.fa`
 - Prefix for the output files generated by this script, e.g. `Sample`
 - Name of the primary shRNA library to which the sequencing reads should be aligned, e.g. `LibraryName`.
 - Optional: additional names of other shRNA library names can be specified, in case your sequenced sample was derived from a cell population transduced with several shRNA libraries.

Example command line:

```
In [2]: run align_primary.py ../cropped/Sample.fa Sample LibraryName
```

- 44 |** The script generates several output files, most importantly a table of the frequencies with which each individual shRNA was detected in the sequencing data. In the above example, this file will be named `SampleLibraryName.counts`, located in the subdirectory `counts`. Once this counts file was successfully generated, files in the directories `cropped` and `maps` can be removed.
- 45 |** Repeat Steps 42–44 for all sequenced samples. Typically, these will consist of sets of the t0 sample, a treated sample and an untreated sample. Corresponding example count files, named `Example_Primary_t0.counts`, `Example_Primary_treated.counts` and `Example_Primary_untreated.counts`, respectively, are provided in the subdirectory `counts`.
- 46 |** The results from the primary screen are analyzed using the script `analyze_primary_screen.py` with the following command-line arguments:
- Prefix for output files, e.g. `../results/Example`
 - Counts file from t0 sample, e.g. `../counts/Example_Primary_t0.counts`
 - Counts file from untreated sample, e.g. `../counts/Example_Primary_untreated.counts`
 - Counts file from treated sample, e.g. `../counts/Example_Primary_treated.counts`
 - File containing growth parameters G and K, e.g. `../growth/Example_Primary_GK.txt`
 - Optional: information on negative controls. If this argument is not provided, the program interprets all shRNA names starting with the prefix “0” as negative controls. Alternatively, custom prefixes can be listed on the command line as consecutive arguments. If no negative control shRNAs are present in the library, the keyword “all” can be

provided to use all shRNAs as an approximation of the “null distribution”. This option is generally not recommended.

Example command line:

```
In [3]: run
analyze_primary_screen.py ../results/Example ../counts/Example_Primary_t0.cou
nts ../counts/Example_Primary_untreated.counts ../counts/Example_Primary_trea
ted.counts ../growth/Example_Primary_GK.txt
```

The script generates output on the terminal screen to summarize statistics of the input data.

Example screen output:

```
Total T0 counts: 15709968
Number of different shRNAs in the T0 sample: 54530
Total untreated counts: 19386703
Number of different shRNAs in the untreated sample: 54630
Total Treated counts: 19907742
Number of different shRNAs in the treated sample: 54252
221 shRNA species found in the untreated sample but not the t0 sample.
121 shRNA species found in the t0 sample but not the untreated sample.
151 shRNA species found in the treated sample but not the t0 sample.
429 shRNA species found in the t0 sample but not the treated sample.
34 shRNA species found in the treated but not the untreated sample.
104 shRNA species found in the untreated but not the treated sample.
```

The script generates the following output files:

- Example.gammas:
Each line summarizes information for one shRNA with the following tab-separated fields: Name of shRNA, growth phenotype in the absence of treatment (gamma), counts in t0 sample, counts in untreated sample.
- Example.taus:
Each line summarizes information for one shRNA with the following tab-separated fields: Name of shRNA, growth phenotype in the presence of treatment (tau), counts in t0 sample, counts in treated sample.
- Example.rhos:
Each line summarizes information for one shRNA with the following tab-separated fields: Name of shRNA, phenotype quantifying the resistance to treatment (rho), counts in t0 sample, counts in treated sample.
- Example_pvalues.txt:

Each gene one line with the following tab-separated fields:

- Entrez Gene ID
- Official Gene Symbol
- Gene information
- Number of shRNAs for which gammas could be calculated
- Gene scored as (P)rotective (i.e. shRNAs were enriched) or (S)ensitizing (i.e. shRNAs were depleted) based on Gamma phenotype
- P value for gene based on gamma phenotype, calculated using Mann-Whitney U test
- P value for gene based on gamma phenotype, calculated using Kolmogorov-Smirnov test
- Number of shRNAs for which taus could be calculated
- Gene scored as (P)rotective (i.e. shRNAs were enriched) or (S)ensitizing (i.e. shRNAs were depleted) based on tau phenotype
- P value for gene based on tau phenotype, calculated using Mann-Whitney U test
- P value for gene based on tau phenotype, calculated using Kolmogorov-Smirnov test
- Number of shRNAs for which rhos could be calculated
- Gene scored as (P)rotective (i.e. shRNAs were enriched) or (S)ensitizing (i.e. shRNAs were depleted) based on rho phenotype
- P value for gene based on rho phenotype, calculated using Mann-Whitney U test
- P value for gene based on rho phenotype, calculated using Kolmogorov-Smirnov test

This file is best opened in a program like Microsoft Excel to allow sorting of genes by P value.

We have previously provided mathematical definitions of the quantitative phenotypes (gamma, tau and rho) and of the approach for calculating P values⁹.

The script also generates the following graphical output in individual pop-up windows:

- Scatter plots of comparing shRNA frequencies in pairs of samples (e.g. Fig. 4a)
- Histograms of the number of shRNA phenotypes obtained for each gene (e.g. Supplementary Fig. 1a).

- Comparison of the phenotypes of all shRNAs, compared to negative control shRNAs (either as cumulative curves, e.g. Supplementary Fig. 1b, or histograms, Fig. 4b)
- Scatter plots comparing gene P values calculated using the Mann-Whitney U (MW) test of the Kolmogorov-Smirnov (KS) test. The two tests generally yield very similar results (Supplementary Fig. 2a)
- Scatter plot comparing gene P values based on growth in the absence of treatment (gamma) with P values based on growth in the presence of treatment (tau) (Fig. 5a).

These graphs are saved as graphics file with the prefix specified on the command line. They can also be explored interactively, e.g. by zooming into areas of the P value scatter plots.

? TROUBLESHOOTING

- 47 |** After running the script, the following interactive functions can be called on the command line to explore the data interactively: `gene_gamma()`, `gene_tau()`, `gene_rho()`.

The functions accept the following arguments:

- Name of gene of interest, in quotes. E.g. 'VPS54'.
- Optional: AllLabels (Boolean):
 - True: Display names of shRNAs targeting the gene of interest
 - False (default): Do not display these names
- Optional: MarkerSize (Integer):
 - Size of circles representing shRNAs. Default: 40
- Optional: ShowLegend (Boolean):
 - True: Display legend identifying shRNAs targeting the gene of interest and negative control shRNAs
 - False (default): Do not display this legend

For example, the following command generates the graph shown in Fig. 5b:

```
In [4]: gene_rho('VPS54')
```

Recloning individual shRNAs for validations and for the genetic interaction map – TIMING 4 – 5 d

- 48 |** Based on the results from the primary screen, choose hit genes to be included in validation experiments and in the genetic interaction map. For each gene, choose the 3–4 shRNAs that showed the strongest phenotypes – ideally in two or more independent experimental replicates of the primary screen. Also choose 12 or more negative control shRNAs.

- 49 |** To clone these individual shRNAs into the barcoded vector pMK1200 (ref. 9) first digest overnight 1 µg of plasmid DNA with 1 µl BstXI according to manufacturer's instructions, run the digestion reaction in a 0.8% (wt/vol) agarose gel and extract using the gel extraction kit according to manufacturer's instructions.

CRITICAL STEP Do not dephosphorylate the vector backbone, since the insert will consist of unphosphorylated oligonucleotides.

- 50 |** Order top and bottom oligonucleotides corresponding to hit shRNA sequences formatted as in the example below for the target site
TTTCTTACTCACCCTAAGAACT. Sequence in red represent unique overhangs that are compatible with BstXI-digested pMK1200; sequence in blue represent the shRNA loop sequence.

Top oligonucleotide:

CGTTCTTAGGGTGAGTAAGAAATAGTGAAGCCACAGATGATTTTCTTACTCACCCT
AAGAACTTGGC

Bottom oligonucleotide:

AGTTCTTAGGGTGAGTAAGAAATACATCTGTGGCTTCACTATTTCTTACTCACCCTA
AGAACGCGCT

- 51 |** Prepare oligonucleotide annealing mix:

Compound	amount per sample	final concentration
water	23 ul	
top oligo	1 ul (100 uM)	2 uM
bottom oligo	1 ul (100 uM)	2 uM
2X annealing buffer	25 ul	1X

- 52 |** Incubate annealing mix at 95°C for 5 min in PCR machine with heated lid, remove to bench and allow it to cool to room temperature for 10 min.
- 53 |** Prepare 1:20 dilution of annealing mixture with H₂O
- 54 |** Assemble the following reaction for ligating the annealed oligonucleotides into the digested barcoding vector:
- 25 ng BstXI-digested, purified pMK1200
- 1 ul 1:10 diluted annealed oligonucleotides
- 1 ul 10X T4 DNA Ligase buffer
- 1 ul T4 DNA Ligase (2000 U/ul)
- 55 |** Ligate for 2 h at room temperature
- 56 |** Transform 2.5 µl ligation mixture into DH5a cells and plate onto LB plates containing 100 µg/mL carbenicillin.

- 57 | To validate correct shRNA inserts, sequence clones with primers 5'pSico-Eco-insert-seq or 3' pSico-Pci-insert-seq (Table 1).
- 58 | As shRNAs are individually cloned and barcoded as described above, they are catalogued in a tab-delimited text file called shRNAs.txt in the subdirectory LUT. An example of such a file is provided. In this file, each line summarizes information for one shRNA in the following tab-separated fields:
- Name of barcoded shRNA construct, in the following format:
EntrezGeneID_GeneSymbol_Number
 - 5'-to-3' sequence of top oligo used to clone this shRNA
 - shRNA name in original pooled library
 - Name of pooled library
 - Plasmid number
 - 5'-to-3' sequence of upstream barcode
 - 5'-to-3' sequence of downstream barcode
- 59 | When the barcodes of a new plasmid of an individually cloned shRNA are identified by sequencing, the scripts `check_upstream_barcode.py` and `check_downstream_barcode.py` are used to check whether the barcodes are acceptable in terms of their Hamming distance to the barcodes of all other previously accepted shRNA plasmids, and whether they are compatible with restriction enzyme digests at later stages in this protocol. The only command-line argument for `check_upstream_barcode.py` and `check_downstream_barcode.py` are the upstream and downstream barcode sequences, respectively, in 5'-to-3' direction. The evaluation of the candidate barcodes is provided as terminal output. For each shRNA construct, one acceptable clone needs to be identified. Usually, sequencing of 1–3 clones per construct is sufficient to identify an acceptable clone.

Examples:

```
In [1]: run check_upstream_barcode.py CTTACTCGAA
Hamming distance to nearest Upstream barcode is 3
No restriction site issues found.
In [2]: run check_downstream_barcode.py GTCATGAAGA
Hamming distance to nearest Downstream barcode is 2
Downstream Barcode contains dam methylation site interfering with XbaI
cleavage.
```

Batch retest of individually cloned shRNAs - TIMING 1–21 d screen (depending on screen design) + 3 d sample preparation

- 60 | To validate the individually cloned shRNAs generated in Steps 48–59, pool them in equal quantities (e.g. 1 µg of each) in a tube, and follow steps 1–38 with

the following substitutions: (a) In Step 2, use the pool of individually cloned shRNAs instead of the library plasmid pool for transfection. (b) In Step 27, use one of the following forward primers: oMK371, oMK372, oMK373, oMK374, oMK375, oMK376, oMK377, or oMK378 (Table 1). For each sample that will be sequenced in the same lane on a flow cell, a different forward primer needs to be chosen to introduce a different index. For all samples, use the same reverse primer, oMCB922 (Table 1). (c) In Step 33, the expected PCR product size is ~170 bp. (d) In Step 38, no custom primer needs to be added to the Illumina primer mix.

? TROUBLESHOOTING

Data analysis for the batch retest – TIMING 1 d

- 61 | Move the sequencing results files, in fasta or Illumina fastq format, to the subdirectory sequencing.
- 62 | On the command line, change to the directory scripts, and enter the IPython console with the following command:

```
ipython --pylab
```

The Enthought python distribution enables interactive graphical data display. If you do not want to use this feature, you can execute all scripts directly from the terminal command line by replacing “run” with “python” in the following command line examples (Steps 63–69). In that case, where graphical output is created as part of the program pipeline, the pop-up window with the graph needs to be closed for the program to proceed.

- 63 | Run the script crop_reads.py to process the sequencing data with the following command-line arguments:
 - Name of the sequencing data file, e.g. ../sequencing/BatchRetestSample.fastq
 - Name of the output file, e.g. ../cropped/BatchRetestSample.fa
 - Number of bases after which the sequencing read should be truncated. For the analysis of batch retest samples, 16 is sufficient.

Example command line:

```
In [1]: run
crop_reads.py ../sequencing/BatchRetestSample.fastq ../cropped/BatchRetestSample.fa 16
```

- 64 | Run the script align_batch_retest.py to align the cropped reads to the shRNA library reference with the following command-line arguments:
 - Cropped sequencing data file, e.g. ../cropped/BatchRetestSample.fa

- Prefix for the output files generated by this script, e.g. BatchRetestSample

Example command line:

```
In [2]: run align_batch_retest.py ../cropped/BatchRetestSample.fa
BatchRetestSample
```

- 65 |** The script generates a table of the frequencies with which each individual shRNA was detected in the sequencing data. In the above example, this file will be named BatchRetestSample.counts, located in the subdirectory counts. Once this counts file was successfully generated, files in the directory cropped can be removed.
- 66 |** Repeat Steps 63–65 for all sequenced samples. For batch retests, these may consist of one t0 sample, an untreated sample and several differentially treated samples. For each treatment condition, a file containing the G and K parameters must be created in the subdirectory growth, as described for primary screens (Step 39).
- 67 |** To calculate gamma phenotypes, use the script batch_retest_shRNA_gammas.py with the following command-line arguments:
- Prefix for output files
 - Counts file for t0 sample
 - Counts file for untreated sample
 - File with growth parameters G and K (K is not used by this script)
 - Optional: information on negative controls. If this argument is not provided, the program interprets all shRNA names starting with the prefix “0” as negative controls. Alternatively, custom prefixes can be listed on the command line as consecutive arguments. If no negative control shRNAs are present in the library, the keyword “all” can be provided to use all shRNAs as an approximation of the “null distribution”. This option is generally not recommended.

This program creates the output file Prefix.gammas, in the same format as the gammas file generated by analyze_primary_screen.py, as well as several graphs corresponding to those generated by analyze_primary_screen.py.

- 68 |** To calculate tau phenotypes, use the script batch_retest_shRNA_taus.py, with the following command-line arguments:
- Prefix for output files
 - Counts file for t0 sample
 - Counts file for treated sample

- File with growth parameters G and K.
- Optional: information on negative controls. If this argument is not provided, the program interprets all shRNA names starting with the prefix “0” as negative controls. Alternatively, custom prefixes can be listed on the command line as consecutive arguments. If no negative control shRNAs are present in the library, the keyword “all” can be provided to use all shRNAs as an approximation of the “null distribution”. This option is generally not recommended.

This program creates the output file Prefix.taus, in the same format as the taus file generated by analyze_primary_screen.py, as well as several graphs corresponding to those generated by analyze_primary_screen.py.

69 | To calculate rho phenotypes, use the script batch_retest_shRNA_rhos.py, with the following command-line arguments:

- Prefix for output files
- Counts file for untreated sample
- Counts file for treated sample
- File with growth parameters G and K.
- Optional: information on negative controls. If this argument is not provided, the program interprets all shRNA names starting with the prefix “0” as negative controls. Alternatively, custom prefixes can be listed on the command line as consecutive arguments. If no negative control shRNAs are present in the library, the keyword “all” can be provided to use all shRNAs as an approximation of the “null distribution”. This option is generally not recommended.

This program creates the output file Prefix.rhos, in the same format as the rhos file generated by analyze_primary_screen.py, as well as several graphs corresponding to those generated by analyze_primary_screen.py.

Example command line:

```
In [3] : run
batch_retest_shRNA_rhos.py ../results/Example_BatchRetest ../counts/Example_B
atchRetest_untreated.counts ../counts/Example_BatchRetest_treated.counts ../g
rowth/Example_BatchRetest_GK.txt
```

This command will create the output file ../results/Example_BatchRetest.rhos

Construction of double-shRNA libraries – TIMING 3 d

- 70 | To construct double-shRNA libraries, select individually cloned shRNAs based on their performance in the batch retest. Only shRNAs that qualitatively reproduce the phenotype observed in the primary screen should be considered for the double-shRNA library. Select at least 3 shRNAs targeting each gene, and 12 or more negative control shRNAs. Pool the plasmids of these shRNAs in equal amounts in one tube, and split the pool to perform 2 restriction digests to generate the insert and backbone fragments. In one, digest 1 µg of the pooled shRNAs with KpnI + AvrII, and purify a 1011 bp-long band (insert) from 0.8% (wt/vol) agarose gel using the Qiagen gel extraction kit according to the manufacturer's instructions. In the other, digest 1 µg of the pooled shRNAs with KpnI + XbaI, and purify a 7808 bp-long band (backbone) from gel.
- 71 | Ligate overnight at 16 °C, 1 µg of the 7808 bp-long fragment to 388 ng of the 1011 bp-long fragment in 10 µl, using 1 µl T4 DNA ligase (2000 U) and 1 µl 10X T4 DNA ligase buffer.
- 72 | Transform 5 µl of this mixture into MegaX bacterial cells by electroporation in a 1-mm gap cuvette at 2 kV, 200 ohms, 25 uF, and recover in 11 mL SOC medium in Erlenmeyer flask 1 h at 37 °C shaking at 250 rpm.
- 73 | Plate bacteria on sufficient numbers of Qtray agar plates to get 1000 colonies per library shRNA. Typically, 500 uL plated from the recipe above yields ~100,000 colonies per plate.
- 74 | Incubate overnight at 37 °C, scrape colonies using 20mL LB per plate and a glass rod.
- 75 | Collect LB from all plates, and proceed to Qiagen gigaprep instructions.
- 76 | Prepare library DNA using Qiagen gigaprep kit; typical yield is ~10 mg.

Pooled screening of double-shRNA library – TIMING 1–21 d screen + 3 d sample preparation

- 77 | To screen the double-shRNA library, follow steps 1–38 with the following substitutions: (a) In Step 2, use the double-shRNA library instead of the primary shRNA library for transfection. (b) In Step 27, use one of the following forward primers: oMK371, oMK372, oMK373, oMK374, oMK375, oMK376, oMK377, or oMK378 (Table 1). For each sample that will be sequenced in the same lane on a flow cell, a different forward primer needs to be chosen to introduce a different index. For all samples, use the same reverse primer, oMCB847 (Table 1). (c) In Step 33, the expected PCR product size is ~190 bp. (d) In Step 38, no custom primer needs to be added to the Illumina primer mix.

? TROUBLESHOOTING

Data analysis double-shRNA screen, GI map construction – TIMING 4 d

- 78 | In subdirectory LUT, create a tab-delimited text file in which each line corresponds to the plasmid number of a plasmid included in the double-shRNA library. An example file, DoubleshRNA_plasmids.txt, is provided. If different plasmids are used in the first and second position, create one file for each set of plasmids.
- 79 | Move the sequencing results files, in fasta or Illumina fastq format, to the subdirectory sequencing.
- 80 | On the command line, change to the directory scripts, and enter the IPython console with the following command:

```
ipython --pylab
```

The Enthought python distribution enables interactive graphical data display. If you do not want to use this feature, you can execute all scripts directly from the terminal command line by replacing “run” with “python” in the following command line examples (Steps 81–93). In that case, where graphical output is created as part of the program pipeline, the pop-up window with the graph needs to be closed for the program to proceed.

- 81 | Run the script crop_reads.py to process the sequencing data with the following command-line arguments:
 - Name of the sequencing data file, e.g. ../sequencing/DoubleshRNASample.fastq
 - Name of the output file, e.g. ../cropped/DoubleshRNASample.fa
 - Number of bases after which the sequencing read should be truncated. For the analysis of double shRNA samples, 26 is sufficient.

Example command line:

```
In [1]: run crop_reads.py ../sequencing/DoubleshRNASample.fastq ../cropped/
DoubleshRNASample.fa 26
```

- 82 | Run the script align_double_shRNAs.py to align the cropped reads to the shRNA library reference with the following command-line arguments:
 - Cropped sequencing data file, e.g. ../cropped/DoubleshRNASample.fa
 - Prefix for the output files generated by this script, e.g. DoubleshRNASample

Example command line:

```
In [2]: run align_double_shRNAs.py ../cropped/DoubleshRNASample.fa
DoubleshRNASample
```

- 83 |** The script generates several output files, most importantly a table of the frequencies with which each double-shRNA was detected in the sequencing data. In the above example, this file will be named DoubleshRNASample.counts, located in the subdirectory counts. Once this counts file was successfully generated, files in the directory cropped can be removed.
- 84 |** Steps 81–83 are repeated for all sequenced samples. For double-shRNA screens, these typically consist of one t0 sample, a treated sample and an untreated sample. Two replicates of the screen should typically be carried out. The following example count files are provided in the subdirectory counts: Rep1_t0.counts, Rep1_treated.counts, Rep1_untreated.counts, Rep2_t0.counts, Rep2_treated.counts, Rep2_untreated.counts.
- 85 |** For each treatment condition, a file containing the G and K parameters must be created in the subdirectory growth, as described for primary screens (Step 39). Example files Rep1_GK.txt and Rep2_GK.txt are provided.
- 86 |** The script double_shRNA_phenotypes.py is called with only one command-line argument, the prefix for the output files, e.g. ../results/Rep1. The remaining is supplied via an interactive dialogue in the terminal window.

Example:

```
In [3]: run double_shRNA_phenotypes.py ../results/Rep1
File with list of plasmids in the first position (XbaI/KpnI
backbone): ../LUT/Double_shRNA_plasmids.txt
File with list of plasmids in the second position (AvrII/KpnI
insert): ../LUT/Double_shRNA_plasmids.txt
Use t0 counts? (y/n): y
Name of the t0 counts file: ../counts/Rep1_t0.counts
Name of the untreated counts file: ../counts/Rep1_untreated.counts
Use treated counts? (y/n): y
Name of the treated counts file: ../counts/Rep1_treated.counts
Name of GK file: ../growth/Rep1_GK.txt
Minimal fraction of combinations required to include shRNA (default: 0.5):
0.5
Count ratio 0.869394877872
WT Log2E -1.1717825642
Expected in position 1 but not sufficiently frequent in rhos:
5861_RAB1A_16
Expected in position 2 but not sufficiently frequent in rhos:
```

```

Count ratio 1.21154456258
WT Log2E 0.428613912177
Expected in position 1 but not sufficiently frequent in gammas:
5861_RAB1A_16
Expected in position 2 but not sufficiently frequent in gammas:
Count ratio 1.05331063702
WT Log2E -0.742951508028
Expected in position 1 but not sufficiently frequent in taus:
5861_RAB1A_16
Expected in position 2 but not sufficiently frequent in taus:

```

The script generates several graphs. These graphs analyze the phenotypes of pairs of shRNAs, A and B in the AB and the BA orientations. AB and BA phenotypes should be very similar; large differences between AB and BA are therefore mostly reflective of experimental noise. The distribution of AB–BA differences in the entire dataset is visualized by a histogram (e.g. Supplementary Fig. 3a). Two-dimensional histograms (based on hexagonally binned heatmaps) explore the dependence of noise on phenotypic strength (Fig. Supplementary Fig. 3b) and abundance of double shRNAs at time t0 (Fig. 6). Less abundant double-shRNAs typically give noisier phenotypes, indicating that data quality can be further improved by scaling up the cell population for the pooled screen. A further internal quality control is the comparison of phenotypes of double-shRNAs containing one shRNA targeting a hit gene and one negative control shRNA in the two possible orientations (Supplementary Fig. 4). A small systematic deviation is observed since shRNAs in the second position of the double-shRNAs are slightly more effective.

The script also generates several output files, which are used by scripts in subsequent steps.

? TROUBLESHOOTING

- 87 |** Genetic interactions are calculated by the script `calculate_GIs.py`. The only command line argument is the same Prefix entered for the previous script, e.g. `../results/Rep1`. In an interactive dialogue, the phenotypes for which genetic interactions should be calculated are specified.

Example:

```

In [4]: run calculate_GIs.py ../results/Rep1
Use rhos? (y/n): y
Use gammas? (y/n): n
Use taus? (y/n): n

```

The script generates several graphs in pop-up windows:

- Comparison of different definitions for the expected double-mutant phenotype. Orange line: Sum definition, green line: Product definition, blue: empirical linear fit for each shRNA (dark blue dots indicate the average phenotypes and slopes for

shRNAs targeting a given gene, light blue error bars indicate standard deviation) (Supplementary Fig. 5a)

- Comparison of slopes for empirical linear fits for shRNAs obtained either along “rows” or “columns” of the double-shRNA phenotype table – both should be identical. This graph can reveal noise or systematic biases. (Supplementary Fig. 5b).

88 | After running the script, several interactive functions can be called on the command line to explore the data interactively:

- `rho_sd()`, `gamma_sd()`. Comparison of single shRNA phenotypes (derived from double shRNAs containing one negative control shRNAs) and double shRNA phenotypes, in combination with one shRNA of interest.

These functions accept the following arguments:

- Name of an shRNA of interest, in quotes. E.g. ‘SEC23B_4’.
- Optional: `LabelAboveZ` (floating point number):
Display names of shRNAs if their genetic interaction with the shRNA of interest is this many standard deviations above the mean. Default is 100, which typically suppresses all shRNA labels
- Optional: `Link` (Boolean):
 - ◆ True (default): Link points representing the same shRNA in the first and second positions of a double-shRNA by a line.
 - ◆ False: Do not display this line

For example, the following command generates the graph shown in Supplementary Fig. 6a:

```
In [5]: rho_sd('SEC23B_4',
             LabelAboveZ=2.5, Link=True)
```

- `-gene_rho_sd()`, `gene_gamma_sd()`. Similar to the previous function, except that data from shRNAs targeting the same gene are averaged. This typically vastly reduces the noise.

These functions accept the following arguments:

- Gene of interest, in quotes. E.g. ‘SEC23B’.
- Optional: `LabelAboveZ` (floating point number):

Display names of genes if their average genetic interaction with the gene of interest is this many standard deviations above the mean. Default is 100, which typically suppresses all shRNA labels

- Optional: Link (Boolean):
 - ◆ True (default): Link points representing the same gene in the first and second positions of a double-shRNA by a line.
 - ◆ False: Do not display this line
- Optional: PlotSD (Boolean):
 - ◆ True (default): Show error bars representing the standard deviation of the values for individual shRNAs targeting the gene of interest.
 - ◆ False: Do not display error bars.

For example, the following command generates the graph shown in Supplementary Fig. 6b:

```
In [6]: gene_rho_sd('SEC23B', LabelAboveZ =
1.5, Link = True, PlotSD =
True)
```

- `ab_rho_sd_yellow_blue()` and `ab_gamma_sd_yellow_blue()`. Similar to `rho_sd()` and `gamma_sd()`, but data for each shRNA are averaged for the shRNA occurring in the first and in the second position within the double-shRNA.

These functions accept the following arguments:

- Name of an shRNA of interest, in quotes. E.g. 'SEC23B_4'.
- Optional: ShowLabels (Boolean):
 - ◆ True: Display shRNA names.
 - ◆ False (default): Do not display shRNA names.

For example, the following command generates the graph shown in Fig. 7:

```
In [7]:
```

```
ab_rho_sd_yellow_blue('SEC23B_4', ShowLabels=True
)
```

- 89 | The script also generates several output files, which are used by scripts in subsequent steps. These include tables of genetic interactions calculated according to the different definitions we described previously⁹. If none of these definitions are a good fit for a given experiment, users can implement additional custom definitions by editing the script `calculate_GIs.py`.
- 90 | Repeat Steps 86–89 for the second replicate of the screen.
- 91 | The script `compare_GIs.py` averages experimental replicates and compares different definitions of genetic interactions. The two command-line arguments are:
- Prefix for the first replicate, e.g. `../results/Rep1`
 - Prefix for the first replicate, e.g. `../results/Rep2`

The phenotypes (rhos, gammas, taus) for which genetic interactions should be compared are specified interactively. Example:

```
In [8]: run compare_GIs.py ../results/Rep1 ../results/Rep2
Use rhos? (y/n): y
Use gammas? (y/n): n
Use taus? (y/n): n
```

The script generates several output files that tabulate genetic interactions averaged between the two replicates. Different methods for calculating genetic interactions are indicated by the following code, which appears in the corresponding file names: *Phenotype Expected Flip*. *Phenotype* can be Rho, Gamma or Tau. *Expected* can be p (for product definition of expected double-shRNA phenotypes), s (for sum definition of expected double-shRNA phenotypes), or f (for empirical fit definition of expected double-shRNA phenotypes). The code for *Flip* can be absent for raw genetic interactions, flip1 for Definition 1 of buffering/synergistic genetic interactions, or flip2 for Definition 2 of buffering/synergistic genetic interactions. We have previously defined these different methods to calculate genetic interactions quantitatively⁹. For example, the above command line will create, among others, the following output file: `../results/Rep1Rep2_Rhofflip2GI.txt`.

The script also generates several graphs as pop-up windows:

- Two-dimensional histograms (based on hexagonally binned heatmaps) visualize the correlation of genetic interactions between shRNA pairs across the two replicates (e.g. Supplementary Fig. 7a)
- Correlation coefficients for genetic interactions across the two replicates are summarized in a bar graph for the different methods for calculating genetic interactions (Supplementary Fig. 7b)

- Mean genetic interaction values are summarized in a bar graph for genetic interactions calculated using the different methods and averaged between replicates (Supplementary Fig. 7c).
- Comparison of the means of Pearson correlation between the genetic interaction patterns of shRNAs targeting the same gene (“Intra-gene correlation”) or different genes (“Inter-Gene correlation”), for genetic interactions calculated using the different methods and averaged between replicates (Supplementary Fig. 7d). For an appropriate definition of genetic interactions, the mean intra-gene correlation should be much larger than the mean inter-gene correlation.

92 | Based on the results produced by the scripts `calculate_GIs.py` and `compare_GIs.py`, users should decide which definition of genetic interactions (as defined in Step 91, e.g. Rhof) is most appropriate for their data. Criteria for this decision are as follows:

- For a given shRNA, most double-shRNAs should show the expected double-shRNA phenotype according to the chosen definition. For example, Fig. 12 reveals that the sum and product definitions are not adequate for most genes in the example data set, therefore the empirical fit for individual shRNAs should be selected here.
- Genetic interaction patterns for shRNAs targeting the same gene should on average be more correlated than genetic interaction patterns for other shRNAs (Supplementary Fig. 7d).

93 | The script `filter_GIs.py` aims to remove shRNAs with possible off-target effects from the dataset. Such shRNAs are detected as shRNAs whose genetic interaction pattern is not sufficiently correlated with that of other shRNAs targeting the same gene⁹. The script requires two command-line arguments:

- Genetic interaction file to be used as input (e.g. `../results/Rep1Rep2_RhofGI.txt`)
- Cutoff for required correlation of genetic interaction pattern with that of other shRNAs targeting the same gene, expressed in standard deviations of the distribution of correlation coefficients between genetic interaction patterns of a given shRNA and all other shRNAs in the data set (e.g. 0.8)

Example command line:

```
In [9]: run filter_GIs.py ../results/Rep1Rep2_RhofGI 0.8
```

The script lists the shRNAs that were removed from the dataset as screen output, for example:

```
Rejected shRNAs: ['C17orf75_3', 'CCT7_2', 'CCT7_3', 'COPB1_1', 'COPB1_2',
```

```
`HMGCR_4', `HMGCR_7', `HMGCR_8', `IGF2R_1', `IGF2R_2', `PDIA3_1', `PDIA3_3',
`SRRM1_2', `SRRM1_3']
```

Two filtered genetic interaction files are generated, one with the shRNA-based genetic interactions (e.g. `../results/Rep1Rep2_KfGI_minZ0.8.txt`), and one with gene-averaged genetic interactions, for which genetic interactions of shRNAs targeting the same gene are averaged (e.g. `../results/Rep1Rep2_KfGI_minZ0.8_GeneGI.txt`).

A heatmap in a popup window visualizes the frequency of different outcomes for the shRNAs targeting a given gene (Supplementary Fig. 8). Only genes targeted by at least two correlating shRNAs are included in the gene-based genetic interaction map.

- 94 | Open Cluster 3.0 (ref. 27) and load the gene-based genetic interaction file generated by the script `filter_GIs.py` (e.g. `../results/Rep1Rep2_KfGI_minZ0.8_GeneGI.txt`). Apply Hierarchical clustering on both “Genes” and “Arrays” using Uncentered Correlation as similarity metric and Average Linkage as clustering method. This will generate three files with the same prefix and the extensions `.atr`, `.cdt` and `.gtr`.
- 95 | Open Java Tree View²⁹ and load the `.cdt` file generated by Cluster 3.0 (e.g. `../results/Rep1Rep2_KfGI_minZ0.8_GeneGI.txt.cdt`) for interactive exploration of the genetic interaction map. Refer to the Java Tree Documentation (<http://jtreeview.sourceforge.net/manual.html>) for instructions.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

TIMING

- Steps 1–7, lentivirus preparation and infection: 7–10 d
- Steps 8–10, conducting a screen: 1–21 d
- Steps 11–38, preparing samples for deep sequencing: 3 d
- Steps 39–47, data analysis primary screen: 1–4 d
- Steps 48–59, recloning individual shRNAs: 4 – 5 d
- Step 60, batch retest of individually cloned shRNAs: 1–21 d screen + 3 d sample preparation
- Steps 61–69, data analysis batch retest: 1 d
- Steps 70–76, construction of double-shRNA library: 3 d
- Step 77, pooled screening of double-shRNA library: 1–21 d screen + 3 d sample preparation
- Steps 78–95, data analysis double-shRNA screen, GI map construction: 4 d

ANTICIPATED RESULTS

The results of the primary screen depend strongly on the biological question. In some cases, a single gene is detected as an exceptionally strong hit³⁰. In other cases, dozens or hundreds of hit genes are detected¹⁰. In the latter case, a genetic interaction map is likely to generate hypotheses for functional connections between genes, such as physical interaction or enzyme-substrate relationships (Fig. 8). These can then be tested using classical cell biological and biochemical approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overview of the experimental procedure. (a) Primary genome-wide shRNA screen, in which hit genes are identified. (b) Cloning of individual shRNAs against hit genes from the primary screen, and confirmation of their phenotype in a batch retest. (c) Construction and screening of a double-shRNA library representing all pairwise combination of selected shRNAs from the batch retest, and calculation of a systematic genetic interaction map.

Figure 2.

Lentiviral plasmids used in the experimental procedure (not drawn to scale). The EF1A promoter drives expression of an mRNA that encodes a puromycin resistance marker, an mCherry fluorescent marker, and either one or two shRNAs embedded in a minimal miR-30 context. (a) Vector pMK1047 contains the pooled, ultracomplex genome-wide shRNA library. The shRNA expression cassette is flanked by PvuII sites that are 1,122 bp apart; this restriction enzyme is used to size-fractionate genomic DNA from cell populations transduced with pMK1047. Primers oMCB800 and one of the primers oMK312 or oMK313 (shown in turquoise; sequences in Table 1) are used to amplify the shRNA cassette from genomic DNA, and primer oMK132 is used as primer for Illumina sequencing to identify the shRNA, starting with the guide strand. (b) Vector pMK1200 is used to clone individual shRNAs. This vector is a pool of plasmids with random N₁₀ barcodes upstream and downstream of the minimal miR-30 context. Annealed oligonucleotides encoding the shRNA of interest are ligated into BstXI-cut pMK1200. The combinations of barcodes and shRNA inserts are identified by sequencing with primers 5' pSico-Eco or 3' pSico-Pci (shown in purple; sequences in Table 1). The shRNA expression cassette is flanked by PvuII sites that are 1,177 bp apart. Primers oMCB922 and one of the primers oMK371 or oMK378 (shown in turquoise; sequences in Table 1) are used to amplify the shRNA cassette from genomic DNA, and the Illumina genomic sequencing primer (shown as SP) is used as primer for Illumina sequencing to identify the downstream barcode. (c) A double-shRNA library is created by digesting a pool of barcoded shRNA cloned in the pMK1200 backbone. This pool is digested with AvrII + KpnI to obtain an insert and with XbaI + KpnI to obtain a

backbone, the ligation of which results in a double-shRNA library. The double-shRNA expression cassette is flanked by PvuII sites that are 1,369 bp apart. Primers oMCB847 and one of the primers oMK371 or oMK378 (shown in turquoise; sequences in Table 1) are used to amplify the barcode junction portion from genomic DNA. Note that although both primers will anneal to two places within the double-shRNA cassette, only one amplicon will be generated by PCR. The Illumina genomic sequencing primer (shown as SP) is used as primer for Illumina sequencing to identify the combinatorial barcode.

Figure 3.

Gel purification steps during sample preparation. (a) Size fractionation of genomic DNA. Genomic DNA isolated from a screen cell population is digested with PvuII and the size range between 1,000 and 1,650 bp, which contains the shRNA expressing cassette for all constructs shown in Figure 2, is excised from an ethidium bromide-stained 0.8% agarose gel, as indicated by the dashed red box, and purified for further use as a PCR template. (b) PCR products are separated on a 20% polyacrylamide gel and visualized by ethidium bromide staining. In addition to the desired PCR product (in the example shown a 190 bp-long product amplified from genomic DNA of cells transduced with a double-shRNA library), shorter products result from long primer dimers (the unwanted band is present in the control reaction lacking template, but not the control reaction lacking reverse primer).

Figure 4.

Graphical output of the script `analyze_primary_screen.py` (screenshot). (a) Each dot represents one shRNA. X-axis: log₂-transformed deep-sequencing counts of cells expressing this shRNA in the t₀ population. Y-axis: log₂-transformed deep-sequencing counts of cells expressing this shRNA in the untreated population at the endpoint of the experiment. Black circles represent negative-control shRNAs. Colored circles represent shRNAs designed to target genes; they are colored according to a heatmap representing the growth phenotype (gamma). (b) Distribution of growth phenotypes (gammas) for negative control shRNAs (blue dots) and shRNAs designed to target genes (red dots). Grey dashed lines indicate the range of phenotypes containing the middle 95% of negative control shRNAs.

Figure 5.

Graphical output of the script `analyze_primary_screen.py` (screenshot) (a) Each dot represents one gene. $\log_{10}$ -transformed P values for an effect caused by gene knockdown were calculated by the Mann-Whitney test. Negative values indicate depletion of cells expressing shRNAs targeting the gene of interest from the population, positive values indicate enrichment. X axis: untreated cell population, Y axis: treated cell population. (b) This graph was generated interactively using the `gene_rho()` command after running the script (Step X). Each dot represents an shRNA. X-axis: $\log_2$ -transformed deep-sequencing counts of cells expressing this shRNA in the untreated population at the endpoint of the experiment. Y-axis: $\log_2$ -transformed deep-sequencing counts of cells expressing this shRNA in the treated population at the endpoint of the experiment. Grey semi-transparent dots represent negative control shRNAs. Solidly colored dots represent shRNAs targeting the gene VPS54; they are colored according to a heatmap representing the resistance phenotype (ρ).

Figure 6.

Graphical output of the script `double_shRNA_phenotypes.py` (screenshot). A two-dimensional histogram (implemented as hexagonally binned heatmap) of AB-BA differences in phenotype (rho) as a function of the average count numbers of the AB and BA double shRNAs in the t0 population.

Figure 7. Graphical output of the script `calculate_GIs.py` (screenshot). This graph was generated interactively using the `ab_rho_sd_yellow_blue()` command after running the script. Each dot represents an shRNA. X axis: average phenotype (ρ) of double-shRNAs containing this shRNA and one of 12 negative control shRNAs. Y axis: phenotype (ρ) of the double-shRNA containing the same shRNA in combination with shRNA SEC23B_4. Values for shRNAs with SEC23B_4 in the first and second position of the shRNA were averaged. Additionally, expected double-shRNA phenotypes according to three different definitions are indicated by solid lines: red, linear fit orange, sum definition; pink, product definition. These definitions are detailed in ref. 9.

Figure 8.

High-density genetic interaction map created by hierarchical clustering of the genetic interaction matrix in Cluster 3.0 (ref. ²⁸), and displayed in Java Tree View²⁹. Genes encoding proteins that interact physically (e.g. COPI complex subunits) or functionally (e.g. ARF1 and its nucleotide exchange factor GBF1) form clusters and typically show buffering genetic interactions with each other.

Table 1

Oligonucleotide sequences

Illumina index sequences are shown in red.

Name	Purpose	Oligonucleotide sequence (5' to 3')
oMK312	Amplification of primary screen samples, forward primer, Index 1	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CATCAGCTCTAGATGACTGACCCCTTG
oMK313	Amplification of primary screen samples, forward primer, Index 2	CCGATGTCTCTAGATGACTGACCCCTTG
oMK314	Amplification of primary screen samples, forward primer, Index 9	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CGATCAGCTCTAGATGACTGACCCCTTG
oMK315	Amplification of primary screen samples, forward primer, Index 4	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CTGACCACTCTAGATGACTGACCCCTTG
oMK316	Amplification of primary screen samples, forward primer, Index 5	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CACAGTGCTCTAGATGACTGACCCCTTG
oMK317	Amplification of primary screen samples, forward primer, Index 6	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CGCCAATCTCTAGATGACTGACCCCTTG
oMK318	Amplification of primary screen samples, forward primer, Index 7	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CCAGATCCTCTAGATGACTGACCCCTTG
oMK319	Amplification of primary screen samples, forward primer, Index 13	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CAGTCAACTCTAGATGACTGACCCCTTG
oMCB800	Amplification of primary screen samples, reverse primer	CAAGCAGAAGACGGCATAACGATGGAC GAGCTGTACAAGTAA
oMK132	Deep sequencing primer for primary screen samples	ACCCCTTGAATTCCGAGGCAGTAGGCA
5' pSico-Eco	Verification of inserts cloned into pMK1200	CCACAACGAGGACTACACCA

Name	Purpose	Oligonucleotide sequence (5' to 3')
3' pSico-Pci	Verification of inserts cloned into pMK1200	TTGTAATCCAGAGGTTGATTATCG
oMK371	Amplification of batch retest and double- shRNA screen samples, forward primer, Index 3	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CTTAGGCggtcagtcacactctttccc
oMK372	Amplification of batch retest and double- shRNA screen samples, forward primer, Index 11	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CGGCTACggtcagtcacactctttccc
oMK373	Amplification of batch retest and double- shRNA screen samples, forward primer, Index 12	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CCTTGTAggtcagtcacactctttccc
oMK374	Amplification of batch retest and double- shRNA screen samples, forward primer, Index 8	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CACTTGAggtcagtcacactctttccc
oMK375	Amplification of batch retest and double-shRNA screen samples, forward primer, Index 10	CTAGCTTggtcagtcacactctttccc
oMK376	Amplification of batch retest and double-shRNA screen samples, forward primer, Index 14	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CAGTTCCggtcagtcacactctttccc
oMK377	Amplification of batch retest and double-shRNA screen samples, forward primer, Index 15	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CATGTCAggtcagtcacactctttccc
oMK378	Amplification of batch retest and double-shRNA screen samples, forward primer, Index 16	aatgatacggcgaccaccgaGATCGG AAGAGCACACGTCTGAACTCCAGTCA CCCGTCCggtcagtcacactctttccc
oMCB922	Amplification of batch retest samples, reverse primer	CAAGCAGAAGACGGCATAACGACATGTCT CGACCTGGCTTACTAG
oMCB847	Amplification of double-shRNA screen samples, reverse primer	caagcagaagacggcatacgaCAGCAAT ATACCTTCTTCTCGAGTC

Table 2

Troubleshooting table.

Step	Problem	Possible Reason	Possible Solution
7	Low rate of infected cells	Problems with virus preparation	Use fresh, low-passage 293T cells in Step 1.
			Use fresh plasmid DNA in Step 2.
			Use a new vial of Mirus LT1 in Step 2.
			Check for mCherry fluorescence in packaging cell line in Step 3.
		Target cells are difficult to infect	Check infection of a control cell line (such as HeLa cells)
			Concentrate virus using spin-filtration or ultracentrifugation after Step 3.
33	No or very faint band for expected PCR product visible on ethidium bromide-stained gel.	More PCR cycles are needed	Increase number of PCR cycles in Step 28. However, the number of PCR cycles should only be as high as necessary to see a clear product band. Excessive numbers of PCR cycles can introduce amplification bias.
		Too much genomic DNA was used as template	Reduce genomic DNA concentration to maximally 1.5 µg per 100 µl in Step 27.
		General problems with PCR reagents or thermocycler	Run a positive control reaction in which the shRNA library plasmid is used as a template. Use fresh reagents in Step 27.
	Unexpected bands are observed	Primer dimers and other template-independent products.	Perform and load a negative control reaction lacking template on the gel next to the sample of interest. Run the gel long enough to separate unwanted bands from the band of interest. Cut out only the desired band in Step 33 (see Fig. 3b)
46	The distribution of negative control shRNA phenotypes is dramatically wider than in the example shown in Fig. 4.	At least one of the cell populations underwent a bottleneck during the screen or sample preparation	Repeat or redesign the screening protocol (Step 8) to avoid population bottlenecks.
	shRNA phenotypes show a bimodal distribution or have a very broad “shoulder” on one side of the distribution.	In at least one of the cell populations, bias was introduced during the sample preparation.	Make sure to use the same number of PCR cycles in Step 28 for all samples that will be compared computationally.
			If the primary shRNA library contains shRNAs of different lengths (e.g. 21mers and 22mers), it is important to avoid introducing size bias when cutting bands from the polyacrylamide gels (Step 33). Therefore, the band should be cut out very generously, leaving a margin of unstained gel around the band.
		At least one of the cell populations was contaminated with a different sample.	Use separate equipment for pre-PCR steps (Steps 11–28) and post-PCR steps (Steps 29–38). In particular, use separate vacuum manifolds.
60	Same problems as for the primary screen (Troubleshooting for Steps 7, 33 above).	See possible reasons for corresponding problem in primary screen (Troubleshooting for Steps 7, 33 above).	See possible reasons for corresponding solution in primary screen (Troubleshooting for Steps 7, 33 above).
77	Same problems as for the primary screen (Troubleshooting for Steps 7, 33 above).	See possible reasons for corresponding problem in primary screen (Troubleshooting for Steps 7, 33 above).	See possible reasons for corresponding solution in primary screen (Troubleshooting for Steps 7, 33 above).
86	Large differences between AB and BA phenotypes for a given combination of shRNAs A, B.	See possible reasons for corresponding problem in primary screen (Troubleshooting for Steps 46 above).	See possible reasons for corresponding solution in primary screen (Troubleshooting for Steps 46 above).